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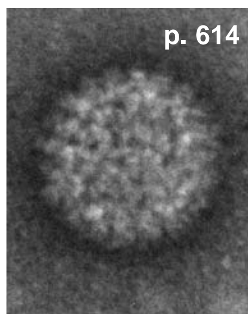
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Human Benefits of Animal Interventions for Zoonosis Control

Jakob Zinsstag,* Esther Schelling,*† Felix Roth,* Bassirou Bonfoh,*‡ Don de Savigny,* and Marcel Tanner*

Although industrialized countries have been able to contain recent outbreaks of zoonotic diseases, many resource-limited and transitioning countries have not been able to react adequately. The key for controlling zoonoses such as rabies, echinococcosis, and brucellosis is to focus on the animal reservoir. In this respect, ministries of health question whether the public health sector really benefits from interventions for livestock. Cross-sectoral assessments of interventions such as mass vaccination for brucellosis in Mongolia or vaccination of dogs for rabies in Chad consider human and animal health sectors from a societal economic perspective. Combining the total societal benefits, the intervention in the animal sector saves money and provides the economic argument, which opens new approaches for the control of zoonoses in resource-limited countries through contributions from multiple sectors.

The economic aspects of controlling zoonoses are rapidly gaining attention in light of challenges, both well-known and new. Wildlife reservoirs of classical and emerging zoonoses (e.g., bovine tuberculosis) persist in many countries and substantially slow control efforts for livestock (*1*). The fast-growing demand for milk and meat in urban centers in resource-limited countries is leading to the intensification of livestock production systems, especially in periurban areas of these countries. However, because efficient zoonosis surveillance and food safety are lacking, the risk for zoonosis transmission is increasing, particularly in rapidly growing urban centers of resource-limited countries (*2*,*3*). Many countries in postcommunist transition face a sharp increase in zoonotic diseases resulting from the breakdown of government-run disease surveillance and control and weak private health and veterinary services (*4*).

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Industrialized countries have responded rapidly to recent zoonosis outbreaks and contained them well (*5*), but many resource-limited and transitioning countries have not been able to respond adequately because they lack human and financial resources and have not sufficiently adapted public health surveillance. In industrialized countries, an important part of successful zoonosis control has been compensating farmers for culled livestock. However, many resource-limited countries would not be able to conduct such programs.

Most zoonoses are maintained in the animal reservoir but can cross over to humans as a result of different risk factors and behavioral traits. For example, brucellosis is transmitted to humans from direct contact with livestock or ingestion of unpasteurized milk or milk products; however, brucellosis is not transmitted from humans to livestock. Hence, elimination of zoonoses such as rabies, echinococcosis, and brucellosis is possible only by interventions that vigorously target animal reservoirs. Control of most zoonoses usually requires interventions outside the public health sector. When one considers health from a point of view independent of species, including humans, domestic animals, and wildlife, zoonoses are part of a broader ecologic concept of health systems (*6*–*8*). To attempt control, and possibly elimination, of zoonoses, benefits to public health and society need to be demonstrated, particularly in countries with scarce resources. We present examples from our work on brucellosis and rabies and demonstrate the circumstances for which zoonosis control would save money for resource-limited countries and likely reduce the occurrence of zoonoses worldwide. Avian influenza is discussed as an additional example.

Diseases

Brucellosis

In Mongolia and central Asian countries after democratic reform and the shift from dependence on the former

Soviet Union in 1990, human brucellosis reemerged as a major, but preventable, disease (*9*). After consultations with experts, the World Health Organization (WHO) raised the question whether mass vaccinations of animals saved money for the public health sector. We used an animal-to-human transmission model to estimate the economic benefit, cost-effectiveness, and distribution of benefit (to society and the public health and agricultural sectors) of mass brucellosis vaccination of cattle and small ruminants (*10*). The intervention consisted of a planned 10-year annual livestock mass vaccination campaign using *Brucella melitensis* Rev-1 for small ruminants and *Brucella abortus* S19 for cattle. In a scenario of achieving 52% reduction of brucellosis transmission between animals, 51,856 human brucellosis cases could be averted, which would add up to a gain of 49,027 human disability-adjusted life years (DALYs; see Appendix). The human death rate from brucellosis is considered to be <2% (*11*) and was not assessed in this study (*4*). Estimated intervention costs were US $8.3 million, and the overall benefit was US $26.6 million (Table 1). This results in a present net value of $18.3 million and a benefit-to-cost ratio for society of 3.2 (95% confidence interval [CI] 2.27–4.37). If the costs of the intervention were shared between the sectors in proportion to the benefit to each, the public health sector would contribute 11%, with a cost-effectiveness ratio of $18 per case averted. However this ratio does not account for illness imposed by brucellosis. When the number of DALYs averted is assumed to be 49,027, the cost-effectiveness ratio is $19.1 per DALY averted (95% CI 5.3–486.8). If costs of vaccinating livestock are allocated proportionally to all benefits, the intervention is cost-saving and cost-effective for the agricultural and the public health sectors (*4*). With such an allocation of costs to benefits per sector, brucellosis control becomes one of the most cost-effective interventions (<$25 per DALY gained) in the public health sector, comparable to cost-effectiveness of vaccinating women and children or treating tuberculosis.

Rabies

Most human deaths from rabies occur in tropical resource-limited countries (*12*). In Africa and Asia, an estimated 24,000–70,000 persons die of rabies each year (*13*). The domestic dog is the main source of exposure and vector for human rabies (*14*). Rabies in humans can be prevented by appropriate postexposure prophylaxis, which is not, however, always available and affordable in resource-limited countries. Human rabies can also be prevented through vaccination of the animal vector. Again, particularly for countries with limited resources, we must ask whether it is cost-saving to the public health sector to prevent human rabies by vaccinating dogs. Bögel and Meslin showed that over 15 years in areas where the virus still circulates in the dog population, dog vaccination combined with postexposure treatment of dog-bite patients is more cost-effective than postexposure prophylaxis alone (*15*). However, in many countries, little is known about the real cost of mass vaccination of dogs, and quantitative data are urgently needed to evaluate the cost-effectiveness of different rabies control strategies in resource-limited countries; rabies control strategies in developing countries are currently under review by WHO (F. Meslin, pers. comm.).

We performed a cost analysis of a pilot dog rabies vaccination campaign in which 3,000 dogs in N'Djaména, Chad, were vaccinated (*16*). The average cost per dog was US $2.14 to the public sector (for vaccine and logistics) and $0.97 to the private-sector dog owner, which brings the full cost to society to $3.11. If all 23,600 dogs in N'Djamena were vaccinated, the average cost per dog would fall to $1.48 for the public sector and $2.45 for society (Table 2). Private sector costs account for 31% of the cost to vaccinate 3,000 dogs and 40% of the cost to vaccinate 23,600 dogs (*17*). The above costs per vaccinated dog

Table 1. Distribution of benefits and suggested allocation of intervention costs for livestock brucellosis mass vaccination campaign in Mongolia*†

Sector	Cost	Benefit	Net present value‡	Benefit cost ratio§
Total agriculture sector	5,174.9	16,611.6	11,436.7	3.2
Human health				
Public health sector				
Central government	1,009.4	3,240.3	2,230.9	3.2
Health Insurance Fund	0.0	0.0	0.0	
Patient				
Out-of-pocket contribution to health costs	1,669.3	5,358.7	3,689.4	3.2
Change in household income	1,103.7	3,542.8	2,439.1	3.2
Total overall human health	3,782.4	12,141.8	8,359.4	3.2
Total private sector	7,947.9	25,513.1	17,565.2	3.2
Total society	8,957.3	28,753.4	19,796.1	3.2

*Scenario for proportion of protected animals at 52% and discount rate at 5%. Costs are in Mongolian tugriks (MNT); 1 US$ ≈1,080 MNT in October 2000.
†Table reproduced from (*4*).
‡Benefits minus costs.
§Benefits divided by costs (minimum 2.27, maximum 4.37).

Table 2. Cost per vaccinated dog extrapolated for the dog population of N'Djaména, Chad*†

Item	Total cost (US$)
Public sector	
Marginal	
Vaccine	14,368
Syringes and certificates	5,079
Fixed	
Furniture and small equipment‡	507
Staff§	8,425
Transportation¶	4,653
Information#	1,806
Total public sector	34,838
Private sector	
Lost work time	22,879
Total private sector	22,879
Total campaign	57,717

*Average population 23,600 dogs.
†Table adapted with permission from (*17*).
‡Tables, chairs, coolers, ice, screens, muzzles, first aid materials.
§Per diem for training, information campaign, vaccination program, lunches.
¶Car, small truck, fuel.
#Campaign, poster distribution.

tally with the estimates by Bögel and Meslin (*15*). The cost estimates provided account for only 1 year of vaccination. In the year before this campaign (*16*,*17*), 69 persons were reported to have been exposed to 29 rabid dogs. If one assumes that human exposure could be avoided by mass vaccination of dogs, the cost-effectiveness of mass dog rabies vaccination would be $837 per averted human exposure ($57,774 per 69 averted exposures). If one assumes that about 16% of the exposed persons would actually become ill with rabies and die (*18*), the cost-effectiveness would be $57,774 per 11 averted deaths ($5,252 per averted death). But if we consider that reported human rabies cases in Africa underestimate the true number of human rabies cases by a factor of 10 to 100 (*18*), then the cost-effectiveness would be $52–$525 per averted death. Hence, mass vaccination of dogs is a comparatively inexpensive and ethical way to control the disease in animals and to prevent human exposure and illness, especially in resource-limited countries. More research is needed to assess the dynamics of dog-to-human rabies transmission and the frequency of revaccination programs needed because of turnover in dog populations and continued risk for reintroduction of rabies from outside sources to unvaccinated dogs.

Avian Influenza

The spread of highly pathogenic avian influenza is a global threat to all countries that have a poultry industry, semicommercial poultry production, or backyard poultry operations and has already caused enormous economic losses (*19*). Moreover, the risk for human pandemic influenza originating from highly pathogenic avian influenza in conjunction with human influenza A virus is very high, with an estimate of >100,000 deaths for the United States alone (*20*). To implement disease prevention and control measures, early identification of emerging patterns of disease is necessary and uses economic methods to determine which mix of measures is most cost-effective. Resource-limited countries in Africa are almost devoid of surveillance capacity and efficient early warning systems, which would be crucial. Surveillance of cross-border diseases cannot be restricted to countries that have the funds. High-income countries would ultimately benefit by providing funding for surveillance and control to low-income countries. Comprehensive economic assessment of this issue are, however, lacking so far.

Awareness, Knowledge, and Information

Many countries, especially those with resource constraints and those in sub-Saharan Africa, lack information on the distribution of zoonotic diseases. Risks for zoonoses are considered negligible compared with those for diseases of higher consequence because the societal consequences of zoonoses are not recognized by the individual sectors. For example, outbreaks of Rift Valley fever in persons in Mauritania were mistakenly identified as yellow fever. The correct diagnosis was made only after public health services contacted livestock services, which informed them of abortions in cattle (*21*). In resource-limited and transitioning countries, many zoonoses are not controlled effectively because adequate policies and funding are lacking. However, transmission of zoonoses to humans can already be greatly reduced by health information and behavior. Authorities in Kyrgyzstan, for example, have started an information campaign to reduce brucellosis transmission to small-ruminant herders by encouraging them to wear gloves for lambing and to boil milk before consuming. Interventions in livestock should always be accompanied by mass information, education, and communication programs.

Financing

Substantial evidence documents that the combined effects of human disease caused by zoonoses, as part of the neglected infectious diseases, are in the same range as the classical diseases of poverty such as HIV/AIDS, tuberculosis, and malaria (*22,23*). On the other hand, the public health component justifies including zoonoses such as bovine tuberculoses in current global programs and initiatives on tuberculosis control (*22*,*23*). Recognition of these facts should result in affected countries applying for funds from the Global Fund to Fight AIDS, Tuberculosis and Malaria (*24*). Surveillance and control of cross-border zoonotic diseases such as highly pathogenic avian influenza cannot be restricted to wealthy countries. According to

Vallat, "One country not able to carry out early detection and rapid response to animal disease outbreaks can represent a threat to all the others" (*25*). To approach these threats, new partnerships (e.g., between resource-limited and industrial countries, public and private sectors, and animal and public health) and permanent dialogue are needed. "It is evident that the interest of the rich countries is to support the others in order to protect themselves" (*25*). Zoonosis control in general should thus be seen from a global perspective and lead to a call for a global subsidiary approach for control. International bodies like the World Organization for Animal Health, the Food and Agriculture Organization, and WHO should foster establishment of global standards for zoonosis surveillance and control. Fostering of global standards is also part of the WHO International Health Regulations that will come into force in mid-2007 and will require all countries to do a better job of surveillance for diseases that can spread between countries (www.who.int/gb/edwha/pdf_files/WHA58-REC1/english/Resolutions.pdf). These efforts should lead to a global fund for the control of zoonoses or become a component of an extended Global Fund to Fight AIDS, Tuberculosis and Malaria. Such a joint facility would allow coherent and integrated control approaches, particularly in the countries with the most serious resources constraints, which in turn would benefit the whole world.

Conclusion

Zoonoses are among the most important animal and public health problems that affect the well-being of societies worldwide, yet they are too often forgotten or neglected. Because most zoonoses go unrecorded, they call for a rethinking of research and control efforts and the economic consequences. The example of brucellosis demonstrates that interventions in livestock against zoonoses, which would never be cost-effective when uniquely assessed from a public health sector point of view, may become cost-saving when considered from a societal perspective. Creating a new global finance facility for the control of zoonoses, similar to or linked with the Global Fund to Fight AIDS, Tuberculosis and Malaria, is timely, is of global interest, and represents a further contribution to successful attainment of the Millennium Development Goals.

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Dr Zinsstag leads a research group at the Swiss Tropical Institute in Basel on the interface of human and animal health. His main interests are cross-sectoral epidemiologic models and economic analyses of zoonoses in resource-limited countries and development of integrated human and animal health services for nomadic pastoralists.

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Appendix. Estimating Disability-adjusted Life Years

Disability-adjusted life years (DALYs) are used in the global comparative assessments of the burden of disease (*26*) and enable costs of interventions to be related to a standardized health outcome across diseases internationally. DALYs is an indicator of the time lived with a disability and the time lost because of premature death (Formula 1).

$$\text{DALYs} = \text{years of life lost} + \text{years of life with a disability} \qquad (1)$$

The duration of time lost due to premature death is calculated by using standard expected years of life lost with model life tables. The reduction in physical capacity due to illness is measured by using disability weights. To calculate the reduction in physical capacity, the following formula is used (Formula 2) (*26*):

$$-\left[\frac{DCe^{-\beta a}}{(\beta+r)^2}\left[e^{-(\beta+r)(L)}(1+(\beta+r)(L+a))-(1+(\beta+r)a)\right]\right] \qquad (2)$$

where a is the age at onset of disease, L is the duration of disability or time lost due to premature mortality, D is the disability weight (or 1 for premature mortality), r is the discount rate, C is the age-weighting correction constant, and β is the parameter from the age-weighting function.

An estimate of the burden of disease for brucellosis is not readily available, so we therefore estimated the DALYs as a result of the disease by assuming that brucellosis is associated with a class II (0.2) disability weight (D), as the disease is perceived as very painful and affects occupational ability even during periods of remission (*27*). Average age at onset was calculated for every age group. For the duration of illness, we considered data by Beklemishev on the duration of clinical cure of 1,000 patients with brucellosis in the Russian Federation (*28*). The frequency distribution of clinical disease duration fits best with an exponential function for an average duration of 4.5 years. For duration of disease, we used @Risk (Palisade Corporation, Newfield, NY, USA) exponential function; β = 4.5 years. For cost-effectiveness, we used the median of the cumulated discounted DALYs, which corresponds to a median duration (L) of brucellosis of 3.11 years (*4*).

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Hantavirus and Arenavirus Antibodies in Persons with Occupational Rodent Exposure, North America

Charles F. Fulhorst,* Mary Louise Milazzo,* Lori R. Armstrong,† James E. Childs,†[1] Pierre E. Rollin,† Rima Khabbaz,† C.J. Peters,†[2] and Thomas G. Ksiazek†

Rodents are the principal hosts of Sin Nombre virus, 4 other hantaviruses known to cause hantavirus pulmonary syndrome in North America, and the 3 North American arenaviruses. Serum samples from 757 persons who had worked with rodents in North America and handled neotomine or sigmodontine rodents were tested for antibodies against Sin Nombre virus, Whitewater Arroyo virus, Guanarito virus, and lymphocytic choriomeningitis virus. Antibodies against Sin Nombre virus were found in 4 persons, against Whitewater Arroyo virus or Guanarito virus in 2 persons, and against lymphocytic choriomeningitis virus in none. These results suggest that risk for infection with hantaviruses or arenaviruses usually is low in persons whose occupations entail close physical contact with neotomine or sigmodontine rodents in North America.

Hantavirus pulmonary syndrome (HPS) is a frequently fatal rodentborne viral zoonosis. Seven species in the virus family *Bunyaviridae*, genus *Hantavirus* (*1*), have been causally associated with HPS: *Sin Nombre virus* (SNV), *New York virus* (NYV), *Black Creek Canal virus* (BCCV), *Bayou virus* (BAYV), and *Choclo virus* (CHOV) in North America (*2–6*), and *Andes virus* (ANDV) and *Laguna Negra virus* (LANV) in South America (*7,8*).

The virus family *Arenaviridae*, genus *Arenavirus*, includes 3 North American species and 14 South American species (*9*). The North American species are *Bear Canyon virus* (BCNV), *Tamiami virus* (TAMV), and *Whitewater Arroyo virus* (WWAV). The South American species include *Guanarito virus* (GTOV), *Junin virus* (JUNV), *Machupo virus* (MACV), and *Sabiá virus* (SABV). These 4 South American species have been causally associated with severe human disease in Venezuela, Argentina, Bolivia, and Brazil, respectively (*10*). The human health importance of the North American arenavirus species has not been rigorously investigated.

Specific members of the subfamilies Neotominae and Sigmodontinae in the rodent family Cricetidae (*11*) are the principal hosts (reservoirs) of the hantaviruses known to cause HPS in North America and the 3 North American arenaviruses. For example, principal hosts and their respective viruses include: the deer mouse (*Peromyscus maniculatus*) in Canada and the western United States, SNV (*12,13*); the white-footed mouse (*Peromyscus leucopus*) in the northeastern United States, NYV (*3*); the hispid cotton rat (*Sigmodon hispidus*) in Florida, BCCV and TAMV (*4,14*); the marsh rice rat (*Oryzomys palustris*) in the southeastern United States, BAYV (*15–17*); the fulvous colilargo (*Oligoryzomys fulvescens*) in Panama, CHOV (*6*); the California mouse (*Peromyscus californicus*) in California, BCNV (*18*); and the white-throated woodrat (*Neotoma albigula*) in New Mexico, WWAV (*19*). *P. maniculatus, P. leucopus*, *P. californicus*, and *N. albigula* are members of the Neotominae and *S. hispidus*, *O. palustris*, and *O. fulvescens* are members of the Sigmodontinae (*11*).

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It is assumed that humans usually become infected with hantaviruses and arenaviruses by inhalation of aerosolized droplets of urine, saliva, or respiratory secretions from infected rodents. Other means of infection include, but are not limited to, inhalation of dust or other organic matter contaminated with infectious virus and contact of infectious materials with mucous membranes.

The purpose of this study was to assess the risk for hantavirus and arenavirus infections among persons who work in North America and have close physical contact with neotomine rodents or sigmodontine rodents through their occupations. These persons include mammalogists, wildlife biologists, scientists whose research concerns the ecology of rodentborne zoonoses, and pest control operators.

Materials and Methods

Study Population

The persons in this study were participants in a survey conducted in 1994 by the Centers for Disease Control and Prevention (CDC). The primary objective of the survey was to assess the risk for hantavirus infections in persons whose occupations expose them to rodents. Participation in the survey was voluntary and entailed completion of a self-administered questionnaire and donation of a small volume of venous blood. Most of the 995 participants were enrolled at 1 of the following: American Society of Mammalogists meeting (Washington, DC, 1994), Wildlife Disease Association meeting (Pacific Grove, California, 1994), Southwestern Association of Naturalists meeting (Emporia, Kansas, 1994), Wildlife Society meeting (Wenatchee, Washington, 1994), 16th Vertebrate Pest Conference (Santa Clara, California, 1994), and Colorado Pest Control Meeting (Denver, Colorado, 1994). The other participants mailed their completed questionnaires and serum samples directly to CDC.

The questionnaire included detailed questions about previous exposure to rodents, use of personal protective equipment to minimize exposure to rodent excretions and secretions, and any previous occurrence of a severe febrile illness that included shortness of breath. The lifetime number of rodents handled by a person was measured categorically: I (1–99), II (100–499), III (500–999), IV (1,000–9,999), V (10,000–49,999), and VI (≥50,000). Use of gloves, protective masks equipped with high efficiency particulate air (HEPA) filters, and protective eyewear also was measured categorically: always (>90% of the time), usually (50%–90% of the time), sometimes (10%–49% of the time), seldom (<10% of the time), or never.

This study was restricted to the 757 participants in the CDC survey who had a history of exposure to rodents in North America and a history of occupational exposure to deer mice, white-footed mice, California mice, woodrats (*Neotoma* spp.), other neotomine rodents, cotton rats (*Sigmodon* spp.), oryzomyine rodents (*Oryzomys* spp. or *Oligoryzomys* spp.), or other sigmodontine rodents. Of the persons included in the study, 699 had worked with rodents only in North America. The 58 others had worked with rodents in North America and in South America. The geographic distribution of exposure to rodents in North America was Canada (n = 36), Alaska (n = 8), the contiguous United States or District of Columbia (n = 726, Table 1), Mexico (n = 91), Guatemala (n = 8), Belize (n = 3), Honduras (n = 3), Costa Rica (n = 21), Nicaragua (n = 4), and Panama (n = 8). Of the persons included in the study, 468 (61.8%) had worked with rodents in >1 state within the contiguous United States.

Persons included in the study had worked with rodents from 1 month to 65 years (mean 12.5 years). The total number of rodents handled by any 1 person ranged from category I (1–99) to category VI (≥50,000); the median was IV (1,000–9,999). Of the 757 persons in the study, 751 (99.2%) had handled deer mice, white-footed mice, cotton rats, oryzomyine rodents, California mice, or woodrats (Table 2).

HPS was first recognized as a clinical entity in 1993 in the southwestern United States (*20*). From March 1, 1993, through September 19, 2006, a total of 453 laboratory-confirmed HPS cases were reported to CDC from the contiguous United States (www.cdc.gov/ncidod/diseases/hanta/hps/noframes/epislides/episl7.htm). Of these, 259 (57.2%) were reported from 6 states in the southwestern United States: Colorado (n = 51), New Mexico (n = 71), Utah (n = 25), Arizona (n = 49), Nevada (n = 18), and California (n = 45). SNV is the only virus known to cause HPS in these 6 states. In this study, 387 (51.1%) persons had worked with rodents in Colorado (n = 124), New Mexico (n = 111), Utah (n = 65), Arizona (n = 90), Nevada (n = 33), or California (n = 169) and had handled deer mice. The total number of deer mice handled by persons in this group ranged from I (1–99) to VI (≥50,000); the median was II (100–499).

The geographic range of BAYV includes Georgia, Louisiana, and Texas (*5,15–17*), BCCV has been found only in Florida (*4*), and the geographic range of NYV includes New York, Pennsylvania, and Rhode Island (*3,21,22*). In this study, 22 persons had worked with rodents in Georgia (n = 1), Louisiana (n = 2), or Texas (n = 20) and handled oryzomyine rodents. The total number of oryzomyine rodents handled by persons in this group ranged from I (1–99) to IV (1,000–9,999); the median was I (1–99). Fourteen persons had worked with rodents in Florida and handled cotton rats. The total number of cotton rats handled by persons in this group ranged from I (1–99) to IV (1,000–9,999); the median was II

Table 1. Occupational exposure of 726 persons to sigmodontine or neotomine rodents within the contiguous United States

State	No. persons exposed*
Alabama	16†
Arkansas	33
Arizona	92†‡
California	178†‡
Colorado	128†‡
Connecticut	19
Delaware	1
Florida	17†‡
Georgia	15
Idaho	21†
Illinois	38†
Indiana	26†
Iowa	17†
Kansas	90†
Kentucky	6
Louisiana	11†
Maine	21
Massachusetts	17
Maryland	27
Michigan	38
Minnesota	21†
Mississippi	6
Missouri	36
Montana	21†
Nebraska	25†
Nevada	39†
New Hampshire	6
New Jersey	9
New Mexico	118†‡
New York	46†
North Carolina	15†
North Dakota	9†
Ohio	25
Oklahoma	41†‡
Oregon	45†
Pennsylvania	45†
Rhode Island	5
South Carolina	16
South Dakota	11†
Tennessee	23
Texas	112†‡
Utah	67†‡
Vermont	6†
Virginia	49†
Washington	73†
West Virginia	11†
Wisconsin	22†
Wyoming	41†
District of Columbia	2

*No. persons who worked with rodents in the state or in the District of Columbia. Of the persons in the study, 468 had worked with rodents in >1 state within the contiguous United States.
†States that have reported ≥1 case of hantavirus pulmonary syndrome to the Centers for Disease Control and Prevention through September 19, 2006 (see www.cdc.gov/ncidod/diseases/hanta/hps/noframes/epislides/episl7.htm).
‡States in which neotomine or sigmodontine rodents are known to be naturally associated with Bear Canyon virus, Tamiami virus, or Whitewater Arroyo virus.

(100–499). Eighty-one persons had worked with rodents in New York (n = 45), Pennsylvania (n = 42), or Rhode Island (n = 5) and handled white-footed mice. The total number of white-footed mice handled by persons in this group ranged from I (1–99) to V (10,000–49,999); the median was II (100–499).

BCNV virus has been found only in California (*18*) and TAMV only in Florida (*14*); the geographic range of WWAV and other arenaviruses naturally associated with woodrats (*Neotoma* spp.) includes Arizona, California, Colorado, New Mexico, Oklahoma, Utah, and Texas (*19,23–27*). In this study, 31 persons had worked with rodents in California and handled California mice (*P. californicus*). The total number of California mice handled by persons in this group ranged from I (1–99) to III (500–999); the median was I (1–99). As indicated previously, 14 persons had worked with rodents in Florida and handled cotton rats. Three hundred and thirty-three persons had worked with rodents in Arizona (n = 87), California (n = 130), Colorado (n = 76), New Mexico (n = 101), Oklahoma (n = 40), Utah (n = 59), or Texas (n = 101) and handled woodrats. The total number of woodrats handled by persons in this group ranged from I (1–99) to V (10,000–49,999); the median was I (1–99).

Lymphocytic choriomeningitis virus (LCMV) is the only Old World arenavirus species that is enzootic in North America. The house mouse (*Mus musculus*) is a member of the subfamily Murinae, family Muridae (*11*) and the principal host of LCMV. In this study, 526 (69.5%) persons had worked with house mice. The total number of house mice handled by persons in this group ranged from I (1–99) to VI ($\geq$50,000); the median was I (1–99).

Of the 757 persons in this study, 735 (97.1%) had worked with rodents before the discovery of HPS in 1993; during that time, 504 (68.6%) of them never or infrequently wore personal protective equipment (gloves, a protective mask equipped with HEPA filters, and protective eyewear) when handling rodents. In contrast, only 267 (36.3%) of these 735 persons never or infrequently wore personal protective equipment when handling rodents after the discovery of HPS. Use of personal protective equipment by the other persons in the study both before and after the discovery of HPS depended on the type of equipment.

All unique identifying information was removed from the serum samples before they were tested for antibodies. Furthermore, all unique identifying information was removed from the computer (electronic) records before analysis of the demographic and serologic data.

Antibody Assays

We tested the serum samples for immunoglobulin G (IgG) against SNV, WWAV, GTOV, and LCMV by using ELISA, as described (*24,28*). The SNV antigen was an

Table 2. Work-related exposure of 757 persons to neotomine and sigmodontine rodents

No. persons*	Rodent†	Exposure‡	
		Range	Median
695	Deer mouse (*Peromyscus maniculatus*)	I–VI	II
487	White-footed mouse (*P. leucopus*)	I–VI	II
34	California mouse (*P. californicus*)	I–III	I
456	Woodrat (*Neotoma* spp.)	I–V	I
392	Other neotomine rodents	I–IV	I
511	Cotton rat (*Sigmodon* spp.)	I–VI	I
51	Oryzomyine rodents	I–IV	I

*No. persons who self-reported occupational exposure to rodents.
†Other neotomine rodents included the pygmy mouse (*Baiomys* spp.), Texas mouse (*Peromyscus attwateri*), brush mouse (*P. boylii*), canyon mouse (*P. crinitus*), Zacatecan deer mouse (*P. difficilis*), cactus mouse (*P. eremicus*), Florida mouse (*P. floridanus*), cotton mouse (*P. gossypinus*), northern rock mouse (*P. nasutus*), golden mouse (*P. nuttalis*), white-ankled mouse (*P. pectoralis*), oldfield mouse (*P. polionotus*), pinyon mouse (*P. truei*), and western harvest mouse (*Reithrodontomys megalotis*) and other harvest mice (*Reithrodontomys* spp.). Oryzomyine rodents included the marsh rice rat (*Oryzomys palustris*), other oryzomine rodents (*Oryzomys* spp.), and oligoryzomine rodents (*Oligoryzomys* spp.).
‡The number of rodents handled by a study subject was categorized as follows: I, 1–99; II, 100–499; III, 500–999; IV, 1,000–9,999; V, 10,000–49,999; VI, ≥50,000.

Escherichia coli–expressed recombinant SNV nucleocapsid protein that is highly cross-reactive with other neotomine rodent-associated hantaviruses and with sigmodontine rodent-associated hantaviruses in the ELISA used in this study (T.G. Ksiazek, unpub. data). The control (comparison) antigen for the SNV IgG ELISA was an *E. coli*–expressed recombinant protein that is antigenically unrelated to the SNV nucleocapsid protein. The arenavirus antigens were detergent lysates of Vero E6 cells infected with WWAV strain AV 9310135, GTOV strain INH-95551, or LCMV strain Armstrong. WWAV is highly cross-reactive with BCNV and TAMV in the ELISA used in this study (M.L. Milazzo, unpub. data). Collectively, WWAV, GTOV, and LCMV represent the 3 major antigen groups in the family *Arenaviridae*, as defined by ELISA (*19*). The control antigens for the arenavirus IgG assays were detergent lysates of uninfected Vero E6 cells. The working concentrations of the SNV, GTOV, and LCMV antigens and the corresponding control antigens were determined by checkerboard titration against convalescent-phase serum samples from humans infected with SNV, GTOV, and LCMV, respectively. The working concentrations of the WWAV antigen and the corresponding control antigen were determined by checkerboard titration against a mouse ascitic fluid against WWAV strain AV 9310135. Serial 4-fold dilutions (from 1:100 through 1:6,400) of each serum sample were tested against the 4 test antigens and 4 control antigens. Antibody bound to antigen was detected by using a goat anti-human IgG (gamma chain–specific) peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical densities (OD) at 405 nm (reference = 490 nm) were measured with a Dynex MRX II microplate reader (Dynatech Industries, Inc., McLean, VA, USA). The adjusted OD (AOD) of a serum-antigen reaction was the OD of the well coated with the test antigen minus the OD of the well coated with the control antigen. A sample was considered positive if the AOD at 1:100 was ≥0.200, the AOD at 1:400 was ≥0.200, and the sum of the AODs for the series of 4-fold dilutions (from 1:100 through 1:6,400) was ≥0.900. These criteria for positivity were based on the results of previous work with the test antigens and control antigens. The antibody titer of a positive sample was the reciprocal of the highest dilution of that sample for which the AOD was ≥0.200.

Results

Antibodies against SNV were detected in 4 (0.5%) of the 757 persons in the study. Antibody titers were 1,600 in 2 persons and ≥6,400 in the other 2 persons. The total years worked with rodents and the lifetime number of rodents handled by the 4 antibody-positive persons were 9.0–30.0 (mean 21.3) and IV (1,000–9,999) to V (10,000–49,999), respectively. Two of the antibody-positive persons had worked with rodents only within the contiguous United States (specifically Arkansas, Arizona, Colorado, Iowa, Kansas, Michigan, Oklahoma, South Carolina, and/or Texas), 1 had worked with rodents in Arizona, Colorado, New Mexico, Utah, Texas, and Mexico, and 1 had worked with rodents in Michigan, Pennsylvania, Mexico, Costa Rica, and Argentina. All 4 antibody-positive persons had handled deer mice, white-footed mice, other neotomine rodents, cotton rats, and other sigmodontine rodents. Those who had worked in South Carolina or Argentina also had handled oryzomyine rodents. All 4 antibody-positive persons reported that they had never worn a protective mask or protective eyewear when handling rodents before the discovery of HPS. One reported having been hospitalized for an illness characterized by fever, headache, and severe shortness of breath (symptoms suggestive of HPS). This person had worked with rodents only within the contiguous United States.

Antibodies against WWAV or GTOV were detected in 2 (0.3%) of the 757 persons in the study. Antibodies against WWAV (antibody titer = 1,600) but not GTOV were detected in a person who had worked with rodents in Texas and Wisconsin and handled woodrats, other

neotomine rodents, and sigmodontine rodents. Antibodies against GTOV (antibody titer = 1,600) but not WWAV were detected in a person who had worked with rodents in Pennsylvania, Utah, and Wyoming and handled white-footed mice, other neotomine rodents, cotton rats, and other sigmodontine rodents. The lifetime number of rodents handled by the 2 antibody-positive persons were III (500–999) and IV (1,000–9,999), respectively. Both reported that they had never worn a protective mask or protective eyewear when handling rodents before the discovery of HPS. Antibodies against LCMV were found in none of the 757 persons in this study.

Discussion

Previously published studies found no antibodies against SNV in 583 persons who worked in Arizona or New Mexico in occupations that potentially exposed them to rodents or rodent droppings (*29,30*) and no antibodies against SNV or WWAV in 72 persons in California whose occupations entailed close physical contact with rodents (*31*). Limited seroprevalence studies found antibodies against LCMV in up to 5.1% of healthy persons in the United States (*32,33*). If one discounts fatal infections and assumes that IgG against SNV and other hantaviruses is measurable many years after recovery from infection, the results of this study indicate that the risk for infection with hantaviruses usually is low in persons whose occupations entail close physical contact with neotomine rodents or sigmodontine rodents in North America. Similarly, the study results indicate that the risk for infection with arenaviruses usually is low in persons whose occupations entail close physical contact with neotomine rodents or sigmodontine rodents in North America

Some hantaviruses and arenaviruses appear to be restricted to small areas within the geographic ranges of the rodent species that serve as their natural reservoirs. For example, BCCV and TAMV have been found only in southern Florida (*4,14*), yet the geographic range of *S. hispidus* extends from Arizona, Nebraska, and Virginia through northeastern Mexico (*11*). Furthermore, the prevalence of infected rodents can vary widely even in a small area (*23,34*). Thus, the low prevalence of antibodies against SNV and against the arenaviruses included in this study could be because few of the rodents handled by the 757 persons in the study were infected with a hantavirus or arenavirus. Other explanations for the low prevalence of antibodies against SNV, WWAV, GTOV, and LCMV in this study are because the circumstances under which or the manner in which the rodents were handled did not favor rodent-to-human virus transmission or because tissues, secretions, and excretions from infected rodents are not highly infectious to humans.

Antibodies against SNV were detected in 3 (0.8%) of the 387 persons in this study who had worked with rodents in Colorado, New Mexico, Utah, Arizona, Nevada, or California and who had handled deer mice. Antibodies to SNV also were detected in 1 (1.2%) of the 81 persons who had worked in New York, Pennsylvania, or Rhode Island and who had handled white-footed mice. The antibodies against SNV in the 3 antibody-positive persons who had worked in the southwestern United States could be a consequence of infection with SNV. The antibodies against SNV in the person who had worked in Pennsylvania could be a result of infection with NYV.

Of the 453 laboratory-confirmed HPS cases mentioned previously, 160 (35.3%) were fatal. Together, the high case-fatality ratio of HPS in North America, the lack of a vaccine against HPS, and the lack of a specific therapy for HPS should motivate persons to minimize their risk for infection while working in the field, classroom, or laboratory with rodents potentially infected with hantaviruses, especially those viruses known to cause HPS. Published guidelines for safely working with rodents potentially infected with hantaviruses include using protective gloves, respirators fitted with HEPA filters, and protective eyewear (*35*). None of the 4 persons in the study who were antibody-positive against SNV had worn gloves, masks, or protective eyewear when handling rodents before the discovery of HPS.

The use of personal protective equipment in the field may seem cumbersome. However, 2 recent HPS cases, 1 fatal, underscore the need to use appropriate personal protective equipment and follow recommended safety procedures when working with rodents potentially infected with hantaviruses that have been causally associated with HPS. The fatal case was in a graduate student who was studying the effects of forest management practices on small mammal populations in West Virginia (*36*). The nonfatal case was in a field technician who was trapping rodents as part of a forest health study in California (*37*). HPS has been reported in other persons whose occupations entailed close physical contact with wild rodents (*38,39*).

The person in this study who was antibody-positive against WWAV had worked with rodents in Texas and handled woodrats. Antibodies against WWAV strain AV 9310135 have been found in southern plains woodrats (*Neotoma micropus*) captured in western Texas and in northern Texas (M.L. Milazzo, unpub. data), and arenaviruses antigenically closely related to WWAV strain AV 9310135 have been isolated from southern plains woodrats captured in southern Texas (*27,40*). Thus, the antibodies against WWAV in this person could be a result of an arenavirus infection acquired from a woodrat captured in Texas.

When examined by antibody-antigen binding assays such as the ELISA, GTOV is distinct from the 3 North American arenaviruses and highly cross-reactive with JUNV, MACV, and SABV (*19*). Thus, the antibodies against GTOV in the person in this study could be a result of an arenavirus infection acquired while traveling in South America. Alternatively, the antibodies could be a result of infection with a North American arenavirus that is antigenically more closely related to GTOV than to BCNV, TAMV, or WWAV.

Recently, antibodies against GTOV but not WWAV or LCMV were detected in 3 peromyscine rodents (*Peromyscus* sp.) captured in southern Mexico (M.L. Milazzo, unpub. data). The antibodies against GTOV in these 3 rodents are the first evidence that an arenavirus antigenically distinct from BCNV, TAMV, WWAV, and LCMV exists in North America and support the idea that the infection in the antibody-positive person in this study was a result of an arenavirus infection acquired in North America.

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This study was approved by the Committee for the Protection of Human Subjects, Centers for Disease Control and Prevention. Written informed consent was obtained from all participants in accordance with Title 45, Part 46 of the Code of Federal Regulations.

Dr Fulhorst is an associate professor at University of Texas Medical Branch. His research interests include the epidemiology and ecology of rodentborne hantaviruses and arenaviruses native to the Americas.

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Influenza (H5N1) Viruses in Poultry, Russian Federation, 2005–2006

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We studied 7 influenza (H5N1) viruses isolated from poultry in western Siberia and the European part of the Russian Federation during July 2005–February 2006. Full genome sequences showed high homology to Qinghai-like influenza (H5N1) viruses. Phylogenetic analysis not only showed a close genetic relationship between the H5N1 strains isolated from poultry and wild migratory waterfowls but also suggested genetic reassortment among the analyzed isolates. Analysis of deduced amino acid sequences of the M2 and neuraminidase proteins showed that all isolates are potentially sensitive to currently available antiviral drugs. Pathogenicity testing showed that all studied viruses were highly pathogenic in chickens; for 3 isolates tested in mice and 2 tested in ferrets, pathogenicity was heterogeneous. Pathogenicity in mammalian models was generally correlated with Lys at residue 627 of polymerase basic protein 2.

Highly pathogenic avian influenza viruses of the H5N1 subtype are zoonotic agents that present a continuing threat to animal and human health. Before 2003, influenza (H5N1) was endemic in poultry in southern China (*1*,*2*) and occasionally caused severe disease in humans (*2*–*4*). The situation changed in late 2003–2004, when the expanded geographic range of subtype H5N1 resulted in unprecedented epizootics in poultry and new human cases in eastern and southeastern Asia (*5*,*6*). The serious pandemic threat associated with these events intensified the urgency of global pandemic preparedness for influenza (H5N1) (*6*).

In May 2005, an outbreak of influenza (H5N1) in migratory waterfowl was observed at Qinghai Lake in western China (*7*,*8*). Possible spread to Europe by overlapping flyways was a concern (*7*). During 2005–2006, influenza (H5N1) spread throughout Mongolia, Kazakhstan, the Siberian and European part of Russia, Ukraine, countries of the European Union, Africa, and the Middle East (*9*). The first human cases of influenza (H5N1) outside Southeast Asia were reported in 2006 in Azerbaijan, Djibouti, Egypt, Iraq, and Turkey (*9*).

The first influenza (H5N1) epizootics in the Russian Federation occurred at the end of July 2005 in the Novosibirsk region (western Siberia) (*10*–*12*), which borders Kazakhstan and is near Mongolia and northwest China. The outbreaks occurred in backyard poultry flocks and small farms near bodies of water where wild birds presumably stop to feed during seasonal migration. Several studies have reported high sequence homology of all gene segments of influenza (H5N1) isolated in 2005 from wild birds (grebe in Novosibirsk Region and mute swan in Astrakhan Region) and from poultry; these studies also examined the relations of outbreaks in poultry to migrations of wild birds (*12*–*14*). However, evidence that wild migratory birds played a role in the spread of influenza (H5N1) was not conclusive.

After the first outbreaks, influenza (H5N1) spread rapidly westward through Russia; several outbreaks in poultry were reported in western Siberia and south and central

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European regions of the Russian Federation in late 2005 and early 2006 (Figure 1). At the beginning of March 2006, the influenza (H5N1) epizootics had resulted in the death or slaughter of >1 million poultry in 13 subjects of the Russian Federation. Most of the outbreaks were similar to those first reported in western Siberia (*12*). No human cases of influenza (H5N1) were associated with these outbreaks.

Russia lies between eastern Asia and Europe. Surveillance of influenza (H5N1) in poultry and wild waterfowl in these regions could provide unique information about the variety of viruses, their evolution, and possible changes. We characterized 7 influenza (H5N1) viruses isolated from poultry in western Siberia and the European part of the Russian Federation during July 2005–February 2006 (Figure 1, Table 1). Full genome sequences were determined and analyzed, and pathogenicity was determined by inoculation of chickens, mice, and ferrets.

Materials and Methods

Virus Isolation and Initial Characterization

From July 2005 through March 2006, ≈300 field samples were collected by a research team from the Federal State Research Institute (FSRI) Research Center for Virology and Biotechnology "Vector" (FSRI "Vector") during 12 outbreaks of influenza (H5N1) in poultry (9 in backyard poultry and 3 at poultry farms) and 1 natural epizootic (mute swans in the Astrakhan Region), ([*14*], Figure 1). Cloacal and tracheal swabs were collected from dead and sick poultry, and internal organs were collected from wild waterfowl found dead near the sites of the outbreaks. Specimen processing and virus isolation were performed at the FSRI "Vector," a certified Biosafety Level 3 laboratory. Aliquots of field samples (0.1 mL of swab media or of 10% [w/v] organ homogenates) were injected into the allantoic cavity of 10-day-old, specific-pathogen–free embryonated chicken eggs. After incubation at 35ºC for 48 h, the allantoic fluid was harvested, and virus was titrated by hemagglutination test with a 0.5% suspension of chicken red blood cells.

Influenza virus was isolated from 60 samples (20%). The subtype of the hemagglutinin (HA) was determined by hemagglutination inhibition test with 0.5% chicken red blood cells and a panel of antiserum against avian HAs (*15*). The neuraminidase (NA) subtype was determined by NA inhibition assay with a panel of anti-NA serum (*15*). All tested viruses belonged to the H5N1 subtype. Because some samples were duplicated (collected from the same backyard or farm or from birds of the same species with similar disease signs), 36 influenza (H5N1) isolates were deposited at the repository of FSRI "Vector." Because most cases of human H5N1 infection are related to direct contact with infected poultry, we chose 7 poultry isolates for further characterization (Table 1, Figure 1).

Virus-containing allantoic fluid was stored at –80ºC. The infectivity of stock viruses was determined in 10-day-old embryonated chicken eggs by the method of Reed and Muench (*16*) and expressed as the $\log_{10}$ 50% egg infective dose (EID_{50})/mL of allantoic fluid.

Pathogenicity Tests in Chickens

The intravenous virus pathogenicity index (IVPI) of the 7 influenza (H5N1) isolates (Table 1) was determined as described by Capua and Mutinelli (*17*). Infective allantoic fluid was diluted 1:10 in sterile phosphate-buffered saline (PBS), and 0.1 mL was injected intravenously into each of ten 6-week-old, specific-pathogen–free chickens. The chickens were examined for clinical signs of disease once a day for 10 days. Pathogenicity was scored as 0 (no signs of illness), 1 (signs of illness), 2 (signs of severe illness), or 3 (death within 24 h of inoculation). The pathogenicity index was then calculated as the mean score per bird per observation. An index of 3 indicated that all birds died within 24 h; an index of 0 meant that no bird showed signs of illness during the 10-day observation period.

Figure 1. Spread of influenza (H5N1) in the Russian Federation, 2005–2006. Locations and dates of outbreaks of disease in poultry and wild waterfowl (1 outbreak in mute swans, Astrakhan region, Nov 2005) investigated by Federal State Research Institute Research Center for Virology and Biotechnology "Vector."

Table 1. Influenza (H5N1) viruses, Russian Federation, 2005–2006

Virus	Date of isolation	Type of operation	Specimen used for isolation	IVPI for chickens*
A/chicken/Suzdalka/06/2005	Jul 22, 2005	Backyard flock	Dead chicken (spleen)	3.0
A/goose/Suzdalka/10/2005	Jul 22, 2005	Backyard flock	Dead goose (lungs)	3.0
A/turkey/Suzdalka/12/2005	Jul 22, 2005	Backyard flock	Sick turkey (cloacal swabs)	3.0
A/chicken/Omsk/14/2005	Aug 12, 2005	Backyard flock	Sick chicken (cloacal swabs)	2.6
A/chicken/Tula/4/2005	Oct 5, 2005	Backyard flock	Dead chicken (lungs)	2.8
A/goose/Krasnoozerskoye/627/2005	Oct 17, 2005	Goose farm (~5,000 geese)	Sick goose (cloacal swabs)	3.0
A/chicken/Krasnodar/123/2006	Feb 12, 2006	Chicken farm (~300,000 chickens)	Dead chicken (lungs)	3.0

*IVPI, intravenous pathogenicity index.

PCR Amplification and Sequencing

Viral RNA was isolated from virus-containing allantoic fluid with the RNeasy Mini kit (QIAGEN, Valencia, CA, USA) as specified by the manufacturer. Uni12 primer was used for reverse transcription. PCR was performed with a set of primers specific for each gene segment of influenza A virus (*18*). PCR products were purified with the QIAquick PCR purification or QIAquick gel extraction kit (QIAGEN). Sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St Jude Children's Research Hospital. DNA sequences were completed by using the Lasergene sequence analysis software package (DNAStar, Madison, WI, USA). The nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers EF205154–EF205209.

Phylogenetic Analysis

For phylogenetic analysis, we chose 2 gene segments encoding the main surface antigens (HA, nt 77–1704; NA, nt 21–1349) and 2 conserved genes encoding internal proteins potentially associated with virulence in mammalian species (polymerase basic protein 2 [PB2], nt 58–2304; nonstructural protein [NS], nt 27–855). To identify related reference viruses, we performed nucleotide BLAST analysis of each virus sequence; sequences were uploaded from the Influenza Sequence Database at Los Alamos National Laboratory (www.flu.lanl.gov) (*19*). Sequences were compared by ClustalW alignment algorithm by using BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/bioedit.html). To estimate phylogenetic relationships, we analyzed nucleotide sequences by the neighbor-joining method with 100 bootstraps by using PHYLIP (the PHYLogeny Inference Package) version 3.65 (http://evolution.gs.washington.edu/phylip.html).

Pathogenicity Tests in Mice and Ferrets

The 50% mouse lethal dose (MLD_{50}), 50% mouse infective dose (MID_{50}), and virus titers and organ tropism of 3 influenza (H5N1) isolates were determined for 8-week-old female BALB/c mice. To determine MLD_{50} and MID_{50}, we anesthetized groups of 4 mice with diethyl ether (inhalation) and inoculated them intranasally with 50 µL of 10-fold serial dilutions of allantoic fluid in PBS. The mice were observed for death (MLD_{50}) for 15 days, or they were killed on day 5 after challenge and tested for pulmonary virus by inoculation of 10-day-old embryonated chicken eggs (MID_{50}). MLD_{50} and MID_{50} were calculated by the method of Reed and Muench (*16*). To determine organ tropism, groups of 3 mice were inoculated intranasally with 50 µL PBS containing 10^3 EID_{50} of virus. In our experience, this viral dose allows the distinction of specific organ tropism among viruses with different pathogenicity patterns in mice. After 5 days, mice were killed and lungs, brain, spleen, liver, and kidneys were collected. Organ homogenates (10% in PBS) were injected into 10-day-old embryonated chicken eggs to detect and titrate virus. Titers were expressed as $\log_{10}$ EID_{50}/0.1 mg of organ tissue.

The pathogenicity and replication of 2 influenza (H5N1) isolates were characterized in a ferret model. Groups of 3 male 8-month-old outbred ferrets were anesthetized by inhalation of diethyl ether and inoculated intranasally with 10^6 EID_{50} of virus in 0.5 mL PBS. This inoculation dose is commonly used to characterize the pathogenicity of influenza (H5N1) in this animal model (*20*,*21*). Ferrets were observed for disease signs for 14 days after inoculation; rectal temperature and body weight were measured daily. Nasal washes were collected on days 1–12 as described (*20*,*21*). Virus titers were determined in 10-day-old embryonated chicken eggs and expressed as $\log_{10}$ EID_{50}/mL of nasal wash fluid.

Results

Pathogenicity in Chickens

Groups of 10 chickens were inoculated with the 7 influenza (H5N1) viruses to determine the IVPI index (*17*). Five of the viruses resulted in the deaths of all 10 chickens during the first 24 hours and therefore had an IVPI index of 3 (Table 1). Two isolates, A/chicken/Omsk/14/2005 and A/chicken/Tula/4/2005 viruses, killed all 10 chickens within 48 hours and had IVPI scores of 2.6 and 2.8, respectively (Table 1). All 7 viruses were highly pathogenic (*17*).

Genetic Characterization

Sequence analysis of PCR products from the 7 isolates demonstrated ≥99% nucleotide identity with the A/bar-headed goose/Qinghai/0510/2005 (H5N1) virus (*7*,*22*) in all gene segments except the NS gene (>98% nucleotide identity). Therefore, all viruses chosen for this study were Qinghai-like influenza (H5N1).

We performed phylogenetic analysis of the HA, NA, PB2, and NS genes of the 7 influenza (H5N1) isolates with sequences uploaded from the Influenza Sequence Database (*19*) (Figure 2). All studied isolates belonged to subclade 2 of clade 2 of H5 HA (Figure 2A) (*23*). Qinghai-like influenza (H5N1) isolated in Asia, Europe, Africa, and the Middle East are closely related in this HA clade. The goose H5N1 isolate from the October 2005 outbreak in the Novosibirsk Region was phylogenetically closely related to virus isolated from a mute swan (*Cygnus olor*) in November 2005 in the southern European part of Russia (Figure 1) (*14*), and virus isolated from a chicken in February 2006 was phylogenetically closely related to virus isolated in 2006 from a swan in Iran (Table 1, Figure 2A).

The NA, PB2, and NS genes of the isolates (Figure 2B, C, D) were phylogenetically related to those of Qinghai-like viruses. The NA gene of A/chicken/

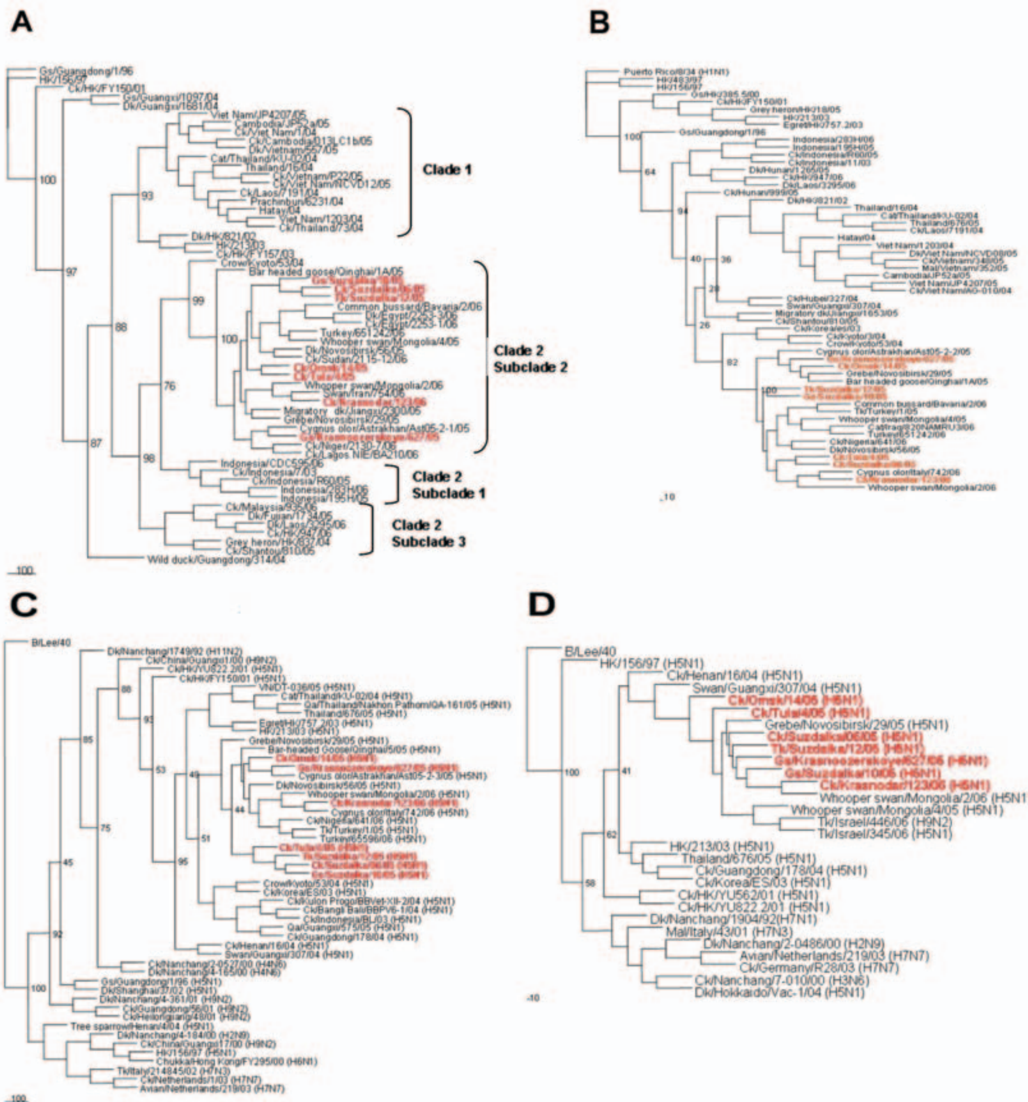

Figure 2. Phylogenetic relationships of the hemagglutinin (HA) (A), neuraminidase (NA) (B), polymerase basic protein 2 (PB2) (C), and nonstructural (NS) (D) genes of the 7 influenza (H5N1) viruses. Nucleotide sequences were analyzed by using the neighbor-joining method with 100 bootstraps. The HA phylogenetic tree was rooted to the HA gene of A/goose/Guangdong/1/96 (H5N1) virus. The NA phylogenetic tree was rooted to the NA gene of A/Puerto Rico/8/34 (H1N1) virus. The PB2 and NS trees were rooted to the PB2 and NS genes of B/Lee/40 virus. For a larger representation of the phylogenetic trees, see online Figure 2, available from www.cdc.gov/EID/content/13/4/539-G2.htm

Krasnodar/123/2006 virus was closely related to that of A/Cygnus olor/Italy/742/2006 (Figure 2B); the PB2 genes of A/goose/Krasnoozerskoye/627/2005 and A/chicken/Krasnodar/123/2006 were closely related to those of A/Cygnus olor/Astrakhan/Ast05-2-3/2005 and A/Cygnus olor/Italy/742/2006, respectively (Figure 2C), and the NS gene of A/chicken/Krasnodar/123/2006 was related to that of A/whooper swan/Mongolia/2/2006 virus (Figure 2D). The phylogenetic distribution of the studied isolates differed for each of these genes and for HA. These findings suggested that reassortment events had occurred among the analyzed isolates within the group of Qinghai-like influenza (H5N1) viruses.

The phylogenetic analysis data demonstrated that influenza (H5N1) viruses in poultry and in wild migratory waterfowl are related. These phylogenetic relationships, together with the temporal and geographic correspondence of the poultry outbreaks and the wild waterfowl migratory patterns (*13*,*14*,*24*), support the involvement of wild birds in the perpetuation and spread of Qinghai-like influenza (H5N1). However, until other possible routes of viral dissemination are analyzed and excluded, whether wild migratory birds are the primary source of influenza (H5N1) virus transmission and infection of poultry cannot be conclusively determined.

Potential Sensitivity to Antiviral Drugs

The H5N1 strains recently isolated in Southeast Asia are resistant to amantadine and rimantadine (*5*,*25*), which target the M2 ion channel protein of influenza A viruses. Influenza (H5N1) viruses resistant to the NA inhibitor oseltamivir have been isolated from oseltamivir-treated patients (*26*,*27*). To determine the potential sensitivity of the studied viruses to these antiviral drugs, we analyzed the amino acid sequences of their M2 and NA proteins.

Amantadine-resistant influenza A variants carry amino acid substitutions at residues 26, 27, 30, 31, or 34 of the M2 protein (*28*,*29*). Our sequence analysis did not show any substitutions at these residues. Therefore, all 7 isolates are potentially sensitive to this class of antiviral drugs.

Amino acid residues 119, 274, 292, and 294 of the NA protein (numbered according to NA of the N2 subtype) are crucial for sensitivity to NA inhibitors (*30*); the substitutions $H_{274}\rightarrow Y$ and $N_{294}\rightarrow S$ were reported to confer resistance to oseltamivir in clinical influenza (H5N1) isolates (*26*,*27*). No amino acid substitutions were observed at the conserved residues in the NA protein of the studied viruses, which suggests that they are sensitive to NA inhibitors.

Molecular Correlates of Pathogenicity in Mammals

The receptor specificity of the HA protein could be crucial for efficient replication and spread of a pandemic strain (*31*). In the HA molecules of all 7 viruses, amino acid residues relevant to receptor binding retained the 2,3-NeuAcGal linkages predicted to confer affinity for avian cell surface receptors (*5*,*32*). A multibasic cleavage site in the H5N1 HA is essential for lethal infection in a mouse model (*33*). We found all 7 isolates contained the multibasic amino acid motif PQGERRRKKR/GL (characteristic of Qinghai-like viruses) at their HA cleavage sites.

Residues in the viral polymerase complex (PB1, PB2, and polymerase acidic protein [PA]) may be associated with the adaptation and virulence of avian viruses in mammals (*33–36*). Sequence analysis of these proteins revealed Lys_{627} in the PB2 of 3 studied isolates: A/chicken/Omsk/14/2005, A/goose/Krasnoozerskoye/627/2005, and A/chicken/Krasnodar/123/2006. In mice, influenza (H5N1) viruses with Lys_{627} are highly virulent and replicate systemically (*33*). Other residues associated with adaptation and virulence, i.e., residues 701 of PB2 (*34*,*35*), 13 of PB1 (*35*), and 615 of PA (*35*), were those typical of avian viruses with low virulence in mammals.

Analysis of NS1, which may also contribute to the virulence of influenza (H5N1), showed a deletion of 5 amino acids that is similar to that found in genotype-Z influenza (H5N1) viruses and that may contribute to increased expression of tumor necrosis factor-α and interferon-γ–inducible protein 10 (IP-10) in primary human macrophages (*2*). No viruses contained Glu_{92} in the NS1, which is associated with the high virulence of H5N1 subtype in 1997 (*37*,*38*), and all contained the "avian-like" PDZ-domain ligand ESEV (*39*).

Pathogenicity and Replication in Mice and Ferrets

The pathogenicity and organ tropism of the 3 influenza (H5N1) isolates A/turkey/Suzdalka/12/2005, A/goose/Krasnoozerskoye/627/2005, and A/chicken/Krasnodar/123/2006 were characterized in a mouse model (Table 2). Of these viruses (isolated in 2005), 2 had a substitution at residue 627 in PB2 that is associated with pathogenicity in

Table 2. Pathogenicity and replication of influenza (H5N1) viruses in mice*

Virus	Log_{10} EID_{50}/mL	MID_{50}†	MLD_{50}†	Titers in mouse organs‡				
				Lungs	Spleen	Brain	Liver	Kidney
A/goose/Krasnoozerskoye/627/2005	9.2	$10^{2.2}$	$10^{2.3}$	6.1±0.3	1.6±0.5	5.2±0.2	1.6±0.3	2.6±0.2
A/turkey/Suzdalka/12/2005	9.3	$10^{5.3}$	$10^{6.3}$	4.1±0.6	<1	2.3±0.5	<1	<1
A/chicken/Krasnodar/123/2006	8.4	$10^{2.1}$	$10^{2.3}$	4.7±0.3	<1	<1	<1	<1

*EID_{50}, 50% egg infectious dose; MID_{50}, 50% mouse infectious dose; MLD_{50}, 50% mouse lethal dose.
† MID_{50} and MLD_{50} are expressed as number of EID_{50}. Values are the means of 2 titration experiments.
‡Mean ± SD from 3 mice, expressed as log_{10} EID_{50}/0.1 mg of organ tissue.

mice (*33*); we chose A/chicken/Krasnodar/123/2006 because it was the only virus isolated in 2006. Isolate A/goose/Krasnoozerskoye/627/2005 was highly pathogenic and replicated systemically in mice. A/chicken/Krasnodar/123/2006 virus had MID_{50} and MLD_{50} values similar to those of A/goose/Krasnoozerskoye/627/2005 virus but was recovered only from mouse lungs, where it replicated to lower titers (Table 2). Virus A/turkey/Suzdalka/12/2005 replicated efficiently in the brains and lungs of mice, although the MID_{50} and MLD_{50} values of this virus indicated low pathogenicity. In general, these data agreed with the results of sequence analysis: the PB2 proteins of both highly pathogenic viruses contained Lys_{627}, which confers high virulence in mice (*33*). However, which molecular determinants restricted the replication of A/chicken/Krasnodar/123/2006 virus to the lungs remains to be determined.

Because the 2 isolates from 2005 (A/turkey/Suzdalka/12/2005 and A/goose/Krasnoozerskoye/627/2005) showed distinct pathogenicity in mice, they were further characterized in the ferret model. The results of pathogenicity studies in ferrets were consistent with those in mice. A/goose/Krasnoozerskoye/627/2005 caused severe disease accompanied by respiratory and neurologic signs previously described in ferrets inoculated with influenza (H5N1) (*20*,*21*). All inoculated ferrets had fever on days 1–7 after inoculation and had substantial weight loss (as much as 23%, data not shown). This virus replicated at high titers in the upper respiratory tract and was recovered from nasal washes until day 11 after inoculation (Figure 3). A/turkey/Suzdalka/12/2005 virus demonstrated low pathogenicity in ferrets. Temperature elevation was observed on days 1 and 2 after inoculation, but no other disease signs or substantial weight loss were noted (data not shown). The virus replicated at low titers in the upper respiratory tract, reached peak titers on day 1 after inoculation, and was cleared by day 6 (Figure 3). The molecular differences between the A/goose/Krasnoozerskoye/ 627/2005 and A/turkey/Suzdalka/12/2005 viruses are shown in Table 3. The 2 viruses differed most in their polymerase proteins. On the basis of available data about the molecular determinants of pathogenicity of influenza (H5N1) in ferrets (*20*,*21*,*36*), we propose that some of these residues underlie the observed differences in pathogenicity.

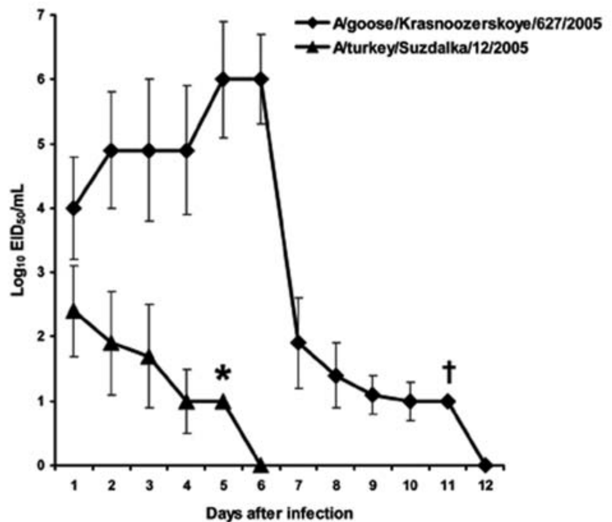

Figure 3. Replication of influenza (H5N1) viruses in ferret upper respiratory tract. Male 8-month-old outbred ferrets were inoculated intranasally with 10^6 50% egg infectious dose (EID_{50}) of virus in 0.5 mL phosphate-buffered saline. Virus titers are the mean ± SD from 3 ferrets, expressed as $\log_{10}$ EID_{50}/mL of nasal wash fluid. *Virus was detected in 2 ferrets. †Virus was detected in 1 ferret.

Discussion

Our findings demonstrate that the influenza (H5N1) viruses isolated from poultry in Russia are Qinghai-like influenza (H5N1) viruses (*22*) and are phylogenetically related to viruses isolated from wild migratory waterfowl (Figure 2). Phylogenetic analysis of these poultry isolates supports the possibility that genetic reassortment had occurred among the Qinghai-like viruses. Kilpatrick and coauthors, in a recent study of the global spread of influenza (H5N1), proposed that influenza (H5N1) viruses were likely introduced into Russia from China by migrating birds and that wild migrating birds play a role in spreading influenza (H5N1) into Europe (*40*). Collectively, our genetic findings, the rapid dissemination

Table 3. Molecular differences between influenza (H5N1) isolates with high and low pathogenicity in mammalian models

Protein*	Position	Virus: A/turkey/ Suzdalka/ 12/2005	Virus: A/goose/ Krasnoozerskoye/ 627/2005
PB2	80	I	T
	473	L	M
	483	M	V
	627	E	K
	666	T	I
PB1	654	S	G
	655	M	I
	744	L	M
PA	377	S	N
	604	R	K
	693	A	V
HA	2	K	E
	8	L	F
	170	N	D
	289	N	S
NA	60	V	L
	266	G	S
NP	389	K	R

*PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein.

of viruses over great distances (Figure 1), and the apparent correspondence between migratory patterns and the sites and timing of poultry outbreaks (*24*) indicate a correlation but do not prove conclusively that wild migrating birds are the primary source of influenza (H5N1) infection of poultry in Russia. Analysis of a greater number of viruses isolated from poultry and wild birds, epidemiologic studies in affected areas, and characterization of other possible human-related modes of virus dissemination and transmission (i.e., trade of poultry or poultry products, spread via rail and motor vehicle routes) might provide confirmatory data.

The studied viruses were highly pathogenic in chickens, but their pathogenicity was heterogeneous in mouse and ferret animal models. The pattern of pathogenicity we observed was generally correlated with known molecular determinants of influenza (H5N1) pathogenicity in mammals.

Influenza (H5N1) outbreaks in poultry in the Novosibirsk Region have caused the deaths of 5,031 birds and the slaughter of 93,620 (a 19% loss) (*12*). In the Russian Federation as a whole, >1 million birds were lost during influenza (H5N1) epizootics from July 2005 through March 2006. Several control measures have been undertaken to prevent the spread of influenza (H5N1) in poultry and potential transmission to humans (*12*). The first is slaughter and disposal of sick poultry and other birds in close contact with them. The second is quarantine of villages and poultry farms where influenza (H5N1) infection is confirmed or suspected. These measures include restriction of the movement of any poultry or poultry products and disinfection of all affected facilities and of any vehicles entering and exiting the area. The third is sanitary and veterinary measures at poultry farms and in backyard flocks in the affected regions to prevent contact of poultry with wild birds and the potential spread of virus by vehicles. The regional spread of influenza (H5N1) and outbreaks at the main poultry production facilities have been halted. No human cases have been reported during or since the 2005–2006 epizootics; therefore, these measures appear to have been effective. February 2006 saw the start of vaccination of poultry at farms and in backyard flocks in the affected areas with inactivated whole-virus influenza (H5N1) vaccines. At present, the effectiveness of the vaccination campaign cannot be assessed.

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Movements of Birds and Avian Influenza from Asia into Alaska

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Asian-origin avian influenza (AI) viruses are spread in part by migratory birds. In Alaska, diverse avian hosts from Asia and the Americas overlap in a region of intercontinental avifaunal mixing. This region is hypothesized to be a zone of Asia-to-America virus transfer because birds there can mingle in waters contaminated by wild-bird–origin AI viruses. Our 7 years of AI virus surveillance among waterfowl and shorebirds in this region (1998–2004; 8,254 samples) showed remarkably low infection rates (0.06%). Our findings suggest an Arctic effect on viral ecology, caused perhaps by low ecosystem productivity and low host densities relative to available water. Combined with a synthesis of avian diversity and abundance, intercontinental host movements, and genetic analyses, our results suggest that the risk and probably the frequency of intercontinental virus transfer in this region are relatively low.

In Alaska, diverse avian hosts from Asia and the Americas overlap in a region of intercontinental avifaunal mixing hypothesized to be an important zone of Asia-to-America virus transfer. Aquatic birds, especially waterfowl and shorebirds, provide a source of influenza viruses for transmission to mammals and poultry (*1–3*). Even without disease, when infected these avian hosts tend to shed high concentrations of virus in their feces (*4–6*). Cross-host infections in wild birds probably occur most frequently when other birds of the same or different species feed, drink, or bathe in waters contaminated by the feces of infected birds. On rare occasions, some of these avian influenza (AI) viruses, generally of low pathogenicity, have crossed species barriers from wild birds to poultry, in which mutations can produce highly pathogenic strains. From poultry, low and high pathogenicity viruses (or genomic segments of these viruses) can be introduced to humans, causing some fatal infections (*7*). This wild-bird reservoir can thus provide the genes for the next pandemic in humans or epizootic in domestic animals and presents an ongoing risk.

The rapid spread of highly pathogenic avian influenza A (H5N1) viruses from Asia across Eurasia (*8,9*) demonstrated how avian vectors can be involved in the distribution of avian and mammalian infections. Key activities for successful global influenza mitigation measures are surveillance, risk assessment, and epidemiologic modeling and prediction of AI virus infection in wild birds (*10,11*). Anthropogenic factors will also affect the evolution and distribution of avian influenza viruses. However, we focused on the natural virus transport system that migratory birds represent in an important high-latitude region with low levels of human presence.

We obtained our baseline data on viruses and vectors by screening wild birds for AI virus in western Alaska, starting in 1998. We focused on western Alaska because of the unparalleled overlap of Old World and New World bird migration systems in this region. To estimate the risk of Asian-origin AI viruses being delivered by migratory birds to North America through Alaska, we evaluated AI virus infection rates, bird movements, and the diversity and degree of intercontinental host overlap.

During the boreal summer, birds come to Alaska to breed from 6 continents: North and South America, Asia, Africa, Australia, and Antarctica. Alaska, thus, has direct, real-time connections with AI virus vectors from much of the world. It is a critical Old World–New World crossover

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zone and almost certainly a region of intercontinental virus transfer. Eurasian birds are common in Alaska during summer. Within 2 of the most important vector groups, waterfowl and shorebirds, at least 43 species regularly found in this region winter primarily in the eastern or southeastern parts of Asia; most are aquatic (online Appendix Table, available from www.cdc.gov/EID/content13/4/547-appT.htm). Additionally, many species are shared with Asia across the Bering Sea (online Appendix Table). This extensive crossover of migratory Old World and New World birds offers excellent potential for virus exchange and transfer to the New World. Outbreaks of avian influenza that have killed persons in southeastern Asia (*7*) and occurrences of highly pathogenic AI (H5N1) infection in migratory birds (*8,9*) highlight the global importance of the Alaska migration system for intercontinental virus transport.

In Alaska, 5 major factors are involved in the natural intercontinental movement of avian-origin viruses: 1) Asian species coming to Alaska; 2) North American migrant birds (Asian-origin migrants that return to North America in autumn) breeding in Asia; 3) individual birds of species that winter on both sides of the Pacific moving between the continents; 4) species that have limited movements or are resident in the region; and 5) water (it has been inferred that live AI virus remains viable in fresh water at high latitudes through the cold northern winters) (*12*).

When we began our study, the role of migratory birds in the transport of highly pathogenic AI was uncertain, but wild birds have been found with these viruses, and with infection several species appear susceptible to severe disease and death (*9,13*). In previously reported cases of infection of wild birds with the highly pathogenic virus, transmission was thought to be from infected chickens, the species in which the shift to increased pathogenicity had originally occurred. Experimental and field studies (*9,14,15*) have identified highly pathogenic AI (H5N1) infection in ducks without clinical disease, which implicates healthy wild birds in transmission. Although in this regard the Asian AI (H5N1) subtype appears unique among highly pathogenic AI viruses, wild birds may be considered as potentially important vectors for strains of low and high pathogenicity.

Methods

Surveillance, Isolation, and Sequencing

We sampled ducks and shorebirds, 2 groups of aquatic birds important as subsistence foods in rural Alaska, widely associated with Alaska waters and common among those species that winter in southeastern Asia (online Appendix Table). We sampled migratory birds that come to North America from southeastern Asian wintering grounds or Asian breeding grounds, as well as North American birds, which mingle with Old World birds on shared breeding and fall staging grounds. Intensive and extensive taxonomic sampling enabled us to obtain the best possible vectorwide prevalence estimates at our sampling sites. Our animal sampling was done according to protocols approved by the Institutional Animal Care and Use Committee.

From May through October, 1998–2004, totals of 7,751 cloacal swabs and 503 fecal samples were collected from waterfowl (Anatidae) and shorebirds (Charadriidae and Scolopacidae), primarily at sites on the Yukon-Kuskokwim Delta, Alaska Peninsula, Seward Peninsula, the North Slope, and the Aleutian Islands. Most samples were obtained after the breeding season (Figure; online Appendix Table). Each of these areas is internationally renowned for its avian diversity and abundance during the migration periods and the boreal summer.

Swabs and fresh fecal samples were placed in sterile medium (brain–heart infusion buffer with 10,000 U/mL penicillin G, 1 mg/mL gentamicin, 20 µg/mL amphotericin B) in the field and cooled before transport to the University of Alaska Museum laboratory. Transportation times were generally short (≈2–14 days), during which time samples were kept cool (mechanically refrigerated or buried above permafrost), frozen at –20°C, or kept on liquid nitrogen. Upon arrival at the laboratory, they were placed in a –70°C freezer. They were then shipped overnight to the Southeast Poultry Research Laboratory in Athens, Georgia, USA, in thick coolers with –70°C ice packs. Samples were not exposed to freeze-thaw cycles but were thawed for analysis.

Samples were processed for virus isolation (1998–2000) or screened by real-time reverse transcriptase–PCR (rRT-PCR) for influenza A virus (2001–2004), and all rRT-PCR–positive samples were subjected to virus isolation. Virus isolation was performed in embryonating chickens eggs as per standard procedures (*16*). For rRT-PCR, RNA was extracted from cloacal swab material with Trizol LS reagent (Invitrogen, Inc., Carlsbad, CA, USA) in accordance with manufacturer's instructions. RNA was tested for avian influenza virus matrix (M) gene, which detects all type A influenza viruses (*17*). The recovery rate was no better when virus isolation was used (of the 5 isolates, 3 were from rRT-PCR and 2 were from direct virus isolation). Several thousand samples have been processed by rRT-PCR at the Southeast Poultry Research Laboratory with an internal positive control; the recovery rate between the methods is equivalent, which indicates that inhibition was not a major factor in our study.

Mapping and Abundance

Delimitation of the Alaska portion of the overlap zone between Old World and New World migration systems in this region was done by using published and unpublished data on Alaska birds from the University of Alaska Museum. Abundance estimates were taken from the literature (*18–23*) and unpublished data (D.D.G., K.W., and University of Alaska Museum). Maps were created and species richness values were calculated by using ArcView 3.3 and ArcGIS 9.1 (Environmental Systems Research Institute, Inc., Redlands, CA, USA).

Genetic Analyses

We used complementary population genetic approaches to assess approximate levels of individual intercontinental movement for 2 vector species of ducks: green-winged teal (*Anas crecca*) and mallards (*Anas platyrhynchos*). Gene flow between green-winged teal populations in eastern Asia (n = 14) and Alaska (n = 40) was determined by using amplified fragment-length polymorphisms (AFLPs). Whole genomic DNA was extracted from tissues by using the DNeasy Tissue Kit (QIAGEN, Valencia, CA, USA). AFLP data were generated by using the Applied Biosystems AFLP Plant Mapping Kit (Foster City, CA, USA) and protocol as described (*24*). Two fluorescently labeled primer pairs were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Electropherograms were scored manually and 218 loci were identified. STRUCTURE 2.1 (*25*) was used to indirectly estimate gene flow by using prior geographic information about each population and determining whether individuals were assigned genetically to their population of origin. Misassigned individuals are likely to have been immigrants or to have had recent immigrant ancestors (*25*). Because AFLPs provide dominant data, each locus is treated as a haploid allele; the no-admixture model with correlated allele frequencies was used. Multiple independent simulations were run at different lengths. Results are based on 100,000 burn-ins and 200,000 subsequent iterations. Pairwise F_{ST} estimates (AFLP-SURV 1.0, [*26*]) gave comparable results (not shown). To estimate gene flow between mallards in Alaska (n = 39) and eastern Asia (n = 105), we used Migrate software (*27*) to estimate the neutral parameter theta ($4N_e\mu$) and the migration rates between continents ($4N_em$) based on sequence data of 256 bp from intron 6 of ornithine decarboxylase (*28*). Multiple factors (e.g., mutation rates, effective vs. census population sizes, and percentage of immigrants successfully breeding) made it difficult to convert estimates based on population genetics to absolute numbers of immigrant individuals. Consequently, we chose a range of values commensurate with the moderate levels of gene flow found (online Appendix Table).

Estimating Asia-to-America Influenza Influxes

To estimate a baseline delivery rate of Asian-origin AI viruses to Alaska through these overlapping migration systems, we considered movement rates (M) of individuals from Asia (i) to Alaska (j) in conjunction with infection rates (I) and the incidence of specific influenza virus strains (V_x) that we detected in this study. Measuring the risk associated with this threat thus becomes $M_{ij} \times I$ = Asian-origin infected bird arrival; strain-specific incidence (V_x) can be added to assess the narrower risk for subtypes, e.g., H5.

Results

Within Alaska, the complexities of bird migration shape the taxonomic and geographic space where Asian-origin AI viruses are most likely to appear. Using Asian species as a guide, we coupled their distributions with those of American migrants (which are necessary to effectively transfer Asian AI virus to the greater New World) to define the extensive overlap of intercontinental avifaunas in northwestern North America (Figure) as the Beringian Crucible. Because of the mingling of intercontinental avifaunas, this area is most likely to harbor host switching and genetic reassortment among AI viruses from Asia and the Americas.

Our surveillance of wild-bird AI virus focused on the eastern, or North American, part of the Beringian Crucible (Figure). We found low rates of infection among the 8,254 samples obtained from the most important host groups, waterfowl (Anatidae) and shorebirds (Charadriidae and Scolopacidae; online Appendix Table). From these samples we obtained only 5 isolates, which represent an infection rate of just 0.061%. These isolates included hemagglutinin subtypes H3, H4, and H6 (*29*). The 5 isolates were found in 3 (0.2%) of 1,477 green-winged teal (*Anas crecca*), 1 (0.76%) of 131 mallards (*Anas platyrhynchos*), and 1 (0.03%) of 3,703 northern pintails (*Anas acuta*). We found neither evidence of a clearly Eurasian origin for any of the virus genes sequenced from these Alaska isolates (*29*) nor H5 subtypes. Our data do show a remarkably close genetic association between avian influenza (H6) virus in Alaska ducks and a poultry outbreak in California in nucleoprotein and nonstructural protein A genes (*29*). This finding reflects real-time connections of migratory ducks between Alaska and California, and this vector connection extends into the Russian Far East (*30*). These findings affirm the intracontinental importance and risk posed by this region.

The number of individuals of the most important host groups (waterfowl and shorebirds) that come to Alaska from Asia is an important and heretofore unknown variable that affects the level of risk posed by these birds. Asian species are easiest to enumerate, because species-level

identity indicates origin. However, many key vector species occur on both sides of the North Pacific and move regularly between Asia and North America (online Appendix Table) and thus represent another important group of species for risk assessment. Within-species intercontinental movements of taxa that are distributed across both Asia and North America are challenging to quantify. Most species-level information is inadequate, and methods such as bird banding have not provided numeric estimates of these movements. We have summarized available data and used population genetics in 2 key vector species to estimate degrees of intercontinental avifaunal interchange in this region (online Appendix Table; an expanded version is available from the authors). Our population genetic work used 2 complementary methods and focused on 2 duck species carrying AI viruses in this region. For green-winged teal, assignment tests using AFLP markers showed that ≈2 (5%) of 40 individuals from Alaska appeared to be recent immigrants from Asia. In mallards, migration-rate values ($4N_em$, the number of immigrants in relation to effective population size) for individuals coming from Asia to Alaska were 1,064–1,727 (95% confidence interval) effective immigrants per generation. In each of these host species, intercontinental gene flow thus appears to be moderate (neither very low nor high), which indicates that thousands of individuals of these species may be coming to Alaska from Asia each year (online Appendix Table). These results corroborate the limited observational evidence from which we understood these movements to be well above zero but not high.

We estimate that 1.5–2.3 million birds from the families Anatidae, Charadriidae, and Scolopacidae come to Alaska from Asia each year (online Appendix Table). Multiplying this vector flow by the 0.061% AI infection rate that we measured among these families in Alaska suggests that 901–1,389 Asian-origin viruses may come from this source. However, our measure of infection rates is based on ducks in autumn, a taxonomic group and time known for increased infection rates (*31,32*). Although a few of our autumn duck samples are probably from birds coming from Asian breeding grounds, we have no isolates from Scolopacidae, perhaps due to fewer samples. Scolopacidae is the numerically dominant host group and is more likely to bring Asian-origin viruses in spring (online Appendix Table, [*32*]). Thus, our estimates of virus coming to Alaska from Asia can be considered to be high. Asian-origin AI virus arrival would most likely occur in the Beringian Crucible (Figure), which in western Alaska is 256,400 km^2, about the size of the United Kingdom or the US states of Wyoming or Oregon. Insofar as we have not detected H5 or H7 subtypes among our 8,254 samples, their incidence has been too low to effectively measure. Given the statistical power of our sample, their delivery rate from Asia through this system appears to be very low.

Discussion

Our surveillance did not show a "hotspot" of AI virus infection among avian hosts. Much higher infection rates are known from other multiyear surveillance studies at lower latitudes, e.g., Delaware Bay (≈4.7%, [*32*]), southern Minnesota (10.8%, [*5*]), and Alberta (22.2%, [*32*]) and British Columbia in Canada (55%, although only a single-year study, [*33*]). The infection rates we found are substantially lower than those found for interior Alaska (9%, [*12*]). Arctic conditions in Alaska prevail well south of the Arctic Circle in the treeless regions of western Alaska, and the US Arctic includes the Alaska Peninsula and Aleutian Archipelago (*34*), a tundra ecosystem where our sampling was concentrated (Figure). Aerial surveys of waterfowl across Alaska show more ponds and fewer ducks per unit area on tundra; the number of ducks per pond on tundra habitat (2.1) is less than half the number found in the boreal-forest dominated interior (5.5, [*18*]). This simple ecologic factor (perhaps due to the lower productivity of these tundra ecosystems), resulting in the dilution of virus in waters with fewer available hosts, may in part explain our results. This is the first geographically and taxonomically extensive Arctic AI surveillance in North America, and it

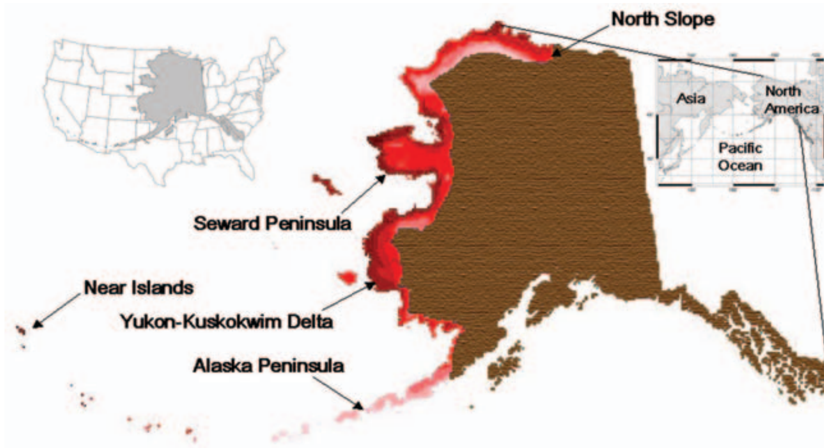

Figure. Composite geographic information system map illustrating the overlap of New World and Old World migration systems among 64 species of waterfowl (family Anatidae) and shorebirds (families Charadriidae and Scolopacidae) in northern and western Alaska (darkness of shade indicates species richness). This overlap between Asiatic and American birds in these families occurs in a zone whose extent is equivalent to a geographic band running from Lake Superior to North Dakota then to Texas and California in the lower 48 US states (left inset).

suggests that some Arctic effect lowers infection rates, thus lowering the risk of intercontinental viral transfer in these high-latitude regions. Our infection rates are low, comparable to those occurring at much lower latitudes (e.g., *9,35*), whereas mid-latitude rates can be 2–3 orders of magnitude higher (*33*).

Human population densities in Alaska are relatively low, especially in the Beringian Crucible, and Alaska lacks a large agricultural sector. However, mammalian carnivores abound and could be susceptible hosts (*36*). Direct human infection from wild birds is possible, but transmission from birds to humans is difficult (*37,38*). Nevertheless, exposure in this region may be considerable; hunters kill ≈99,000 waterbirds for food each year on the Yukon-Kuskokwim Delta alone (*39*).

Although the existence of North American and Eurasian viral lineages is well established in the literature, evidence from other regions of North America has shown that geographic structure has been insufficient to prevent sporadic intercontinental exchange of some hemagglutinin subtypes (*29,40*). Our results can be considered to confirm the comparative rarity of such events in this important region of Alaska. Despite high diversity of host species and high numbers of individual birds in Alaska making intercontinental movements, the low AI infection rates and the genetic attributes of virus isolates (*29*) suggest that at most only small numbers of Asian-origin AI viruses or genes likely arrive in Alaska annually. Although AI viruses from Alaska have a clear link with other viruses in the lower 48 US states (*29*), the predominance of Arctic ecologic conditions and the lack of agriculture in the Alaska region most affected suggest a low risk for intercontinental viral transfer in this region.

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Diagnosis of Tuberculosis by an Enzyme-Linked Immunospot Assay for Interferon-γ

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We evaluated an enzyme-linked immunospot assay for interferon-γ (T SPOT-TB) for rapid diagnosis of active tuberculosis (TB) in a disease-endemic area. From January to June 2005, patients whose clinical symptoms and radiographic findings were compatible with TB were recruited, and a blood sample was obtained for T SPOT-TB assay within 7 days of microbiologic studies. Sixty-five patients were studied, including 39 (60%) with active TB. Thirty-five (53.8%) patients had underlying medical conditions. Thirty-seven patients had positive cultures for *Mycobacterium tuberculosis*, and 11 patients had positive cultures for nontuberculous mycobacteria. The sensitivity, specificity, positive predictive value, and negative predictive value of the T SPOT-TB assay were 87.2%, 88.5%, 91.9%, and 82.1%, respectively. The accuracy of this test in diagnosing active TB is >80%, even in an area with a high incidence of nontuberculous mycobacterial disease.

Tuberculosis (TB) is one of the most important infectious disease in the world. In 2003, the incidence and death rates for TB in Taiwan were 62.38 and 5.80 per 100,000, respectively (*1*). Successful control of TB depends on prompt detection of patients with *Mycobacterium tuberculosis* infection. The conventional methods for laboratory diagnosis of TB, including acid-fast staining and culture, are either insensitive (*2*,*3*) or time-consuming (*4*). Although new diagnostic methods that use nucleic acid amplification and detection may provide quick and specific results for identifying the *M. tuberculosis* complex (*5*,*6*), their sensitivities are considerably less than those of culture (*6*).

Recent studies demonstrated that an enzyme-linked immunospot (ELISPOT) assay for interferon-γ (IFN-γ) produced by activated T cells after exposure to antigens of *M. tuberculosis*, early secretory antigenic target 6 (ESAT-6), and culture filtrate protein 10 (CFP-10) is a specific method for identifying *M. tuberculosis* infection (*7–9*). However, its performance in rapid diagnosis of active TB in disease-endemic areas is still unknown. We evaluated the ELISPOT (T SPOT-TB) assay in clinically suspected cases of TB.

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Methods

Patients

This study was conducted from January to June 2005 in northern Taiwan at a tertiary care referral center with 2,000 beds; the study was approved by the ethics committee of the hospital. Patients with fever or respiratory symptoms (cough, dyspnea, or hemoptysis) for ≥2 weeks and compatible radiographic findings were considered to have clinically suspected cases of TB. The compatible findings included fibroexudative or fibrotic lesions over upper lung, pulmonary nodules with or without cavitation, multiple patches of alveolar infiltrates, miliary shadowing, and pleural effusion with lymphocytotic and exudative characteristics. Patients were invited to provide informed consent and were interviewed and examined. A blood sample was obtained for ELISPOT within 7 days of microbiologic studies (including acid-fast smears [AFS] and mycobacterial culture). Twelve healthcare workers (HCWs) in the hospital were included as a healthy control group.

Laboratory Procedures

AFS for respiratory samples and mycobacterial culture were performed as previously described (*5*). If the primary care physician deemed it necessary, samples were screened for infection with HIV type 1 or type 2 viruses by

using competitive ELISAs (Wellcome Laboratories, Beckenham, UK), and infection was confirmed by Western blotting (Diagnostics Pasteur, Marnes-la-Coquette, France).

ELISPOT Assay

Five milliliters of blood was obtained from each patient and processed within 2 hours. ELISPOT was performed by using a commercial kit (T SPOT-TB; Oxford Immunotec Ltd, Oxford, UK) as previously described (*10*). Briefly, peripheral blood mononuclear cells were separated by using Ficoll-Paque centrifugation. Cells were washed, resuspended, and counted. Ninety-six–well polyvinylidene fluoride–backed plates (MAIPS4510; Millipore, Billerica, MA, USA) were coated with 15 µg/mL of monoclonal antibody 1-D1K against IFN-γ (Mabtech, Nacka Strand, Sweden). Cells (250,000/well) were added to duplicate wells containing antigen (ESAT-6 or CFP-10) or mitogen. No antigen was added to the background control wells. After incubation for 18 h, plates were washed, 100 µL (1 µg/mL) of biotinylated monoclonal antibody 7-B6-1-biotin against IFN-γ (Mabtech) was added, and plates were incubated for 2 h. Plates were then washed, streptavidin-alkaline phosphatase toxoid (Mabtech) was added and incubated for 1.5 h; plates were washed again and 100 µL of chromogenic alkaline phosphatase substrate (Bio-Rad Laboratories, Hercules, CA, USA) was added. After 10–15 min, the plates were washed and spots were enumerated with a stereomicroscope independently by 2 observers. Mean values determined by the 2 observers and both duplicate wells were used in all calculations. The number of spots in the background control wells was subtracted from the number in the test wells, and a response was considered positive if the number of spots per test well was ≥10 and at least twice the value found in the background control wells.

Clinical Evaluation of Patients

All medical records including history, symptoms, signs, radiologic, pathologic, and microbiologic results, and follow-up observations were carefully reviewed to obtain data for generating a clinical diagnosis. On the basis of clinical findings, 2 categories of patients were considered to have active TB: those whose clinical specimens were culture-positive for *M. tuberculosis* and those whose biopsy specimens had caseating granulomas that showed marked improvement after treatment.

Results

Patient characteristics are summarized in Table 1. All patients were previously vaccinated with bacillus Calmette-Guérin (BCG). Laboratory tests for HIV infection were performed for 42 patients and results were positive for 3 patients. Of 23 patients with an unknown HIV status, all had an initial lymphocyte count >1 × 10⁹/L and did not have AIDS-defined illness (*11*). Thirty-nine (60%) had active TB; 37 had culture-confirmed TB and 2 had histopathology-proved TB with marked improvement of their clinical conditions after treatment. Thirty-one patients had pulmonary TB, 3 had TB pleurisy (*M. tuberculosis* was isolated from pleural effusion), and 2 had concomitant pulmonary TB and TB pleurisy. The remaining 3 patients had pulmonary and extrapulmonary TB. Of the 26 non-TB patients, all were culture negative for *M. tuberculosis* for multiple specimens (mean 8.3, range 3–20).

Eight (12.3%) fulfilled the diagnostic criteria for non-tuberculosis mycobacterial (NTM) disease (*12*). Of these 8 patients, 2 infected with *M. avium-intracellulare* complex responded to treatment with clarithromycin, ethambutol, and rifampin; 1 infected with *M. kansasii* responded to treatment with isoniazid, rifampin, and ethambutol; and 3

Table 1. Clinical characteristics of 65 patients suspected of having tuberculosis, Taiwan, 2005*

Characteristic	Value
Mean age, y (range)	52.2 (2–84)
Male:female (% male)	37:28 (56.9)
Underlying medical condition	35 (53.8)
Malignancy	17 (26.2)
Diabetes mellitus	12 (18.5)
Other†	11 (16.9)
Symptoms‡	
Cough or dyspnea [no. with hemoptysis]	44 (67.7) [2]
Fever	35 (53.8)
Mean duration of symptoms (range)	23.4 wk (2 wk– 5 y)
Radiographic finding	
Upper lobe fibroexudative lesions [no. with cavitation]	25 (38.5) [2]
Multiple patches of alveolar infiltrates [no. with cavitation]	20 (30.8) [1]
Multiple nodules or mass [no. with cavitation]	10 (15.4) [3]
Upper lobe fibrotic change	5 (7.7)
Pleural effusion [no. with upper lobe fibrotic change]	3 (4.6) [1]
Miliary lesion	2 (3.1)
Acid-fast smear positive	33 (50.8)
Mycobacterial culture	48 (73.8)
Mycobacterium tuberculosis	37 (56.9)
M. avium-intracellulare complex	2 (3.1)
M. chelonae	2 (3.1)
M. abscessus and *M. chelonae*	2 (3.1)
M. abscessus	1 (1.5)
M. marinum	1 (1.5)
M. kansasii	1 (1.5)
Unidentified species	2 (3.1)

*Values are no. (%) unless otherwise indicated.
†Underlying disease was alcoholism in 4 patients, AIDS in 3 patients, end-stage renal disease in 2 patients, Sjogren syndrome in 1 patient, and hepatitis B–related liver cirrhosis in 1 patient.
‡Two patients were asymptomatic and had abnormal chest radiographs noted incidentally.

infected with *M. abscessus* responded to treatment with clarithromycin. Clinical conditions and radiographic abnormalities improved in 9 patients after treatment with antimicrobial drugs and in 2 patients after treatment with antifungal drugs. Three other patients provided biopsy specimens, which showed malignancy in 2 patients and a benign tumor in 1 patient. Another patient died of *Staphylococcus aureus* pneumonia and bacteremia. Three other patients showed no clinical and radiographic improvement after empiric treatment for 2 weeks. Specimens from 2 these patients were tested by a nucleic acid amplification assay (BD ProbeTec ET DTB system; Becton Dickinson Instrument Systems, Sparks, MD, USA) and showed negative results. Nine of 12 patients with diabetes and the 3 patients infected with HIV had active TB. In the 48 patients with mycobacteria isolated from respiratory specimens, the average interval between the date when microbiologic studies were performed and the date when the result of mycobacterial culture was available was 49.9 days (range 14–77 days). However, the average interval for the ELISPOT assay for these patients was 4.5 days (range 1–8 days) after microbiologic studies were performed.

Table 2 shows the correlation between ELISPOT results and the final diagnosis for the 65 patients. Of the 22 patients with AFS-positive TB, 19 (86.4%) were ELISPOT positive. Three showed false-negative results in the ELISPOT, including a 41-year-old HIV-positive man, a 47-year-old HIV-negative man with diabetes mellitus, and a 78-year-old woman with diabetes mellitus and Sjogren syndrome. Of the 11 non-TB patients with positive AFS, mycobacterial culture showed NTM disease in 8 patients. Three showed false-positive results in the ELISPOT, including a 74-year-old man with diabetes who was culture positive for *M. chelonae*, a 50-year-old previously healthy man who was culture positive for *M. marinum*, and a 21-year-old previously healthy woman who was culture positive for *M. avium-intracellulare* complex. The positive predictive value (PPV) of ELISPOT for AFS-positive patients was 86.4% (Table 2).

Of the 17 patients with AFS-negative TB, 2 (11.8%) showed negative results in the ELISPOT (Table 2). Both were previously healthy and had culture-positive TB pleurisy with pleural effusions with lymphocytotic and exudative characteristics. Chest radiographs for these 2 patients showed pleural effusion without parenchymal lesions. Their sputum cultures were negative for *M. tuberculosis*. HIV status was tested in only 1 patient. For the 15 non-TB patients with negative AFS, all showed negative results in the ELISPOT, i.e., the specificity and PPV of the ELISPOT were 100% (Table 2).

Among the 28 ELISPOT-negative patients, 3, including 1 with culture-confirmed TB, were retested 2, 4, and 5 weeks later, respectively. All were again ELISPOT negative. Among the 12 HCWs, all were ELISPOT negative except 1 who previously had culture-confirmed TB and had been treated for 10 months.

Discussion

Delayed diagnosis and treatment can increase the risk for dissemination of *M. tuberculosis* and decrease survival for some subgroups of TB patients (*13–15*). Thus, new technologic developments, which facilitate rapid diagnosis, are needed for successful control of this disease. Besides the development of nucleic acid amplification assays for rapid detection of *M. tuberculosis* complex, attempts have been made to exploit the T-cell response for rapid diagnosis of *M. tuberculosis* infection (*16,17*). The major problem with tuberculin skin testing (TST) is cross-reactivity with antigens in other mycobacteria, such as the *M. bovis* BCG vaccine strain and environmental mycobacterial species. This cross-reactivity leads to false-positive results and decreased PPV, especially in BCG-vaccinated persons and in areas of high incidence of NTM disease, such as Taiwan. In Taiwan in 2001, 2.74% of preschool children were TST positive, whereas active TB developed in only 2.29/100,000 children 5–9 years of age (*1*). Use of ESAT-6 and CFP-10, two antigens encoded in the region of difference 1, which distinguishes *M. tuberculosis* from other mycobacteria, has increased the specificity and PPV of IFN-γ ELISPOT assays (*10,18–22*). Our study showed that the sensitivity, specificity, PPV, and negative predictive values (NPV) of the ELISPOT assay were >80% in the diagnosis of active TB in clinically suspected patients. Results were also available ≈45 days earlier than those obtained with mycobacterial culture.

The genes coding for ESAT-6 and CFP-10 are absent from most environmental mycobacteria, except for *M. kansasii*, *M. marinum*, *M. szulgai*, *M. flavescens*, and *M. gastrii* (*23–25*). Whether ESAT-6 or CFP-10 is present in

Table 2. Correlation between results of enzyme-linked immunospot (ELISPOT) assay and diagnosis of 65 patients suspected of having tuberculosis (TB), Taiwan, 2005

Results of acid-fast smears (no. samples)	No. samples, TB (n = 39), ELISPOT (+)	TB (n = 39), ELISPOT (–)	No TB (n = 26), ELISPOT (+)	No TB (n = 26), ELISPOT (–)	Sensitivity, %	Sensitivity, %	Predictive value, %, Positive	Predictive value, %, Negative
Positive (33)	19	3	3	8	86.4	72.7	86.4	72.7
Negative (32)	15	2	0	15	88.2	100	100	88.2
Total (65)	34	5	3	23	87.2	88.5	91.9	82.1

M. chelonae and the *M. avium-intracellulare* complex has not yet been determined. Although PPV is associated with pretest probability of active TB in a cohort, our results showed that the ELISPOT can accurately discriminate TB from NTM disease and other respiratory diseases. All 3 patients with false-positive ELISPOT results had NTM disease. The 3 AFS-positive TB patients with false-negative ELISPOT results had other diseases (2 had diabetes mellitus and 1 had AIDS), which could weaken the T-cell response (*26,27*). However, neither of the 2 AFS-negative ELISPOT false-negative TB patients had another disease. Because HIV status was not routinely tested, the possibility of asymptomatic HIV infection that potentially influenced the ELISPOT results cannot be excluded.

Consistent with previous reports (sensitivity 80.7%–94.4%) (*20,28–30*), assays detecting secretion of IFN-γ caused by stimulation with ESAT-6 or CFP-10 for diagnosis of TB have a sensitivity >80%. However, specificity (45.5%–69.2%), PPV (65.4%–85.4%), and NPV (53.6%– 90.0%) were highly variable, which was probably due to different criteria for patient selection and diagnosis of active TB. In a study conducted in Japan (*20*), only patients with culture-confirmed infection were considered to have active TB. Thus, culture-negative TB patients would be classified into a non-TB group, but some showed positive test results, which resulted in decreased specificity and PPV. In the study conducted in Denmark (*28*), several risk factors predisposing persons to recent *M. tuberculosis* infection were observed in the 10 patients with false-positive results, including a history of recent exposure, immigration from a highly disease-endemic area, intravenous drug use, and HIV positivity. In the study conducted in Brazil (*30*), controls were medical students, who were at high risk for nosocomial exposure; 50% of them were ELISPOT positive, which resulted in low specificity and PPV.

Many of our patients without active TB were ELISPOT negative. In a study in Taiwan in 2001, 2.74% of preschool children were TST positive, and the annual estimated infection rate was 0.43% (*1*). Therefore, it is unlikely that all of our ELISPOT-negative patients had never been infected with *M. tuberculosis*. Furthermore, the results with samples from HCWs decrease the possibility that acute illness caused a false-negative result. Previous studies with sequential testing showed that responses of ESAT-6– or CFP-10–specific T cells decay progressively with treatment for TB (*9,22,31–34*). Our ELISPOT-negative patients may not have been recently infected with *M. tuberculosis*; thus, levels of their circulating ESAT-6– or CFP-10–specific effector T cells, rather than memory T cells, decreased and failed to yield a positive ELISPOT result (*35,36*). Further long-term follow-up study of ELISPOT-positive TB patients is needed to better understand the dynamic changes in ESAT-6– or CFP-10–specific effector T cells.

Patients with AFS-negative TB should be further investigated because this type of TB is usually diagnosed late and has been reported to be responsible for ≈17% of TB transmission (*37,38*). Our study showed that all AFS-negative ELISPOT-positive patients had true cases of TB, i.e., PPV = 100%. Only 2 patients with TB pleurisy and negative sputum culture for *M. tuberculosis* showed false-negative ELISPOT results. The cause of this finding is not known because the current hypothesis for the pathogenesis of TB pleurisy is that the caseous material from a subpleural focus ruptures into the pleural space 6–12 weeks after a primary infection. This material then interacts with previously sensitized T cells, which results in a delayed hypersensitivity reaction and accumulation of fluid (*39,40*). The 2 patients with false-negative ELISPOT results might have been at a early stage of primary TB, and their sensitized T cells had not yet returned to the systemic circulation before sampling was conducted. Further investigation is needed to assess the performance of the ELISPOT assay in patients suspected of having TB with negative AFS results.

The resurgence of TB has prompted the need for sensitive, accurate, and fast methods for laboratory detection of *M. tuberculosis* infection. Although previous studies demonstrated that the ELISPOT assay for INF-γ is a powerful tool for detecting latent *M. tuberculosis* infection, our results showed that in patients who were previously vaccinated with BCG, the diagnostic value of this test in detecting active TB approached 90% in sensitivity, specificity, PPV, and NPV, even in an area with a high incidence of NTM disease.

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Global Emergence of Trimethoprim/Sulfamethoxazole Resistance in *Stenotrophomonas maltophilia* Mediated by Acquisition of *sul* Genes

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Trimethoprim/sulfamethoxazole (TMP/SMX) resistance remains a serious threat in the treatment of *Stenotrophomonas maltophilia* infections. We analyzed an international collection of 55 *S. maltophilia* TMP/SMX-sensitive (S) (n = 30) and -resistant (R) (n = 25) strains for integrons; *sul*1, *sul*2 and *dhfr* genes; and insertion element common region (IS*CR*) elements. *sul*1, as part of a class 1 integron, was detected in 17 of 25 TMP/SMX-R. Nine TMP/SMX-R strains carried *sul*2; 7 were on large plasmids. Five TMP/SMX-R isolates were positive for IS*CR2,* and 4 were linked to *sul*2; 2 others possessed IS*CR3*. Two IS*CR2*s were adjacent to *flo*R. Six TMP/SMX-S isolates harbored novel IS*CR* elements, IS*CR9* and IS*CR10.* Linkage of IS*CR3*, IS*CR9,* and IS*CR10* to *sul*2 and *dhfr* genes was not demonstrated. The data from this study indicate that class 1 integrons and IS*CR* elements linked to *sul*2 genes can mediate TMP/SMX resistance in *S. maltophilia* and are geographically widespread, findings that reinforce the need for ongoing resistance surveillance.

Nosocomial *Stenotrophomonas maltophilia* are intrinsically resistant to a plethora of antimicrobial agents that severely limit commonly used empiric standard antimicrobial therapies. *S. maltophilia* is resistant to many β-lactams, β-lactamase inhibitors, and aminoglycosides (*1*,*2*). A recent survey of SENTRY (www.jmilabs.com) Antimicrobial Surveillance Program isolates indicated that the newer fluoroquinolones demonstrated good efficacy; the most active were levofloxacin (6.5% resistance) and gatifloxacin (14.1%) (*3*). Furthermore, the resistance to the polymyxins (20%–32%) is higher than observed in *Pseudomonas aeruginosa* (*3*,*4*). Because of low resistance levels (≈5%), trimethoprim/sulfamethoxazole (TMP/SMX) remains the therapy of choice worldwide. A recent study encompassing data from Europe, Latin America, and North America indicates that the level of resistance to TMP/SMX is 3.8%; however, previous studies indicate that the level is higher in Latin America than North America (*5*,*6*). Although surveillance studies are few, resistance to TMP/SMX appears to be emerging, and recent in vitro modeling studies have shown that combination therapies of TMP/SMX plus ciprofloxacin and TMP/SMX plus tobramycin exhibit a greater killing capacity then TMP/SMX alone (*7*,*8*).

S. maltophilia exhibits an array of mechanisms that singularly or collectively contribute to its multidrug resistance status. Intrinsic resistance includes inducible efflux pumps (*2*) and multiple β-lactamase expression (*1*) but not mutations in the quinolone resistance–determining region (*9*). In addition, *S. maltophilia* can acquire resistance through integrons, transposons, and plasmids (*10*). Recently, class 1 integrons have been characterized from *S. maltophilia* strains isolated in Argentina and Taiwan, which indicates that they contribute to TMP/SMX resistance through the *sul*1 gene carried as part of the 3′ end of the class 1 integron (*10*).

In addition to class 1 integrons, other mobile elements are associated with *sul* genes. For example, *Vibrio cholerae* serogroup 0139 is resistant to several antimicrobial agents, including SMX, and it has been recently shown that the *sul*2 gene was part of a cluster located on a novel genetic

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element of the integrative conjugative element group named SXT. The resistance genes harbored by SXT are embedded in a composite transposon-like structure and were probably acquired recently (*11*). Within this antimicrobial drug resistance region, an insertion element common region (IS*CR*) sequence, IS*CR2,* is adjacent to a *sul*2 gene that moves by 1-ended transposition. Thus, the possibility exists that *sul*2 genes can transfer intra- and intergenerically, including into *S. maltophilia*. Herein, we describe the molecular characterization of an international collection of *S. maltophilia* isolates and determine their mechanism of resistance to TMP/SMX, including the first report of *sul*2 genes and the first description of insertion element common region (IS*CR*) elements carried in *S. maltophilia*.

Methods and Materials

Bacterial Strains

During 1998–2003, a total of 1,744 *S. maltophilia* isolates collected worldwide were forwarded to the SENTRY Program (Europe, USA, and Australia) and tested for antimicrobial drug susceptibility. A TMP/SMX resistance phenotype was demonstrated for 71. From these isolates, 25 nonclonal strains from patients in North America, Latin America, and Europe were analyzed by using molecular methods together with 30 representative isolates that were TMP/SMX-susceptible. Isolates were identified by using the Vitek System and confirmed by using API20NE (bioMérieux, Hazelwood, MO, USA).

Susceptibility Methods

Isolates were tested for susceptibility to TMP/SMX according to procedures of the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) (*12*,*13*) by using broth microdilution methods (TREK Diagnostics, Cleveland, OH, USA). MIC results were confirmed with TMP/SMX. Etests were performed according to the manufacturer's directions (AB Biodisk, Solna, Sweden).

Molecular Materials

PCR primers were purchased from Sigma-Genosys Ltd. (Pampisford, UK) and are listed in the Table. General reagents for DNA manipulation were obtained from Invitrogen (Groningen, the Netherlands). All other reagents were obtained from Sigma Chemical Co. or BDH (both of Poole, England, UK).

Strain Typing

Clonality among the *S. maltophilia* isolates was assessed by pulsed-field gel electrophoresis (PFGE) followed by *Xba*I digestion of genomic DNA. This assessment was conducted according to the standard 1-day protocol (*16*).

Plasmid Isolation

Bacterial plasmids were isolated by the alkaline lysis method described by Grinsted and Bennett (*17*). Essentially, an overnight 10-mL culture was centrifuged (12,000× *g*) and suspended in water (250 µL) before 200 µL of lysis solution (0.2 mol/L NaOH, 1% sodium dodecyl sulfate [SDS]) was added. After lysis, 125 µL of neutralizing solution (0.3 mol/L potassium acetate, 1 mmol/L EDTA) was added. After precipitation, the suspension was centrifuged (12,000× *g*) and washed twice with 500 µL of a 50/50 (v/v) phenol/chloroform solution. The DNA was precipitated from the solution with the addition of 0.7 volumes of iso-amyl alcohol. The DNA/RNA pellet was washed twice in 1 mL 70% ethanol before being dried. The DNA was dissolved in 30 µL with 0.1 U RNase.

Southern Hybridization

IS*CR* and *sul*2 PCR amplicons generated with primers CRF/CRFF-r were labeled with P^{32}-CTP by random primer extension by using a commercially available kit (Stratagene, Amsterdam, the Netherlands) according to the manufacturer's instructions. Unincorporated nucleotides were removed by passing the labeled DNA through a Sephadex column (Nick column, Pharmacia Bio-tech, Uppsala, Sweden).

Agarose gels used for Southern transfer were denatured for 45 min in denaturing solution (0.5 mol/L NaOH, 1.5 mol/L NaCl) before being neutralized in 0.5 mol/L Tris-HCl, pH 7.5, 1.5 mol/L NaCl for 30 min. DNA was then transferred to Hybond (Amersham, Buckinghamshire, UK) nylon membrane by vacuum by using a custom-made Southern blotting apparatus. The nylon filter was prehybridized for at least 2 h with a blocking solution (6× SSC [1× SSC is 0.15 mol/L NaCl plus 0.014 mol/L sodium citrate], 0.1% [w/v] polyvinylpyrrolidone 400, 0.1% Ficoll [v/v], 0.1% bovine serum albumin, 0.5% SDS, 150 µg/mL denatured calf thymus DNA) at 65°C. The labeled denatured probe was then added to the solution and incubated overnight at 65°C. Finally, the filter was washed (300 mL 2× SSC, 0.1% [w/v] SDS followed by 0.1× SSC 0.1% SDS) at 65°C. Autoradiographic images were recorded on Hyperfilm-MP (Pharmacia Bio-tech), which was exposed overnight with intensifying screens.

PCR Analysis

The presence of class 1 integrons in each strain was assessed by using class 1 specific primers. Gene cassettes embedded within the class 1 integrons were determined by using primers listed in the Table. Isolates were also

screened for *sul*1, *sul*2, and *sul*3 by using sul1-F and -R, sul2-F and -R, and sul3-F and -R, respectively. Seven positive class 1 integron PCR products were chosen randomly, extracted from agarose gels after size separation, and sequenced with IntF, IntR, and custom-made oligonucleotide primers (Table).

The presence of IS*CR* elements in each strain was also determined by using primers CRF/CRFF-r designed to amplify the same 700-bp fragment internal to the open reading frames (ORFS) of IS*CR1–5* (Table). Full-length IS*CR2* elements were amplified with primers designed to target the ends of IS*CR2*. Primers used to amplify genes often associated with IS*CR2* or IS*CR3* are also given (Table). Because *dhfr* genes are associated with IS*CR* elements, we also performed molecular analysis of them.

PCRs were conducted in a final volume of 20 µL by using 10 µL ABgene Expand Hi-fidelity Master Mix (ABgene House, Surrey, UK). Primers were used at final concentrations of 10 µmol/L, and 1 µL of an overnight bacterial culture (optical density 1.0 at 600 nm) was added as source of DNA template. The cycling parameters were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 68°C for 1–4 min, depending on the sequence to be amplified, and ending with a 5-min incubation at 68°C.

DNA Sequencing and Analysis

Sequencing was conducted on both strands by the dideoxyl-chain termination method with a Perkin-Elmer Biosystems 377 DNA sequencer (Perkin-Elmer, Waltham, MA, USA). Sequence analysis was performed with the Lasergene DNASTAR software package (SelectScience Ltd., Bath, UK). Sequence alignments were conducted with the ClustalW program (www.ebi.ac.uk/clustalw) and the PAM 250 matrix.

The sequence of IS*CR2*, together with the adjacent *sul*2 region and the novel IS*CR9* and IS*CR10,* has been deposited in GenBank. The genetic locus IS*CR2*-*glmM*/*sul*2 from isolates 5232, 4647, 3800, and 2107 has been attributed the accession nos. AM182031, 182030, 182029, and 181666, respectively. IS*CR9* and IS*CR10* have been given the numbers AM182033 and AM182032, respectively.

Table. Oligonucleotide primers used in this study, Cardiff, 2007

Primer	Sequence (5′→3′)	GenBank accession no.	Target	Reference
Ina-F	GCCTGTTCGGTTCGTAAGCT		*intI*	(*14*)
Int-R	CGGATGTTGCGATTACTTCG		*intI*	(*14*)
sul1-F	ATGGTGACGGTGTTCGGCATTCTGA		*sul1*	(*15*)
sul1-R	CTAGGCATGATCTAACCCTCGGTCT		*sul1*	(*15*)
sul2-F	GAATAAATCGCTCATCATTTTCGG	AJ289135	*sul2*	(*15*)
sul2-R	CGAATTCTTGCGGTTTCTTTCAGC	AJ289135	*sul2*	(*15*)
aacA4-F	AACTTGCGAGCGATCCGATG		*aacA4*	(*14*)
aacA4-R	ATGTACACGGCTGGACCATC		*aacA4*	(*14*)
aacA7-F	AATGGATAGTTCGCCGCTCG		*aacA7*	This study
aacA7-R	TTCCGGAAGCAGCGTACTTG		*aacA7*	This study
CRF	CACTWCCACATGCTGTKKC	AF231986	All IS*CR*	This study
CRF-r	GMMACAGCATGTGGWAGTG	AF231986	All IS*CR*	This study
CRFF	GGRYGCAACGSCTCAAGCG	AF231986	All IS*CR*	This study
CRFF-r	CGCTTGAGSCGTTGCRYCC	AF231986	All IS*CR*	This study
LECR2	CACTGGCTGGCAATGTCTAG	AF231986	IS*CR2*	This study
RECR2	CTTTGGACCGCAGTTGACTC	AF231986	IS*CR2*	This study
FloF	TCGACATCCTGGCTTCACTG	AF231986	*floR*	This study
FloR	ATTACAAGCGCGACAGTGGC	AF231986	*floR*	This study
dfra20f	GGGAAACACCGAGAAATGGG	AJ605332	*dfrA20*	This study
dfrA20R	TTCTTCTTCCCATTCTCCCC	AJ605332	*dfrA20*	This study
dfrA9F	CAGATTCCGTGGCATGAACC	X57730	*dfrA9*	This study
dfrA9R	GACCTCAGATACGAGTTTCC	X57730	*dfrA9*	This study
dfrA10F	TGTAGCGCGTGGTGTAAACG	AY055428	*dhfr10*	This study
dfrA10R	ACGTCTACGTGAGTATCCGC	AY055428	*dhfr10*	This study
strAF	TCTGTCGCACCTGCTTGATC	AY055428	*strA*	This study
strAR	CATTGCTGATGAACTGCGCG	AY055428	*strA*	This study
tetAF	CGCTGTTTGTGATTACACCC	AJ250203	*tetA*	This study
tetAR	CAGCGAGATGCGATATATCC	AJ250203	*tetA*	This study
glmmR	GAGTCAACTGCGGTCCAAAC	AJ289135	*glmM*	This study
glmmF	ACGGTATTCGTGGCAAAGCC	AJ289135	*glmM*	This study

Results

TMP/SMX MICs

TMP/SMX MICs separated the isolates into an obvious bimodal distribution. The TMP/SMX-resistant isolates possessed MICs >32 mg/L, whereas the sensitive controls used as molecular comparators possessed TMP/SMX MICs ranging from 0.5 to 2 mg/L (Online Appendix Table, available from http://www.cdc.gov/EID/content/13/4/559-appT.htm).

Detection and Determination of Class 1 Integrons

Of the 25 TMP/SMX-resistant *S. maltophilia* isolates that we analyzed, 17 possessed the *sul*1 gene as part of the 3′ end of a class 1 integron. None of the TMP/SMX-susceptible *S. maltophilia* isolates yielded positive *sul*1 PCR products. PFGE analysis (data not shown) showed that only 2 isolates (9189 and 12221 from Chile) are clonally related (online Appendix Table). To our knowledge, this is the first report of *sul*1-positive *S. maltophilia* isolates from North America and Europe. The *sul*1-positive isolates are widespread, being from Europe, North America, and South America. Most (5) were isolated from Brazil. The integrons associated with the *sul*1 gene vary in size; however, when 2 strains were isolated from the same country (e.g., 3438 and 3444, 9189 and 12221, and 98 and 14469), they possessed integrons of the same size, despite not being clonally related (Online Appendix Table). Seven of these integrons were randomly selected to examine their gene cassettes. The genetic context of the class 1 integrons and procured gene cassettes are shown in Figure 1. Strains 1893 (Germany) and 9431 (Brazil) possessed only the *int* and *sul*/*qac* genes. The class 1 integrons from strains 4891 (USA), 9189 (Chile), and 12221 (Chile) contained an embedded *aacA4* gene cassette. The 2 Mexican strains (3438 and 3444) contained 2 aminoglycoside-modifying genes (*aacA7* and *aadA5*) and an unknown ORF (Figure 1) yet were clonally unrelated, as judged by PFGE profiling. None of the integrons were the same as those characterized from strains isolated from Argentina (*10*).

Detection and Location of *sul*2 Genes

All 55 isolates (both TMP/SMX resistant and sensitive) were screened for *sul*2 genes with the primers listed in the Online Appendix Table. Nine of the isolates gave PCR products for *sul*2. None of the TMP/SMX-susceptible *S. maltophilia* isolates displayed positive *sul*2 PCR products. Sequence analysis showed 100% identity with previous *sul*2 sequences.

Given that *sul*2 is normally located on medium-to-large sized plasmids, plasmids were isolated and characterized for *sul*2 carriage. Plasmid DNA was prepared from each isolate and used as a template for PCRs by using the *sul*2 primer detection set. In every case, a product of the size expected of *sul*2 sequence amplification was obtained. The purity of each plasmid preparation was evaluated by attempted PCR amplification of the host cell chromosomal *gyrA* gene. In no case was an amplification product obtained when plasmid DNA was used as template; in contrast, a *gyrA* amplification product of the correct size was obtained from genomic DNA. These data were later confirmed by Southern hybridization that used the labeled *sul*2 gene as a probe (data not shown). Unsurprisingly, in most cases *sul*2 was found on a large plasmid of ≈120 kb; however, in 2 of 9 *sul*2-positive isolates, *sul*2 gene was chromosomally encoded.

Detection of IS*CR* Elements in TMP/SMX-sensitive and -resistant Strains

The s*ul*2 gene and *dhfr* genes are often found on plasmids and in close association with class 1 integrons or IS*CR* mobile genetic elements (*10,15,18,19*). Accordingly, we investigated the 55 *S. maltophilia* isolates for IS*CR* elements. Seven of the 25 TMP/SMX-resistant isolates yielded PCR products of the expected size (≈700 bp) when the IS*CR* specific primers CRF/CRFF-r were used, and 6 of 23 TMP/SMX-sensitive *S. maltophilia* isolates also yielded the correct-sized amplification products.

To determine whether the locations of the IS*CR* sequences in the *S. maltophilia* isolates are chromosomal or plasmid mediated, plasmid DNA was prepared from each isolate and used as a template for IS*CR*-PCR and Southern hybridization analysis in a similar manner as described for *sul*2. In every case, a product of the size expected of IS*CR* sequence amplification was obtained. Hence, in those isolates that possess an IS*CR* element, the element is located on a plasmid (data not shown). The PCR

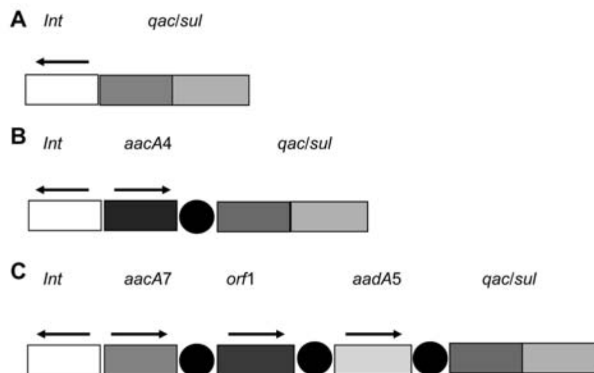

Figure 1. Schematic diagram of class 1 integrons from *Stenotrophomonas maltophilia* isolates. A) isolates 1893 and 9431; B) isolates 489, 9189, and 12221; C) isolates 3438 and 3444. Arrows depict direction of transcription, and shaded boxes represent gene cassettes found within the integron. The dark circles represent the 59-bp region immediately 5′ to the incorporated gene cassette.

IS*CR* amplification products were recovered, purified, and ligated into the cloning vector, PCR-Topo-2.1 (Invitrogen) and recombinant plasmids were recovered by transformation of *Escherichia coli* DH5α. One clone from each transformation was chosen for further study.

Sequence analysis showed that 5/7 amplicons obtained from TMP/SMX-resistant *S. maltophilia* isolates were identical to the equivalent sequence of IS*CR2;* the other 2 amplicons were identical to that of IS*CR3* (Online Appendix Table). IS*CR2* sequences were identified in isolates originating from North and South America, as well as from Europe. In contrast, the IS*CR3* sequence was identified only in isolates that originated from Spain.

The IS*CR*-like elements carried by the sensitive isolates, while clearly related to IS*CR1–5*, differed markedly from known IS*CR* sequences (*15*). Two variants were found, which we have designated IS*CR9* and IS*CR10*. The putative amino acid sequences of IS*CR9* and IS*CR10* are ≈95% identical to each other and display 30%, 48%, and 74% identity to IS*CR2*, IS*CR,3* and IS*CR5*, respectively (Online Appendix Figure, available from http://www.cdc.gov/EID/13/4/content/559-appG.htm). These novel IS*CR*s are harbored in isolates from several different regions, including South American countries, the United States, and Turkey (Table).

Identification of Resistance Genes and Sequences Adjacent to IS*CR* Elements

IS*CR2* is often associated with various antimicrobial resistance genes, not least, genes mediating TMP/SMX resistance (Figure 2) (*15*). These and other genes normally associated with IS*CR2* were therefore analyzed; these included *dhfr*A10, *dhfr*A9, *dhfr*A20, *flo*R, *tet*R, *str*A, *sul*2, and *glmM* encoding a truncated phosphoglucosamine mutase. Pairs of oligonucleotides were used (Table) to genetically characterize all those *S. maltophilia* isolates that possessed an IS*CR* element.

The *flo*R gene was detected in isolates 2139 and 2170 (which also contains IS*CR2*) from Turkey and the United States, respectively, and in isolates 12044 and 12049 (which also contains IS*CR3*) from Spain. A truncated *glmM* allele (Δ*glmM*) was detected in all IS*CR2*-containing isolates, and *sul*2 was found in all IS*CR2*- and IS*CR3*-containing isolates. The *dhfr, tetR,* and *strA* genes were not detected.

Linkage of the IS*CR* element to Δ*glmM*, *sul*2, or *flo*R was then investigated by PCR analysis, i.e., the oligonucleotide pair CRFF/*sul*2F is expected to generate a product if the IS*CR* sequence is close to *sul*2 and downstream of it (Figure 2). Using this strategy, we found that IS*CR2* was linked to Δ*glmM* and *sul*2 in all isolates that possess IS*CR2*. The *flo*R gene was also found to be linked to IS*CR2*, on the opposite side from Δ*glmM* and *sul*2, in isolates 2139 and 2170 (Figure 2). Linkage of IS*CR3* to either *sul2* or *floR* was not demonstrated.

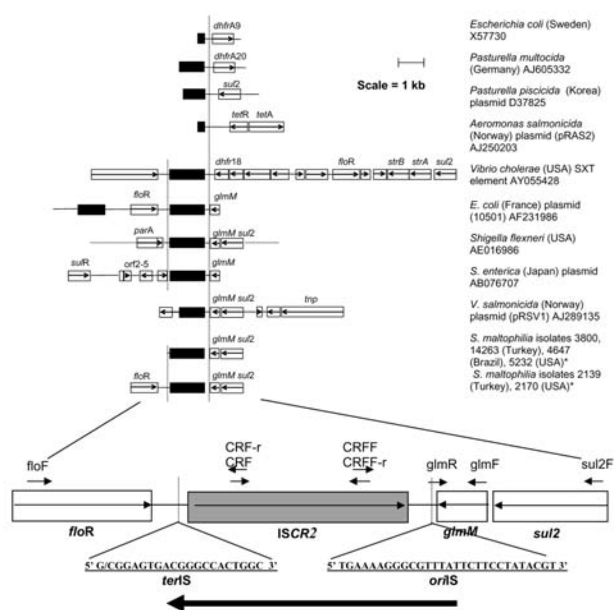

Figure 2. Genetic context of various insertion element common region (IS*CR2*) elements. Schematic of various GenBank sequences that harbor the IS*CR2* element. Open reading frames (ORFs) are depicted as open boxes with the direction of transcription of the various ORF indicated with arrows. The ORF of the IS*CR2* transposase is highlighted, and the limits of the IS*CR2* element are shown with vertical lines. The IS*CR2* element consists of an ORF of 1491 bp together with ≈120 bp of upstream sequence and ≈240 bp of downstream sequence. The IS*CR2* element identified in this study associated with the *flo*R resistance gene is amplified to show primer sites and positions of the putative *ori*IS and *ter*IS of the IS*CR2* element. The **bold arrow** indicates the direction of rolling circle replication of the IS*CR2* element. GenBank accession numbers of the various sequences are indicated. *S. enterica*, *Salmonella enterica*; *S. maltophilia*, *Stenotrophomonas maltophila.* *This study.

Discussion

We report *sul*2 genes being present in *S. maltophilia* and contributing to TMP/SMX resistance. In most cases, *sul*2 was carried on large plasmids (≈120 kb), but as judged by Southern hybridization data, a few appear to be chromosomally encoded. This study also supports the findings of Barbolla et al. that *sul*1 present in *S. maltophilia* is associated with class 1 integrons (*10*). Herein, we have characterized *S. maltophilia sul*1 genes from North America, South America, Spain, Turkey, Italy, and Germany, and observed that all of them were associated with class 1 integrons.

Most studies of the location and dissemination of *sul*2 genes have concentrated on *Enterobacteriaceae*, such as *E.*

coli and *Salmonella enterica*. A recent study by Antunes et al. found *sul*1, *sul*2, or *sul*3 genes in most Portuguese isolates (*18*); 24 of 200 isolates contained both *sul*1 and *sul*2. *sul*2 has also recently been identified in *S. enterica* from Brazil (*20*). Similar results have been reported from *E. coli* urinary tract isolates in which ≈26% of strains possessed both *sul*1 and *sul*2 genes (*21*). A biased study examining TMP/SMX-resistant *E. coli* recently reported that 15 of 20 isolates possessed *sul*2 and that 6 of those also carried *sul*1 on a class 1 integron (*14*). Additional studies of *E. coli* have shown the intercontinental predominance of *sul*1 through class 1 integrons (*22*). A study by Pei et al. demonstrated the correlation of anthropogenic activity with the presence of *sul* genes in environmental samples (*23*). However, none of the studies demonstrated the genetic origin of the *sul*2.

In addition to *sul* genes associated with plasmids and class 1 integrons, we investigated whether the *S. maltophilia* isolates possessed IS*CR* elements and whether these could be linked to *dhfr* or *sul* genes, as has been shown (*18*). Of the 25 TMP/SMX-resistant isolates, 6 harbored *sul*2 linked to IS*CR2*. However, we could not detect any *sul*3 genes. In the isolates with IS*CR2*, the element was directly linked to a deleted version of a phosphoglucosamine mutase gene, Δ*glm*M, as has been reported on other occasions (Figure 2). This arrangement is identical to those of 5 other sequences in the EMBL database, in *E. coli* isolated from cattle in France and Germany (*24*), in the plasmid pRVS1 isolated from a strain of *Vibrio salmonicida* from Norway, in a plasmid from a strain of *S. enterica* isolated in Japan, and on the chromosome of *Shigella flexneri* isolated in the United States (*18*,*24*). In all cases, Δ*glmM* and *sul*2 are linked to the end of IS*CR2* that accommodates the IS*91 ori*IS equivalent (Figure 2). The dual arrangement of Δ*glm*M and *sul*2 is also found in plasmids of marine psychotrophic bacteria isolated in Norway (GenBank accession no. AJ306553/4), but in these cases the IS*CR2* element appears not to be present.

Two of the isolates harbored a copy of the *flo*R gene immediately upstream of a copy of IS*CR2* (Figure 2), an arrangement identical to that reported on plasmids found in isolates of *E. coli* from cattle in France and Germany (*24*). The *S. maltophilia* isolates investigated in this study came from Turkey and the United States. Two isolates from Spain also carry the *flo*R gene but not IS*CR2*. Instead, the isolates possess copies of IS*CR3,* which do not appear to be linked to *flo*R. The finding of florfenicol-resistant traits on plasmids in different bacterial species from different countries highlights the wide geographic spread of this resistance mechanism. The location of *flo*R next to IS*CR2* is such that it is possible, if not probable, that the resistance gene can be cotransposed with the IS*CR* element.

The findings within this study are important for several reasons. First, this is, to our knowledge, the first report of IS*CR* elements being found in *S. maltophilia* isolates. In 6 cases, these were linked to *sul*2 genes responsible for the TMP/SMX-resistant phenotype. Moreover, these isolates were unrelated strains found in different countries. Second, since TMP/SMX is the mainstay therapy for *S. maltophilia* infections, the mobilization of *sul* genes by means of class 1 integrons and IS*CR* elements is likely to increase with TMP/SMX consumption. Third, most *sul*2 genes in this study have been found on plasmids, and *sul*2-containing plasmids can potentially confer an increase in bacterial "fitness" (*25*). As yet, such phenomena have only been explored in *Enterobacteriaceae*, and it has yet to be established whether *sul*2-carrying plasmids have such an additive effect in *S. maltophilia* or for that matter, other nonfermenting gram-negative bacilli.

These data suggest that microbiology laboratories need to carefully monitor *S. maltophilia* TMP/SMX resistance, which has the potential to increase by means of mobile elements. We also advocate the continued international surveillance of antimicrobial drug resistance that may act as early warning systems for this kind of resistance. Furthermore, yearly monitoring with molecular probes is advisable.

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Dr Toleman is currently working as a research fellow at the Medical School, Cardiff University, Wales. His interest is the dissemination of antimicrobial-drug–resistant genes among clinical and environmental bacteria.

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Flinders Island Spotted Fever Rickettsioses Caused by "marmionii" Strain of *Rickettsia honei*, Eastern Australia

Nathan B. Unsworth,* John Stenos,* Stephen R. Graves,* Antony G. Faa,† G. Erika Cox,‡ John R. Dyer,§ Craig S. Boutlis,¶ Amanda M. Lane,# Matthew D. Shaw,# Jennifer Robson, and Michael D. Nissen††**

Australia has 4 rickettsial diseases: murine typhus, Queensland tick typhus, Flinders Island spotted fever, and scrub typhus. We describe 7 cases of a rickettsiosis with an acute onset and symptoms of fever (100%), headache (71%), arthralgia (43%), myalgia (43%), cough (43%), maculopapular/petechial rash (43%), nausea (29%), pharyngitis (29%), lymphadenopathy (29%), and eschar (29%). Cases were most prevalent in autumn and from eastern Australia, including Queensland, Tasmania, and South Australia. One patient had a history of tick bite (*Haemaphysalis novaeguineae*). An isolate shared 99.2%, 99.8%, 99.8%, 99.9%, and 100% homology with the 17 kDa, *ompA*, *gltA*, 16S rRNA, and Sca4 genes, respectively, of *Rickettsia honei*. This Australian rickettsiosis has similar symptoms to Flinders Island spotted fever, and the strain is genetically related to *R. honei*. It has been designated the "marmionii" strain of *R. honei*, in honor of Australian physician and scientist Barrie Marmion.

Australia has several endemic rickettsial diseases. In addition, epidemic typhus arrived with the first fleet in 1788 (*1*), but the disease did not become established in Australia. The current endemic rickettsial diseases are murine typhus (*Rickettsia typhi*), scrub typhus (*Orientia tsutsugamushi*), and the spotted fever group (SFG) diseases—Queensland tick typhus (QTT; *R. australis*) and Flinders Island spotted fever (FISF; *R. honei*) (*2*).

QTT, first described in 1946, was characterized as a relatively mild disease with symptoms of fever, headache, malaise, enlarged lymph nodes, and a maculopapular (sometimes vesicular) rash. Most patients have an eschar and some have a slight cough, myalgia, and chills (*3*,*4*). Cases of QTT have been detected only on the eastern seaboard of mainland Australia, with most originating in late winter (*5*). FISF was described in Australia, in 1991. It is found in southeastern Australia and is characterized by fever, headache, myalgia, transient arthralgia, maculopapular rash, and cough in some cases (*6*,*7*). Most cases occur in summer. Both QTT and FISF are transmitted to humans by tick bites. Ticks of the genus *Ixodes*, especially *I. holocyclus*, are the main arthropod hosts of QTT and *Bothriocroton hydrosauri* (formerly *Aponomma hydrosauri*) are the main hosts of FISF (*8*–*10*).

We describe 7 cases of a rickettsial disease similar to FISF, which occurred in the eastern half of Australia. The etiologic agent of this disease is an SFG rickettsia, genetically related to *R. honei* and less closely related to *R. australis*. The etiologic agent of the rickettsiosis has been designated the "marmionii" strain of *R. honei*.

Case Reports

Patient 1

A 37-year-old woman from Port Willunga, South Australia, sought treatment in February 2003, with a 2-

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week history of headache, fever, and sweats. No rash or eschar was seen, and she had no recollection of arthropod exposure. She had traveled to Kangaroo Island 2–3 weeks before the onset of illness. Laboratory tests showed elevated levels of liver function test enzymes, mild leukopenia, and thrombocytopenia. Her health improved after receiving oral doxycycline for 5 days. Rickettsial serology later showed an increase in antibody titer. Both PCR and culture results were positive for an SFG rickettsia (Table 1).

Patient 2

A 9-year-old girl sought treatment at the Darnley Island Health Clinic, Torres Strait, Queensland, in February 2003. She was febrile (38.5°C) and reported headache, nausea, and abdominal pain. She had no eschar or rash. She was initially thought to have a viral illness; however, after 3 days she was still febrile (39.0°C), and the provisional diagnosis was changed to scrub typhus; a regimen of oral doxycycline, 100 mg per day, was begun. She was not seen by medical or nursing staff between day 3 and 8 of the illness, but was afebrile and well by day 8. Her SFG title increased, despite a 6-month delay in obtaining the convalescent-phase serum. Results of culture and PCR of the blood sample taken on day 8 were positive for an SFG rickettsia (Table 1).

Patient 3

A 27-year-old man sought treatment at the Darnley Island Health Clinic in March 2003. His temperature was 37.4°C, and he reported headache, arthralgia, and cough. He exhibited no eschar or rash. The provisional diagnosis was of viral upper respiratory tract infection. He was seen again on days 3 and 4 with persisting symptoms and a sore throat. On the latter visit his condition was diagnosed as tonsillitis, and treatment with penicillin V was begun. Blood tests for malaria and scrub typhus were initiated. He returned on day 29 with fever (37.6°C), cough, pharyngitis, and arthralgia. Results of serologic investigations for *Plasmodium falciparum* and rickettsia (taken on day 3) were negative. Antibiotics were not given because the illness was thought to be viral. His symptoms resolved within the following 2 weeks. Antirickettsial antimicrobial agents were not given at any stage during the illness. Day 3 serum and follow-up serum specimens obtained 6 months later were both negative for rickettsial antibodies; however, results of PCR and culture on the day 3 blood specimen were positive for SFG rickettsiae (Table 1).

Patient 4

A 10-year-old boy was brought to the Yam Island Health Clinic, Torres Strait, Queensland, in May 2003, five days into an illness with manifestations of fever (38.1°C), headache, and cough. Diagnostic tests for scrub typhus, malaria and leptospirosis were initiated but he was given no specific antimicrobial therapy. Two days later, he seemed improved, and a provisional diagnosis of viral upper respiratory tract infection was made. However, when he was seen on day 14, some symptoms remained (cough and headache), and treatment with amoxicillin was begun. He was well when examined on day 22. At no stage was he given antirickettsial therapy. His day 5 blood sample was negative for SFG/typhus group (TG) rickettsial antibodies, but results of PCR and culture were positive for a SFG rickettsia. Follow-up serum taken 14 months later was negative for rickettsial antibody (Table 1).

Patient 5

A 50-year-old man was admitted to Innisfail Hospital, Innisfail, Queensland, in June 2003. He reported a 7-day history of fever and rigors and a 4-day history of maculopapular rash. He also reported myalgia, arthralgia, conjunctivitis, swollen hands, dry cough, and constipation. An eschar was found on the right side of his neck. His temperature was 38.5°C and blood pressure 95/60 mm Hg. Serum chemistry showed elevated levels of total bilirubin (23; normal range 2–20 μmol/L), alkaline phosphatase (276; normal range 30–115 units/L), gamma-glutamyl transpeptidase (199; normal range 0–70 units/L), aspartate

Table 1. Rickettsial serology, PCR, and culture results from 7 cases of FISF*

		1st serum sample			2nd serum sample			Microbial detection of FISF agent by			
Patient	Location	Day after disease onset	SFG titer	TG titer	Day after disease onset	SFG titer	TG titer	Sero-positivity	Serocon-version	PCR	Culture
1	Port Wilunga, SA	14	128	<128	190	256	<128	+	–	+	+
2	Darnley Is, QLD	8	256	<128	186	512	<128	+	–	+	+
3	Darnley Is, QLD	3	<128	<128	179	<128	<128	–	–	+	+
4	Yam Is, QLD	5	<128	<128	515	<128	<128	–	–	+	+
5	Innisfail, QLD	7	<128	<128	17	4,096	2,048	+	+	+	+
6	Launceston, TAS	34	256	128	60	256	128	+	–	+†	–
7	Iron Range, QLD	5	<128	<128	18	1,024	<128	+	+	+‡	ND

*FISF, Flinders Island spotted fever; SFG, spotted fever group; TG, typhus group; SA, South Australia; Is, island; QLD, Queensland, TAS, Tasmania; ND, not done.
†PCR positive at both 34 and 60 d after disease onset.
‡Performed on serum and real-time PCR positive sample only.

transaminase (AST) (301; normal range 5–40 units/L), alanine transaminase (ALT) (129; normal range 5–40 units/L), and lactate dehydrogenase (LDH) (701, normal range 100–225 units/L). Further investigation showed proteinuria, moderate thrombocytopenia (59; normal range 150–400×10^9/L), mild neutrophilia with left shift (7.9; normal range 2.0–7.5 × 10^9/L), and lymphopenia (0.7; normal range 1.0–4.0 × 10^9/L). Examination of convalescent-phase serum showed seroconversion to SFG rickettsia. Results of rickettsial PCR and culture were positive for a member of the SFG (Table 1). He recovered after treatment with oral doxycycline (100 mg twice per day) for 5 days.

Patient 6

A 33-year-old man from Lilydale, a small town in northeastern Tasmania, sought treatment from his general practitioner in May 2003 (day 1) after a recent fishing trip. His symptoms included fever (38.3°C) and headache. On day 6 the patient was improving but had developed cervical lymphadenopathy. His illness was thought to be viral in origin so he was not treated with any antibiotics. The patient's condition improved, and he had a symptom-free period of ≈10 days. Fever developed again 33 days after onset of the earlier illness with the same symptoms including aches and pains. Three days later, he was admitted to Launceston General Hospital. He appeared markedly ill with a blanching maculopapular rash that had not been evident before, over his trunk, inguinal lymphadenopathy, neutropenia (0.9; normal range 2.0–7.5 × 10^9/L) and slightly elevated levels of C-reactive protein (10; normal range 0–8 mg/L). At this time the possibility of rickettsial disease was raised, and appropriate tests were performed.

On day 7 after the second onset of fever, the patient was able to work but still felt ill and had a slight fever (37.6°C). On day 27 after the second onset of fever, more rickettsial tests were performed before he received treatment with a 14-day course of doxycycline. He made a complete recovery without further relapse. He showed a raised rickettsial SFG titer and a positive SFG rickettsial PCR results for both blood samples tested (days 34 and 60) (Table 1).

Patient 7

A 55-year-old man, an entomologist, at Iron Range, Cape York Peninsula in far north Queensland, removed a tick from the left ventrolateral side of his abdomen in late May 2002. Five days after removing the tick (day 5), an influenzalike illness with myalgia and arthralgia developed. On day 6, a high fever developed, and on the following day, he experienced persisting severe lethargy and severe muscle cramps in major muscle groups of his upper and lower legs. On day 8, an eschar appeared at the site of the tick bite. It was oval in shape and ≈150 mm by 75 mm. A widespread maculopapular/petechial rash also appeared over his body. High fever, severe lethargy, and myalgias continued. On day 9, he visited his doctor in Brisbane where the examination confirmed a widespread maculopapular/petechial rash with generalized lymphadenopathy and myalgias affecting large muscle groups. A large eschar was found on his left lower abdomen. An SFG illness was suspected, and treatment with doxycycline, 100 mg twice per day, was begun His doctor reexamined him on day 20, and his condition had improved. His myalgia had decreased, and the rash faded over 5 weeks.

Laboratory testing on day 10 showed lymphopenia (0.8; normal range 1.0–4.0 × 10^9/L) and mild thrombocytopenia (146; normal range 150–400 × 10^9/L). Liver function tests showed slightly elevated AST (48; normal range 5–40 units/L) and ALT (44, normal range 5–40 units/L) and mildly elevated LDH (325; normal range 100–225 units/L). Rickettsial serology was negative on day 10 but convalescent-phase serology on day 23 showed an SFG seroconversion. A real-time PCR on the day 10 serum specimen showed a positive result for the SFG/TG *gltA* gene, but the 17-kDa PCR result was negative (Table 1).

The removed tick was subsequently identified as *Haemaphysalis novaeguineae.* DNA was extracted from the tick and PCRs performed targeting the rickettsial *rrs*, *ompA* and *ompB* genes. PCR products were sequenced, aligned, searched with BLAST (available from http://130.14.29.110/BLAST/), and submitted to GenBank (accession nos. AJ585043, AJ585044, and AJ585045 for the *rrs*, *ompA,* and *ompB* genes, respectively). Phylogenetic analysis of all 3 genes showed that the closest relatives were *R. honei* strain TT-118 (Thai tick typhus) and *R. honei* strain RB (FISF) (*11*).

Methods

Rickettsial Serology

Serologic testing was performed on human serum specimens by using a goat anti-human IgM, IgG, and IgA fluorescein isothiocyanate–labeled secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), by an indirect immunofluorescence assay (IFA) as described (*7*). Antigens used included *R. honei*, *R. australis*, *R. akari*, *R. conorii*, *R. sibirica,* and *R. rickettsii* from the SFG; and *R. typhi* and *R. prowazekii* from the TG. All titers >128 were considered positive.

Rickettsia Isolation from Blood

Rickettsial isolation was performed with Vero cell cultures as previously described (*12*). Cultures were observed microscopically weekly for a cytopathic effect and monthly by immunofluorescence. IFA-positive cultures had their DNA extracted and their rickettsial status confirmed by

PCR. Positive cultures were passaged onto confluent XTC-2 cell monolayers and grown at 28°C in Leibovitz L-15 media (Invitrogen, Melbourne, Victoria, Australia) supplemented with 5% heat-inactivated fetal bovine serum, 0.4% tryptose phosphate (Oxoid, Basingstoke, UK), and 200 mmol/L-glutamine (Invitrogen).

Rickettsial PCR on Blood

Rickettsial real-time PCR was performed on buffy coat (except for serum for case 7). DNA was extracted by using a DNA extraction kit (Gentra, Minneapolis, MN, USA) and the primers CS-F and CS-R and the probe CS-P (Table 2; Biosearch Technologies Inc., Novato, CA, USA) as previously described (*13*).

Confirmatory PCR was performed on the 17-kDa gene (*orf17*) by using the primers MTO-1 and MTO-2 (Table 2; Invitrogen) (*14*), with an annealing temperature of 51°C and a total of 45 cycles. PCR products were visualized by electrophoreses on a 1% Tris-acetate EDTA agarose gel (Amresco, Solon, CA, USA) stained with ethidium bromide. PCR-positive samples had their DNA cleansed using the QIAquick DNA clean up kit (QIAGEN, Düsseldorf, Germany) and were sequenced at Newcastle DNA (Newcastle University, Newcastle, New South Wales, Australia). Phylogenetic analysis of DNA sequences was performed with DNADIST and NEIGHBOR computer programs of the PHYLIP version 3.63 software package (available from http://evolution.genetics.washington.edu/phylip.html). Sequences were compared to those of the rickettsial strains considered to be valid species (*19*). Phylogenetic trees and bootstrap analyses were performed with 100 alignments by using the SEQBOOT and CONSENSE programs of PHYLIP.

Rickettsial Molecular Characterization

Rickettsial isolates had portions of their *gltA*, 16S rRNA, ompA, and Sca4 antigen genes amplified and sequenced to supplement the 17-kDa gene analysis done on buffy coat and cultures. The primer pairs CS-162-F with CS-731-SR and CS-398-SF with RpCS1258 (Table 2) were used to amplify the 5′ and 3′ ends of *gltA,* respectively (*15*).

The 16S rRNA gene (*rrs*) was amplified by using the primer pairs rRNA1 with rRNA3 and rRNA2 with rRNA4 (Table 2) (*17*). The PCR contained 1 µmol of each respective primer, 200 µmol/L of each dNTP, 10× reaction buffer, 2 mmol/L $MgCl_2$, 2 U Taq polymerase, and 4 µL of rickettsial DNA extract. The amplification was performed in a thermocycler (Rotor-Gene 3000, Corbett Research, Sydney, New South Wales, Australia) with an initial denaturation of 95°C for 3min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 1 min; with a final extension of 10 min. PCR products were visualized and sequenced as described above.

Table 2. Oligonucleotide primers used for PCR amplification and sequencing of products

Primer	Nucleotide sequence (5′→3′)	Gene	Reference
CS-F	TCG CAA ATG TTC ACG GTA CTT T	*gltA*	*13*
CS-R	TCG TGC ATT TCT TTC CAT TGT G	*gltA*	*13*
CS-P*	TGC AAT AGC AAG AAC CGT AGG CTG GAT G	*gltA*	*13*
MTO-1	GCT CTT GCA ACT CTA TGT T	*orf17*	*14*
MTO-2	CAT TGT TCG TCA GGT TGG CG	*orf17*	*14*
CS-162-F	GCA AGT ATC GGT GAG GAT GTA ATC	*gltA*	*15*
CS-398-SF	5′ATT ATG CTT GCG GCT GTC GG	*gltA*	*15*
CS-731-SR	AAG CAA AAG GGT TAG CTC C	*gltA*	*15*
RpCS1258p	ATT GCA AAA AGT ACA GTG AAC A	*gltA*	*16*
rRNA1	AGA GTT TGA TCC TGG CTC AG	*rrs*	*17*
rRNA2	AAG GAG GTG ATC CAG CCG CA	*rrs*	*17*
rRNA3	CCC TCA ATT CCT TTG AGT TT	*rrs*	*17*
rRNA4	CAG CAG CCG CGG TAA TAC	*rrs*	*17*
Rr190.70p	ATG GCG AAT ATT TCT CCA AAA	*ompA*	*16*
Rr190.602n	AGT GCA GCA TTC GCT CCC CCT	*ompA*	*16*
D1f	ATG AGT AAA GAC GGT AAC CT	*sca4*	*18*
D928r	AAG CTA TTG CGT CAT CTC CG	*sca4*	*18*
D767f	CGA TGG TAG CAT TAA AAG CT	*sca4*	*18*
D1390r	CTT GCT TTT CAG CAA TAT CAC	*sca4*	*18*
D1219f	CCA AAT CTT CTT AAT ACA GC	*sca4*	*18*
D1876r	TAG TTT GTT CTG CCA TAA TC	*sca4*	*18*
D1738f	GTA TCT GAA TTA AGC AAT GCG	*sca4*	*18*
D2482r	CTA TAA CAG GAT TAA CAG CG	*sca4*	*18*
D2338f	GAT GCA GCG AGT GAG GCA GC	*sca4*	*18*
D3069r	TCA GCG TTG TGG AGG GGA AG	*sca4*	*18*

*5′ end labeled with 6-FAM; 3′ end labeled with BHQ-1.

The ompA gene (*ompA*) was amplified by using the primers Rr190.70p and Rr190.602n (Table 2) by using the above protocol but with an annealing temperature of 48°C (*16*). The Sca4 antigen gene (*sca4*) was amplified by using the primer pairs D1f and D928r, D767f and D1390r, D1219f and D1876r, and D1738f and D2482r, following the specified protocol (*18*). The final segment of the gene was amplified with the primers D2338f and D3069r following the same protocol and an annealing temperature of 48°C (*18*).

Results

Seroconversion, defined as a 4-fold increase in antibody titer, occurred in only 2 of the 7 patients (patients 5 and 7), although positive titers were seen in 5 of 7 patients (Table 1). In 5 of 6 patients a rickettsia was isolated from blood (in EDTA-vacutainers; Table 1) in Vero cell culture, however, 4 of these 5 isolates did not persist in cell culture after their third passage. The remaining isolate, from patient 5, has been maintained in continuous culture in only the XTC-2 cell line.

Patients 1–6 had rickettsial DNA detected in their buffy coat DNA extracts by real-time PCR. Patient 7 had rickettsial DNA detected in his serum by using real-time PCR. Of the 7 cases, all but 1 (patient 7), were PCR positive for the 17-kDa gene and all 5 positive rickettsial cultures were also PCR positive for the same gene (Table 1). The 17-kDa PCR sequences for the buffy coats of cases 1–6 and cultures of patients 1–5 were found to be 100% homologous to one another and to the Japanese *Haemaphysalis* tick sequences Hf151 and Hl550 (*20*) (GenBank accession nos. AB114816 and AB114807, respectively). A 399-bp sequence also exhibited 99.2% homology with *R. honei* strains RB and TT-118 (GenBank accession nos. AF060704 and AF060706, respectively) as shown in the phylogenetic tree (Figure).

Analysis of a 1082 bp *gltA* sequence from the KB strain exhibited 99.7 and 99.8% homology with *R. honei* strains RB and TT-118, respectively (GenBank accession nos. AF018074 and U59726, respectively) (Figure). An 1142 bp *rrs* sequence exhibited 100% homology with the Australian *Haemaphysalis novaeguineae* tick sequence AL2003 (*11*) (GenBank accession no. AJ585043) and a 1,388-bp sequence exhibited 99.6% and 99.9% homology with the *R. honei* strains RB and TT-118, respectively (GenBank accession nos. U17645 and L36220, respectively) (Figure). A 511-bp sequence of *ompA* exhibited 100% homology with the *H. novaeguineae* sequence AL2003 (*11*) (GenBank accession no. AJ585044) and a 513-bp sequence had 99.8% homology with the *R. honei* strains RB and TT-118 (GenBank accession nos. AF018075 and U43809, respectively). Only 1 nucleotide substitution was found in a 2,961-bp sequence of the Sca4 gene (100%

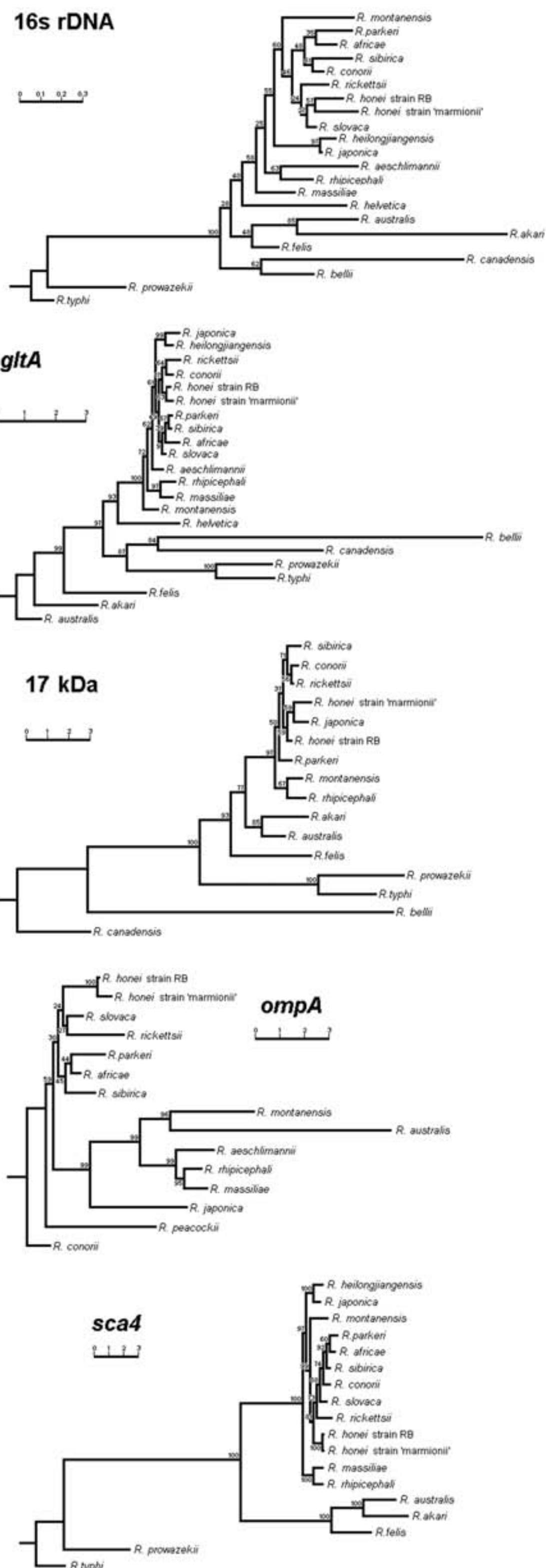

Figure. Phylogenetic trees obtained by a neighbor-joining analysis of the 16s RNA, *gltA*, 17-kDa, *ompA*, and Sca4 antigen genes. Bootstrap values from 100 analyses are shown at the node of each branch.

homology) with *R. honei* strain RB (GenBank accession no. AF163004) (Figure).

These *R. honei* strain "marmionii" sequences have been submitted to GenBank with the accession nos. AY37683 for the 17-kDa gene, AY37684 for the *gltA* gene, AY37685 for the 16S rRNA gene, DQ309095 for the Sca4 gene, and DQ309096 for the *ompA* gene.

Discussion

These 7 cases of FISF are an example of many newly emerging rickettsial diseases (*21*). Its symptoms are consistent with a relatively mild rickettsial SFG disease. The most frequent acute symptoms observed were fever (100%), headache (71%), arthralgia (43%), myalgia (43%), cough (43%), rash (maculopapular/petechial) (43%), nausea (29%), pharyngitis (29%), and lymphadenopathy (29%). In only 2 patients was an eschar evident. The rash did not appear on the palms or soles, unlike previously reported FISF cases (*6*,*12*). One patient (patient 7), had a history of a *H. novaeguineae* tick bite, which may imply an incubation period of 5 days. The cases in this report occurred between February and June (late summer and autumn), in contrast to previously described cases of FISF and QTT, which have their peak onsets in summer and late winter, respectively (*5*,*6*).

The biphasic illnesses seen in patients 3 and 6 were unusual for SFG rickettsial diseases. Because no specimens were taken during the initial phase of either patient's illness, that this phase was rickettsial in nature cannot be confirmed. Patient 6's illness may have been rickettsial in nature because of the appropriate incubation time after a fishing trip in an area endemic for ticks. His illness had the longest duration of all the reported cases, with rickettsiae still detectable 27 days after the onset of the second febrile illness. This is possibly the first report of an SFG rickettsia being associated with a chronic infection in a human. Relapsing rickettsial diseases are known to exist, such as Brill disease, a recurrent form of epidemic typhus (*22*). Rickettsiae persisting in human and animal organs after illness have been reported with scrub typhus and SFG rickettsia (*23*,*24*). An Australian case of recurrent rickettsial illness was diagnosed serologically as QTT (*25*).

The isolation of rickettsiae from patient 2 after antimicrobial drug therapy and while she was clinically well is unusual. The presence of rickettsiae may be due to the bacteriostatic nature of the patient's treatment, which allowed a small number of rickettsiae to survive before being eliminated by her immune system. This phenomenon may also have been the beginning of a chronic infection, as described above in patients 3 and 6.

Apart from patients 5 and 7, antibody levels of paired serum specimens (Table 1) did not show a marked rise in titer. Because the second serum sample from 4 of the case-patients was received in excess of 6 months after illness, the antibody levels may have subsided, explaining the apparent lack of seroconversion in patients 3 and 4. Because most rickettsioses are diagnosed through serologic tests, some cases of rickettsial disease are likely being missed due to a lack of seroconversion, as we have observed with these cases of FISF. This demonstrates the usefulness of PCR for diagnosing acute rickettsial diseases. Cases of rickettsioses without seroconversion or positive serology titers have been previously described with "*R. sibirica mongolotimonae*" (*26*). Despite the initial isolation of *R. honei* strain "marmionii" in Vero and L929 cells at 35°C, no isolate could be continuously grown in these cell lines. This may be due partially to temperature-dependent growth kinetics, similar to those of *R. felis* (*27*).

The 7 described cases were distributed widely throughout eastern Australia. Cases have appeared on the eastern seaboard of Australia (including the Torres Strait), Tasmania, and in South Australia. Cases are yet to be reported in Victoria, New South Wales, the Northern Territory, or Western Australia. The discovery of FISF cases in the Torres Strait suggests its possible presence in Papua New Guinea. In comparison, QTT is found only down the eastern seaboard and not south or west of Wilson's Promontory in Victoria. Traditionally, FISF has only been found in the southeastern states, including Tasmania and South Australia (*6*,*12*,*28*).

At present, *R. honei* has been found on 2 other continents, with potential reservoirs in *Ixodes* and *Rhipicephalus* ticks in Asia and in *Amblyomma cajennense* in North America (*29*). The only known vector/reservoir of *R. honei* in Australia is *Bothriocroton hydrosauri* (*10*). *R. honei* strain "marmionii" has not been found in any *B. hydrosauri* ticks, although *H. novaeguineae* may be a vector/reservoir, as a *H. novaeguineae* tick was removed from patient 7 before the onset of illness. Rickettsial *rrs* and *ompA* gene sequences within the tick demonstrated 100% homology with *R. honei* strain "marmionii" (*11*). *H. novaeguineae* is known to bite numerous animals including humans and is found in both northern Australia and Papua New Guinea (*30*). The vectors and reservoirs of *R. honei* strain "marmionii" in southern Australia are not known.

When compared phylogenetically to other rickettsiae, *R. honei* strain "marmionii" has the closest homology with Australian *R. honei* strain RB, which had been isolated from a febrile patient on Flinders Island. When the *gltA*, *rrs*, *ompA*, *orf17*, and *sca4* genes are compared between *R. honei* strains RB and "marmionii," they are 99.7%, 99.6%, 99.6%, 99.0%, and 100% homologous, respectively. Homologies of 99.8% and 99.9% are seen with the *gltA* and *rrs* genes, respectively, when *R. honei* strains TT-118 and "marmionii" are compared. An 811-bp *ompB* gene

sequence from the *H. novaeguineae* tick removed from patient 7 also showed 100% homology with *R. honei* (*11*). This supports its description as an SFG rickettsia but not a new species by using previously proposed criteria (*19*). Further analysis is needed to further define the taxonomic position of *R. honei* strain "marmionii."

The 7 cases of an illness similar to FISF demonstrate that new emerging rickettsioses are present in Australia. These described cases encompass a geographic distribution larger than those of FISF and QTT. The only known tick host of *R. honei* strain "marmionii" is *H. novaeguineae*, a tick not previously recognized as a transmitter of human pathogens. Genetically, the etiologic agent of these 7 cases is closely related to *R. honei*. We propose to name the agent *Rickettsia honei* strain "marmionii," in honor of the Australian physician and scientist Barrie P. Marmion, for his research into Q fever, another important rickettsial disease.

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Dr Unsworth is a postdoctoral research associate at Texas A&M University, College Station, Texas, USA. His interests include the epidemiology of Australian rickettsiae and Q fever pathogenesis.

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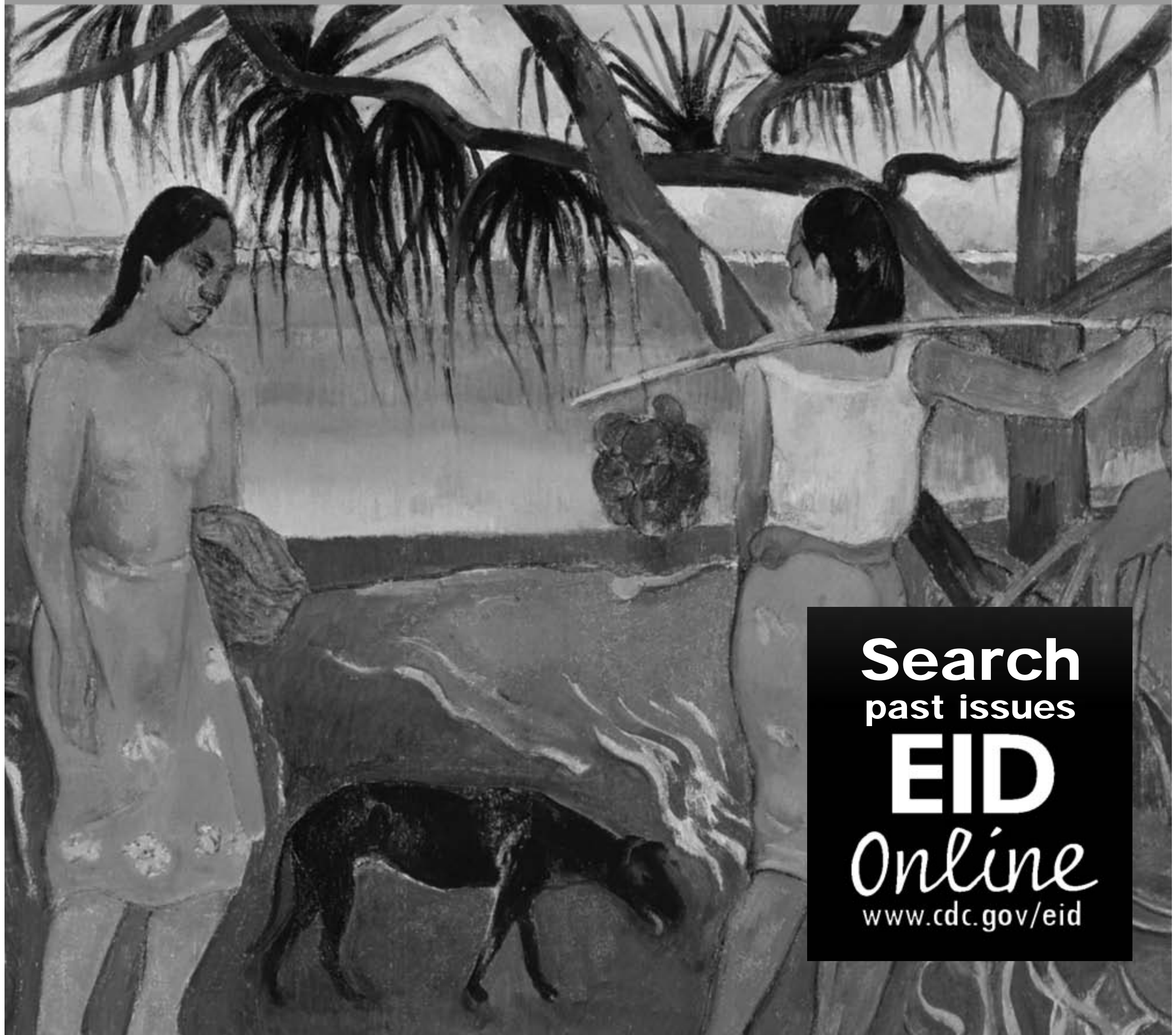

Symptomatic and Subclinical Infection with Rotavirus P[8]G9, Rural Ecuador

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During the past decade, rotavirus genotype G9 has spread throughout the world, adding to and sometimes supplanting the common genotypes G1–G4. We report evidence of this spread in a population sample within rural Ecuador. A total of 1,656 stool samples were collected from both patients with diarrhea and from asymptomatic residents in 22 remote communities in northwestern Ecuador from August 2003 through February 2006. Rotavirus was detected in 23.4% of case-patients and 3.2% of controls. From these 136 rotavirus-positive samples, a subset of 47 were genotyped; 72% were of genotype G9, and 62% were genotype P[8]G9. As a comparison, 29 rotavirus-positive stool samples were collected from a hospital in Quito during March 2006 and genotyped; 86% were of genotype P[8]G9. Few countries have reported P[8]G9 rotavirus detection rates as high as those of the current study. This growing prevalence may require changes to current vaccination programs to include coverage for this genotype.

Rotavirus is the most important cause of acute gastroenteritis and death in infants and young children worldwide, causing an estimated 352,000–592,000 deaths in children <5 years of age (*1*). Although the incidence of infection in children in industrialized and developing countries is similar, outcomes vary widely. In countries classified by the World Bank as high-income, the risk of dying from rotavirus before age 5 is 1 in 48,680; the equivalent risk in low-income countries is 1 in 205 (*1*).

The rotavirus genome is made up of 11 double-stranded RNA segments; each segment encodes a unique structural or nonstructural protein. A 3-layered protein coat encloses the genetic material. The VP2 proteins form the innermost layer, which is in turn surrounded by a sheet of VP6 proteins. The outer layer consists of 2 antigenic proteins, VP7 and VP4, also referred to as the G (glycoprotein) and P (protease-sensitive) proteins, respectively (*2,3*). To date, 15 G-protein genotypes and 24 P-protein genotypes have been identified (*3*), of which 10 G and 12 P types are known to infect humans. The combination of these 2 proteins constitutes the viral genotype (*4*). Because of the segmented nature of the rotavirus genome, the genes for the external structural proteins may segregate independently during coinfection (genetic reassortment), thus increasing the genetic diversity of rotaviruses.

Data from 1994 through 2003 indicate that the 4 most prevalent human rotavirus genotypes worldwide were P[8]G1 (52%), P[4]G2 (11%), P[8]G4 (8%), and P[8]G3 (3%), which together represented ≈74% of the global isolates (*2*). In Latin America during the same period, the prevalence of these 4 viral types was similar (*3,5*).

More recent data suggest that the G9 genotype has gained global importance during the past 10 years (*3*,*6–9*). The P[8]G9 type, the most common combination, may have resulted from a reassortment event between the most prevalent type P[8] and a strain carrying G9. From 1990 through 2004, P[8]G9 rotaviruses caused <5% of rotavirus infections worldwide but 15% of infections in South America (*2,3*). A less common reassortment is P[6]G9 (*2,6*).

Genotyping of circulating strains has epidemiologic importance and relevance for vaccination planning. The most efficacious vaccination protocols are those that use viral serotypes similar to those circulating in a given community (homotypic responses) (*3*). Vaccines with serotypes distinct from those circulating (heterotypic) are less effective (*9,10*).

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Although rotavirus infections have been reported in Ecuador (*11*), to our knowledge, this is the first report of circulating genotypes. The data presented here are unique in that they are community-based and include all symptomatic community residents as well as asymptomatic controls. This approach differs from most rotavirus genotyping studies, which focus on patients in a clinical setting. The data thus document the total illness rate associated with rotavirus infection from 22 remote, rural communities on the northern coast of Ecuador.

Methods

Study Population and Design

As part of a larger community-based case-control study, fecal samples were collected from persons in 22 remote communities located in Esmeraldas, the northernmost province on the coast of Ecuador. Each of 21 small, rural communities was visited 4 times, each time for 15 days, from August 2003 through February 2006. Fecal samples were also collected from the region's largest town, Borbón, for 15 days in July 2005. During each 15-day visit, health workers visited every household and interviewed residents to identify every case of diarrhea. For each identified case of diarrhea, 3 asymptomatic controls were selected, 1 from the case-patient's household and 2 randomly selected from the community. A total of 1,656 stools samples were collected, 411 (25%) from patients with diarrhea.

To determine whether the results in these remote communities were representative of rotavirus infections in other Ecuadorian locations, 29 fecal samples from children <5 years of age with rotavirus-associated diarrhea rotavirus were collected at the Hospital de Niños Baca Ortiz in Quito, which is an urban environment ≈200 km from the study area. Protocols were approved by the bioethics committee at the Universidad San Francisco de Quito and the Internal Review Board at the University of California, Berkeley, California, USA.

Rotavirus Detection and Testing

All of the 1,656 samples (symptomatic and nonsymptomatic) were analyzed for the presence of rotavirus with a commercial immunochromatographic test (RIDA Quick Rotavirus, R-Biopharm AG, Darmstadt, Germany). All rotavirus-positive samples collected from February 2005 through February 2006 (n = 47) were preserved in liquid nitrogen and transported to Quito for PCR genotyping. The double-stranded rotavirus RNA was extracted from the stool specimens by using TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA, USA) or the UltraClean Tissue RNA Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. RNA was stored at –80°C until further use. A 2-step, seminested multiplex reverse transcription–PCR was carried out for G- and P-genotyping based on a protocol provided by the US Centers for Disease Control and Prevention (J. Gentsch, pers. comm.). Briefly, primers 9con1 and 9con2 were used for the first amplification of the VP7 gene and primers 9T-1, 9T-2, 9T-3P, 9T-4, and 9T-9B were then used to ascertain the G genotype (*12*). Primers Con3 and Con2 were used for the partial amplification of the VP4 gene and primers 1T-1, 2T-1, 3T-1, 4T-1, 5T-1, and ND2 were then used to ascertain the P genotype (*13*).

Viral RNA was denatured for 5 min at 97°C. Retrotranscription and the first amplification were carried out by using a SuperScript III RT/Platinum Taq polymerase kit (Invitrogen Corp.). Primers were used at 200 nmol/L each, and the 1× buffer provided by the manufacturer contained 1.6 mmol/L $MgSO_4$ and 200 µmol/L of each deoxynucleotide triphosphate. The retrotranscription was carried out at 42°C for 45 min and stopped at 96°C for 2 min. The first amplification consisted of 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The second amplification was carried out by using PuReTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ, USA) and primers at a final concentration of 400 nmol/L. The cycling parameters were 30 cycles at 94°C for 30 s, 42°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 1 min. Electrophoresis of the PCR product was conducted on 1.8% agarose gels at 60 volts and visualized under ultraviolet light.

Nucleotide Sequencing

For sequencing purposes, samples were transferred directly onto chromatography paper strips treated with sodium dodecyl sulfate–EDTA, dried overnight at room temperature, and sent to Belgium by standard postal service (*14*). From the community samples, 22 PCR products that were identified as P[8]G9 were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and sequenced with the ABI PRISM BigDye Terminator Cycle sequencing reaction kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 automated sequencer (Applied Biosystems). Primers Beg9 and End9 were used for the VP7 gene (*15*) and primers 1-17F (*16*) and Con2 were used for the VP4 gene. The sequencing reaction conditions were 25 cycles at 94°C for 15 s, 50°C for 15 s, and 72°C for 4 min, and a final extension of 72°C for 7 min.

Sequence Analysis

Partial VP7 DNA sequences from 22 G9 community samples and 65 additional G9 VP7 sequences obtained from GenBank for comparison purposes were aligned by using ClustalW (*17*). A phylogenetic tree was constructed

by using a maximum likelihood algorithm as implemented by DNAML in PHYLIP (*18*). A VP7 sequence from a G3 genotype rotavirus was used to root the tree. Bootstrap support was calculated by using 500 bootstrapped data replicates as implemented by SEQBOOT in PHYLIP. VP7 gene sequences from the 22 G9 community isolates were deposited in GenBank under accession nos. DQ848566–DQ848587.

Results

Of 1,656 fecal samples from remote communities analyzed for rotavirus, 136 (8.2%) were determined to be positive by the commercial immunochromatographic test. Of these positive samples, 96 were from the 411 patients with diarrhea and 40 were from the 1,245 asymptomatic controls. Diarrhea was significantly associated with being infected with rotavirus (odds ratio = 9.2; 95% confidence interval 6.1–13.9). Rotavirus RNA was detected at the highest rates from symptomatic infants and, surprisingly, persons >40 years of age (Table 1). No pronounced seasonality of rotavirus infection was determined, and incidence was not significantly associated with month of collection or with the observed 30-day rainfall for 15 days before the visit and during the visit (data not shown). This lack of seasonality in the tropics has been reported previously (*19*).

From the 136 rotavirus-positive community samples, a subset of 47 samples were genotyped for the VP4 and VP7 genes. This subset represented all samples collected from February 2005 through February 2006, from 14 rural communities and Borbón. Of these 47 samples, 35 (74%) yielded successful PCR typing results for the VP4 gene and 37 (79%) yielded successful PCR typing results for the VP7 gene. An additional 6 (13%) yielded successful PCR amplification at 1 of the 2 genes. The remaining 8 (17%) samples were not typeable. Six of these untypeable RNA samples, along with 6 typeable samples, were subjected to electrophoresis on an agarose gel and visualized by staining with ethidium bromide in an attempt to detect rotavirus RNA. None of the untypeable samples produced rotavirus RNA banding patterns, whereas 3 of 6 typeable samples could be visualized.

Among the successfully typed samples, genotypes P[8] and G9 predominated. A small proportion of the samples produced patterns corresponding to P[6]G1 and P[6]G9 (Table 2). Table 3 summarizes the percentage of patients infected with P[8], G9, and P[8]G9 based on 2 assumptions of the 13 samples in which 1 or both of the VP4 and VP7 genes were not typeable. The first assumption was that samples were nontypeable because they were degraded sometime between testing positive by immunochromatographic tests in the field and the sample's arrival in the laboratory in Quito. In this case, we assumed that those samples were missing data. The second assumption was that the samples were nontypeable because they were novel strains, and we therefore included them in the dataset. G9 genotype was identified in 34 samples, resulting in a 72%–92% infection rate; P[8] genotype was identified in 31 samples, a 66%–89% infection rate; and the combination of P[8]G9 was found in 29 samples, a 62%–88% infection rate.

To determine whether the genotypes in remote communities corresponded to strains circulating elsewhere in Ecuador, we analyzed 29 rotavirus-positive samples from Hospital de Niños Baca Ortiz in Quito. Again, genotypes P[8] and G9 overwhelmingly predominated (Table 2). Electrophoretic evidence for 5 mixed infections was found among these urban samples, but this was not seen among the rural samples (Table 2).

Of the 29 P[8]G9 community samples, 22 were sent for sequencing to the University of Leuven in Belgium. Approximately 750 bp of high-quality nucleotide sequence data for the VP7 gene was obtained from each sample. The 22 P[8]G9 samples were remarkably homogenous at the sequence level, with only 3 single nucleotide polymorphisms found in the 22 sequences. Phylogenetic analysis of the sequences (Figure) showed that the Ecuadorian sequences grouped together monophyletically and were part of the large clade composed of most of the recently isolated G9 rotavirus sequences worldwide.

Discussion

The present study reports a high rate of infection (72%–96%) with rotavirus G9 genotype among persons in 2 geographically distinct regions within Ecuador, a remote coastal rain forest and an urban Andean hospital. To our knowledge, it is the first description of rotavirus genotypes in Ecuador, and the results support the observation that the

Table 1. Age distribution of 411 case-patients (those with diarrhea) and 1,245 controls (those without diarrhea) from rural communities of Esmeraldas, Ecuador*

	Age group, y					Total
	<1	1–<5	5–<20	20–<40	≥40	
Case-patients +/n (%)	20/69 (29.0)	33/181(18.2)	16/64 (25.0)	0/17 (0)	11/34 (32.4)	96/411 (23.4)
Controls +/n (%)	0/35 (0)	6/142 (4.2)	17/493 (3.4)	13/282 (4.6)	4/251 (1.6)	40/1,245 (3.2)
OR (95% CI)	– (3.6–∞)	5.1 (2.0–15.2)	9.3 (4.1–20.9)	– (0–4.8)	29.5 (7.8–133.9)	9.2 (6.1–13.9)

*Samples collected Aug 2003–Feb 2006. + indicates number of positive results on immunochromatographic test. OR, odds ratio; CI, confidence interval. CI bands for the <1 y and 20–<40 y age groups were obtained by using the Fisher exact test.

Table 2. Distribution of G and P types of rotavirus in rural communities of Esmeraldas and in Quito, Ecuador*

Location	P[6]G1	P[6]G9	P[8]G9	P[8]G_{NT}	P_{NT}G9	$P_{NT}G_{NT}$	P[4]/P[8] G2	P[4]/P[8] G2/G9	P[8] G2/G9	P[6]/P[8] G9	Total
Esmeraldas	3	1	29	2	4	8	0	0	0	0	47
Quito	1	0	21	0	1	1	1	2	1	1	29

*As determined by genotype specific, multiplex reverse transcription–PCR. Samples were collected from 14 rural communities and Borbón in Esmeraldas and from Quito, Feb 2005–Feb 2006. Undetermined types are designated NT. Coinfection with different genotypes is designated with a slash.

G9 genotype, particularly P[8]G9, is spreading throughout Latin America. Also, the present study appears to be one of the few community-based descriptions of rotavirus infection (*20–22*). Symptomatic persons were actively identified in the community, recruited into the study, and matched with 3 asymptomatic controls each. This approach presents a more complete picture of rotavirus infection in rural communities than would be possible with the clinical sampling used in most previous studies that presumably focused on more urban environments. The high rate of rotavirus infection among symptomatic persons >40 years of age may be due to this age group's lack of exposure to the emerging rotavirus genotype and is an observation that might have been missed in a purely clinical study.

The G9 genotype has been documented since the early 1980s (*23,24*). Throughout much of the 1980s and 1990s, G9 was considered very rare; however, recent reports have described it as increasingly important (*2,3,25*). In the United States, the G9 genotype was detected in a 1995–1996 outbreak (*8*) and maintained its presence in the subsequent 2 years, with an average detection rate across 10 US cities of 7% (*26*). In Australia, the overall G9 detection rate, averaged across 3 population centers, increased from <1% in 1997 to 29% in 2001 (*27*). In Japan, G9 was essentially undetected throughout the mid-1980s and 1990s until it suddenly appeared in several cities in 1998–1999 (*28*). In India, G9 strains were detected for the first time in the late 1980s and throughout the early 1990s were usually found in combination with the P[11] or P[6] genotypes at a detection rate of about 20% (*12*). A study of 6 population centers across India during 1996–1998 found an overall G9 detection rate of 17% but found G9 as the major strain (and for the first time associated mainly with P[8] genotype) in New Delhi in late 1998 (*29*). At 17 sites throughout the African continent during 1996–1999, the G9 detection rate was generally ≤5% (*30*), with the exception of Ghana (1997–1999), where it comprised 28% of rotavirus positive samples, and Nigeria (1998–1999), where it comprised 37% (*31*). In Europe, many instances of G9 detection have been reported from the late 1990s through the early 2000s (*16,32–34*).

Latin America, in particular, has seen a surge in dominance of this genotype in recent years. In Rio de Janeiro, Brazil, during 1997–1999, the detection rate was ≈15% (*6,7*). In São Paulo, Brazil, during 1996–2003, the rate was 17% overall but in the last 2 years, G9 accounted for 30%–50% of rotavirus infections (*35*). To our knowledge, only 4 studies have reported G9 detection rates as high as those in our study: 75% in Paraguay, 2000 (*36*); 75%–90% in Salvador, Brazil, 1999–2002 (*9*); 92% in Chiang Mai, Thailand, 2000–2001 (*37*); and 73% in Alice Springs, Australia, 2001 (*27*).

A potential source of bias in this study comes from the incomplete typing of 13% of the putatively rotavirus-positive specimens and the inability to type an additional 17%. These incomplete or untypeable samples, which were positive by immunochromatographic tests, may be the result of inappropriate handling or storage of some fecal samples, which can be complicated in remote community studies such as this. Typing failure because of sample degradation or false-positive ELISA results is not likely to result in biased results, and the lack of visualizable rotavirus RNA bands among the untypeable samples suggests that degradation is a likely cause of typing failure. However, in any PCR-based typing scheme, typing failure may be caused by primer–template mismatch, which could bias results, especially with novel strains (*16,38*). A nonsystematic review of 16 recent studies suggests that failure of PCR-based G-typing is relatively widespread, although the failure rate varies. Four studies report <5% typing failure (*16,33,35,37*), 3 report 5%–10% failure (*9,12,26*), 2 report 10%–20% failure (*29,36*), 2 report 20%–30% failure (*27,30*), and 2 report >30% failure (*31,34*). An additional 3 studies do not explicitly state whether all typed samples yielded results (*6,7,32*). A more complete picture of global rotavirus diversity should facilitate efforts to improve molecular typing techniques. In this study, the maximum possible bias would affect the Esmeraldas community results by lowering the G9 infection rate from 92% to 72% and the P[8]G9 from 88% to 62%. In the Quito samples the effect is much less pronounced, potentially lowering the G9 infection rate from 96% to 90% and P[8]G9 rate from 93% to 86%. Further studies are required to narrow this uncertainty; however, even the lower end

Table 3. Estimated percentages of rotavirus-positive persons infected with P[8], G9, and P[8]G9, Ecuador*

Location	P[8], n (%)	G9, n (%)	P[8]G9, n (%)
Esmeraldas	31 (66–89)	34 (72–92)	29 (62–88)
Quito	26 (90–96)	26 (90–96)	25 (86–93)

*Assumes that all nontypeable samples were either degraded (upper bound) or a novel strain (lower bound).

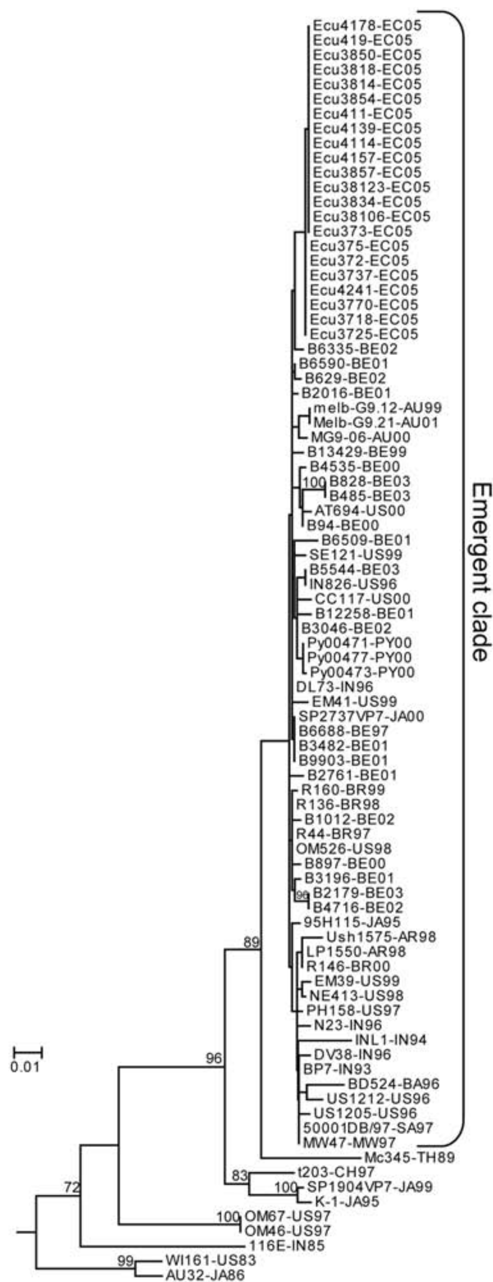

Figure. Maximum likelihood phylogenetic tree constructed from VP7 nucleotide sequences of G9 genotype rotavirus isolates. Taxa included are the 22 sequences from the current study and 65 sequences obtained from GenBank that represent global G9 rotavirus diversity. Taxa labels indicate isolate name followed by the country and year of collection. Country abbreviations: AU, Australia; BA, Bangladesh; BE, Belgium; BR, Brazil; CH, China; EC, Ecuador; IN, India; JA, Japan; MW, Malawi; PY, Paraguay; SA, Republic of South Africa; TH, Thailand; US, United States. The 22 Belgian isolates were selected from the GenBank PopSet AY487853-AY487895. Bootstrap values >70 are shown on internal branches. The tree was rooted with the VP7 sequence of a G3 genotype rotavirus (AY740736). GenBank accession nos. for the diversity isolates are listed in the online Figure legend, available from www.cdc.gov/EID/content/13/4/574.htm

estimates indicate that P[8]G9 is the predominant strain in Ecuador.

Additional evidence that G9 rotavirus is spreading through Latin America comes from comparing our nucleotide sequences to other sequences reported in GenBank. The sequences from the current study cluster into a large clade, which includes most of the recently isolated G9 rotavirus reported in the literature. This "emerging clade" is relatively homogenous: most isolates within the clade have ≤1% sequence divergence, an observation about G9 that has been made previously (*39*). However, the more recent regional isolates do tend to cluster together, as is the case for subclades composed of strains from Australia, Paraguay, or Ecuador (Figure). The low bootstrap support for these subclades is due to the small number of single nucleotide polymorphisms differentiating them.

The increasing prevalence of G9 rotavirus is particularly relevant given that many countries, including Ecuador, have approved the use of 2 rotavirus vaccines (*10,40*), but despite the wide distribution of G9 during the past 9 years (*2,3,6–9*), neither vaccine formulation includes the serotype G9 antigen (*10,40*). Studies have shown that some vaccines that do not contain the G9 antigen may still be capable of eliciting protective immunity against the G9 serotype (*10*). This immunity is most likely attributable to the G9 genotype's common association with P[8], which is included in both vaccines. However, cross-immunity may not be universal, as has been seen with type P[4]G2 (*2,3,10*). Continual surveillance of circulating types, therefore, should be carried out before to the introduction and during the implementation of rotavirus vaccination programs.

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Effectiveness of Interventions to Reduce Contact Rates during a Simulated Influenza Pandemic

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Measures to decrease contact between persons during an influenza pandemic have been included in pandemic response plans. We used stochastic simulation models to explore the effects of school closings, voluntary confinements of ill persons and their household contacts, and reductions in contacts among long-term care facility (LTCF) residents on pandemic-related illness and deaths. Our findings suggest that school closings would not have a substantial effect on pandemic-related outcomes in the absence of measures to reduce out-of-school contacts. However, if persons with influenzalike symptoms and their household contacts were encouraged to stay home, then rates of illness and death might be reduced by ≈50%. By preventing ill LTCF residents from making contact with other residents, illness and deaths in this vulnerable population might be reduced by ≈60%. Restricting the activities of infected persons early in a pandemic could decrease the pandemic's health effects.

Three influenza pandemics have occurred during the 20th century (in 1918, 1957, and 1968), and another pandemic is inevitable (*1*). The requirements for a pandemic virus include the existence of a new influenza A hemagglutinin for which there is little immunity, the ability of this strain to infect humans efficiently, and person-to-person transmission. Such viruses are likely to arise in densely populated agricultural communities where contact between humans and birds or pigs are close and persistent (*2*). In 1997, a highly pathogenic avian influenza A (H5N1) virus was transmitted from live poultry to humans in Hong Kong Special Administrative Region, People's Republic of China, killing 6 of 18 infected persons (*3*). From December 2003 through June 6, 2006, the World Health Organization confirmed 225 human cases and 128 deaths associated with influenza A (H5N1) infections in humans (*4*), and in October 2005, influenza A (H5N1) infections among birds were identified for the first time in Europe. Currently circulating influenza A (H5N1) viruses appear to infrequently infect humans, and person-to-person transmission, if it occurs, is certainly not efficient. However, international health officials are concerned that, as human exposure to such viruses increases, so does the possibility that a pandemic virus might appear.

The next influenza pandemic in the United States could result in 89,000 to 207,000 deaths, 314,000 to 734,000 hospitalizations, and 18 to 42 million outpatient visits, with a direct economic effect between US $71 and $166 billion, according to 1 set of estimates (*5*). Others have described the possible effects of vaccine and antiviral interventions. One study estimated that vaccinating 60% of the population would be necessary to achieve optimal cost benefits, assuming that development and mass production of a vaccine would require 6–8 months after the pandemic virus was characterized (*5*). Longini et al. (*6*) estimated the effectiveness of rapid targeted antiviral prophylaxis of persons early in a pandemic by using epidemic stochastic simulations. They found that if the next pandemic virus had a similar virulence to that of the 1957–58 pandemic virus, then delivering prophylaxis to 80% of exposed persons for up to 8 weeks could reduce attack rates by 2%–33% and death rates by 0.04–0.58/1,000 persons. However, such a strategy would require a stockpile of 1.9 billion doses of antiviral agents, which exceeds the current production capacity for these drugs for at least the next 5 years.

In the absence of adequate supplies of vaccines and antiviral agents, at least during the first wave of an influen-

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za pandemic, public health officials should consider using interventions designed to reduce the number of contacts between infected or exposed persons and susceptible persons. The US Department of Health and Human Services Influenza Pandemic Plan discusses several possible containment strategies, including those directed to single persons or entire communities (*7*). We used new stochastic simulation models to estimate the effects of several interventions of this kind. These models represented the spread of a pandemic in an urban US community, allowing for contacts in different settings (or mixing groups), including households, daycare centers, schools, workplaces and long-term care facilities (LTCFs). By using the age distribution of the US population (*8*), we placed each person in the community in a stratum, defined by age group and (if ≥65 years of age) by residence in the community or in an LTCF. Person-to-person transmission probabilities depended on the daily duration of contacts. Contact rates and their duration varied by each person's stratum and mixing groups. By using these models to simulate an influenza pandemic, we estimated the effects of school closings, home confinement of ill persons (i.e., isolation) or their household contacts (i.e., quarantine), and reduction of contacts among residents of LTCFs on overall illness attack rates, hospitalization rates, and mortality rates.

Materials and Methods

Simulation Model

We simulated an influenza outbreak in a small urban US community. The simulation model used data from the Asian influenza A (H2N2) pandemic in 1957–58 (*6*) and from studies on US influenza-related excess rates of hospitalizations and death (*9–11*). The simulation process begins with the generation of a community of households, where the distributions of sizes of the households and ages of the household members follow the 2000 US Census. Every person in the community belongs to 1 of 5 age-dependent strata: preschool children (ages birth–4 years), schoolchildren (ages 5–18 years), adults (ages 19–64 years), seniors (ages ≥65 years) living at home, and seniors (ages ≥65 years) living in an LTCF. In addition, each person belongs to ≥1 mixing groups, according to his or her stratum: households, daycare centers, schools, workplaces, LTCFs, and the community. The mixing matrix is presented as Table 1.

On any given day, a susceptible person, A, makes contacts with other persons that may lead him or her to become infected. These contacts take place in each of A's mixing groups. The probability that person A becomes infected depends on the following input parameters: 1) the number of different persons with whom person A has contact in each mixing group, 2) the total duration, in minutes, of all the contacts with each of these persons, and 3) the per-minute rates of infection transmission if the contacted person is infectious. The number and duration of contacts may be different on weekdays and weekend days. The values of the parameters that were used in this study are presented in the online Supplemental Materials Appendix (available at http://www.cdc.gov/eid/content/13/4/581.htm). Once person A becomes infected, he or she undergoes a latent period, followed by a period in which he or she is infectious. The mean length of the latent and infectious periods are input parameters.

This model has 3 new features that are not shared by the commonly used simulation models (such as the model in [*6*]) for transmission of influenza: 1) the probability of transmission depends on the total duration of all contacts between 2 persons, rather than on the number of times they make a contact, 2) the transmission parameters do not depend on the population size, and 3) different contact parameters can be specified for weekdays and weekend days. Technical details of the simulation model are presented in the Supplemental Materials Appendix. The basic reproductive number (R_0) for this model is 2.7. This value is within the range (2.0–3.0) estimated by Mills et al. (*12*) for the 1918 influenza pandemic.

Interventions

The interventions we examined in this simulation study were school closings, confinement of ill persons and their household contacts to their homes, and reduction in contact rates among residents of LTCFs. Interventions were implemented at the start of the outbreak.

School Closings

When this intervention was implemented, schools closed when the prevalence of illness among children in

Table 1. Mixing matrix for the simulation model

Age stratum, y	Mixing group					
	Household	Daycare center	School	Workplace	Community	LTCF*
<1–4	+	+			+	
5–18	+		+		+	
19–64	+			+	+	
≥65, at home	+				+	
≥65, in LTCF						+

*LTCF, long-term care facility.

the school exceeded a predetermined threshold, set to 10%, 15%, or 20% in the simulations. A school remained closed for a predetermined period (7, 14, or 21 days). On weekdays, household and community contact parameters of children whose school was closed were assigned their weekend levels; their contacts with other children who continued to attend school and with working adults did not change.

Confinement to Home

When this intervention was implemented, a given fraction of households were assumed to comply. If a household complied, then all of its members followed the confinement rules unless they had been previously ill and had recovered. We considered 2 types of confinement: ill persons only, and ill persons and all the members of the same household. Confinement began after a given number of days of illness (1, 2, or 3 days) and did not depend on the severity of illness. If symptoms were severe, then the person reduced his or her duration of contacts with other household members by 50%.

When a person was confined on a weekday (because of his or her illness or illness of another household member) and did not withdraw due to severe symptoms, then the duration of contacts with household members who continued to go to school or work did not change. Durations of contacts with household members who stayed at home and were not withdrawn were the same as on a weekend day.

When ill persons were confined, they returned to school or work 1 day after their illness ended. When ill persons and other household members were confined, a person returned to school or to work 1 day after his or her illness ended (even if other ill persons remained in the household). A person who did not become ill returned to school or work on the third day after the last day of illness of any household member (because the length of the latent/incubation period was assumed to be 2 days).

Reduction of Contacts in LTCFs

We examined the effects of 2 interventions on LTCF residents: reduction in duration of contacts with other residents who were ill, and reduction in duration of contacts with visiting family members. Contacts with LTCF staff did not change.

Effectiveness of Interventions

We first ran a set of 200 simulations using the baseline settings for all the parameters, without any interventions (online Supplemental Materials Appendix). The average rates for the 3 outcomes of interest—overall illness rate, hospitalization rate, and death rate—were calculated for 200 simulations and used as baseline rates. For each intervention, we ran a set of 200 simulations and used the averages of these simulations as estimates of the expected rates under this intervention. The effectiveness of each intervention was defined as follows:

Effectiveness = [(baseline rate) – (rate with intervention)]/baseline rate

Sensitivity Analysis

We performed a sensitivity analysis to assess the robustness of our findings regarding the effectiveness of the 3 modeled interventions. In common with all simulation studies, our findings depended on several parameters for which we have estimated values that we believe are reasonable starting points. These values included baseline contact rates, the probability of illness given infection, the relative infectiousness of an infected person without influenza symptoms, the probability of withdrawal to home because of severe symptoms, and the reduction in contact rates due to severe symptoms. We varied the values of these parameters and examined the effects of these changes on estimates of the effectiveness of school closings and confining ill persons to their homes.

Results

Baseline Rates

Based on the 200 simulations conducted with the baseline values of the pandemic parameters, the baseline rate of illness was 32.1%, (95% confidence interval [CI] 31.2%–32.9%), the baseline rate of hospitalization was 196.9/100,000 (95% CI 183.2–210.6) and the baseline rate of death was 63.4/100,000 (95% CI 56.2–70.6). These results were based on the assumption that the illness rates would be similar to their values in the 1957 influenza pandemic.

School Closings

Two parameters affected the effectiveness of school closings: the percentage of ill schoolchildren required to close a school and the number of days the school remained closed. The effectiveness of the intervention varied as a function of the percentage of ill persons required for closing a school and the duration of the closure (Figure 1). For example, if each school were closed for 7 days when the proportion of ill children exceeded 10%, then the overall illness rate was 0.288 (95% CI 0.278–0.297). The baseline illness rate was 0.321; therefore, the effectiveness of this intervention was (0.321–0.288)/0.321 = 0.103 (95% CI 0.075–0.131). As expected, effectiveness usually decreased as the percentage of ill children required to close a school increased. The effect of the length of closure was less clear (Figure 2). When schools were closed, transmission in households and in the community increased; thus, school closings could increase death and illness rates in

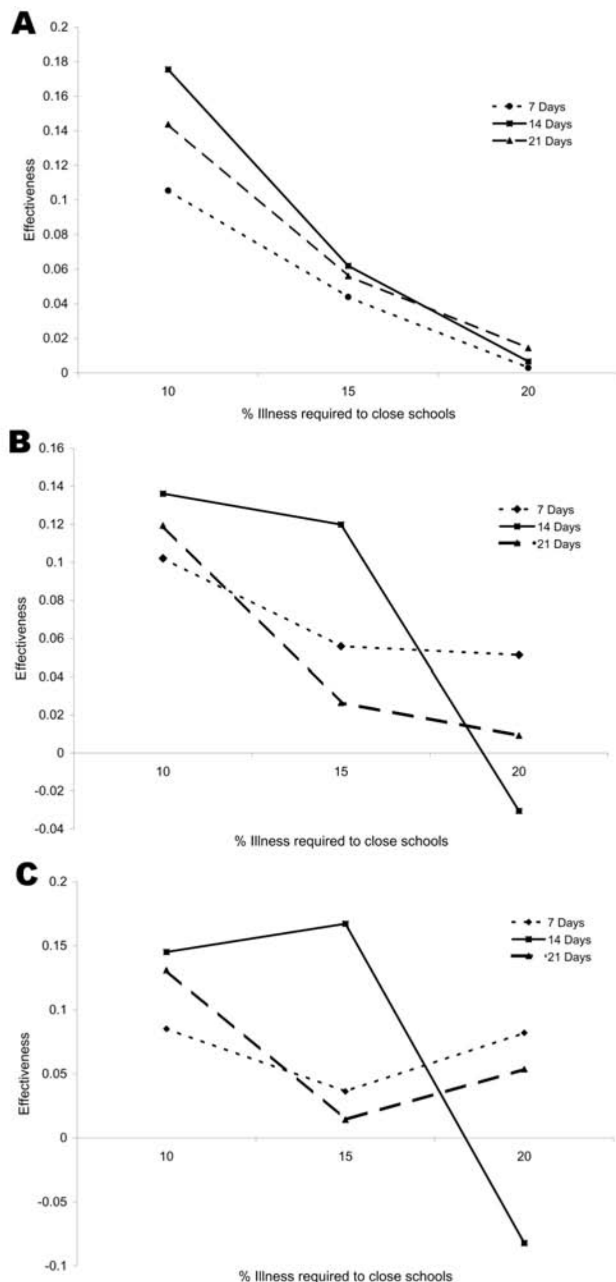

Figure 1. Estimated effectiveness of closing schools on illness (A), hospitalization (B), and death (C) rates during a simulated pandemic.

some groups. For example, when the illness rate required for school closing was 10%, then closing schools for 14 days had the largest effect on hospitalization rates, compared with closings of 7 or 21 days. However, when the rate for closing was 20%, then closing schools for 14 days had a smaller effect on hospitalization rates than closing for 7 or 21 days.

Confinement to Home

In our models, confinement to home took place after a person showed symptoms of influenza. A delay of 1, 2, or 3 days occurred between onset of symptoms (which coincided with the onset of infection) and the beginning of the confinement period. This delay and the proportion of households that complied with the confinement rules affected the effectiveness of the intervention. Figure 2 presents the effectiveness of these interventions as a function of the percentage of households that comply (between zero and 80%) for a delay of 2 days. As expected, effectiveness usually increased with the compliance percentage. Confining the ill persons and their household members was more effective than confining the ill persons only. For example, given a delay of 2 days and 60% compliance, the effectiveness of these interventions on illness rates was 0.33 for confining the ill only and 0.80 for confining ill persons and their household members. Effectiveness decreased when the length of the delay was increased.

Reducing Contacts in LTCFs

Reducing contacts with ill residents of LTCFs decreased the rates of illness, hospitalization, and death for LTCF residents by >50% (Table 2). Reducing contacts also decreased the rates of hospitalization and death in the general population by up to 14% and 24%, respectively.

Effect of Intervention on Dynamics of the Pandemic

Figure 3 presents the dynamics of the pandemic (A) without any intervention, (B) when schools are closed for 14 days as the proportion of ill children exceed 10%, and (C) when ill persons and all their household contacts are confined after the second day of illness of the index case-patient and compliance is 40%. We see that these interventions do not affect the time to the peak of the pandemic (around week 5). The rate of decline following the peak does not change under confinement to home, while it slightly decreases under school closing.

Sensitivity Analysis

The value of the basic reproductive number (R_0) for the baseline setting of our parameters is 2.7. Because this value is higher than values used in recent simulation studies (*13*,*14*), we evaluated the effectiveness of the interventions under smaller values of R_0. We found that reducing R_0 resulted in an increase in the effectiveness of confinement to home and a decrease in the effectiveness of school closings. Thus, our findings regarding the effectiveness of confinement and the lack of effectiveness of school closings remain valid for smaller values of R_0. The results of additional sensitivity analyses were as follows.

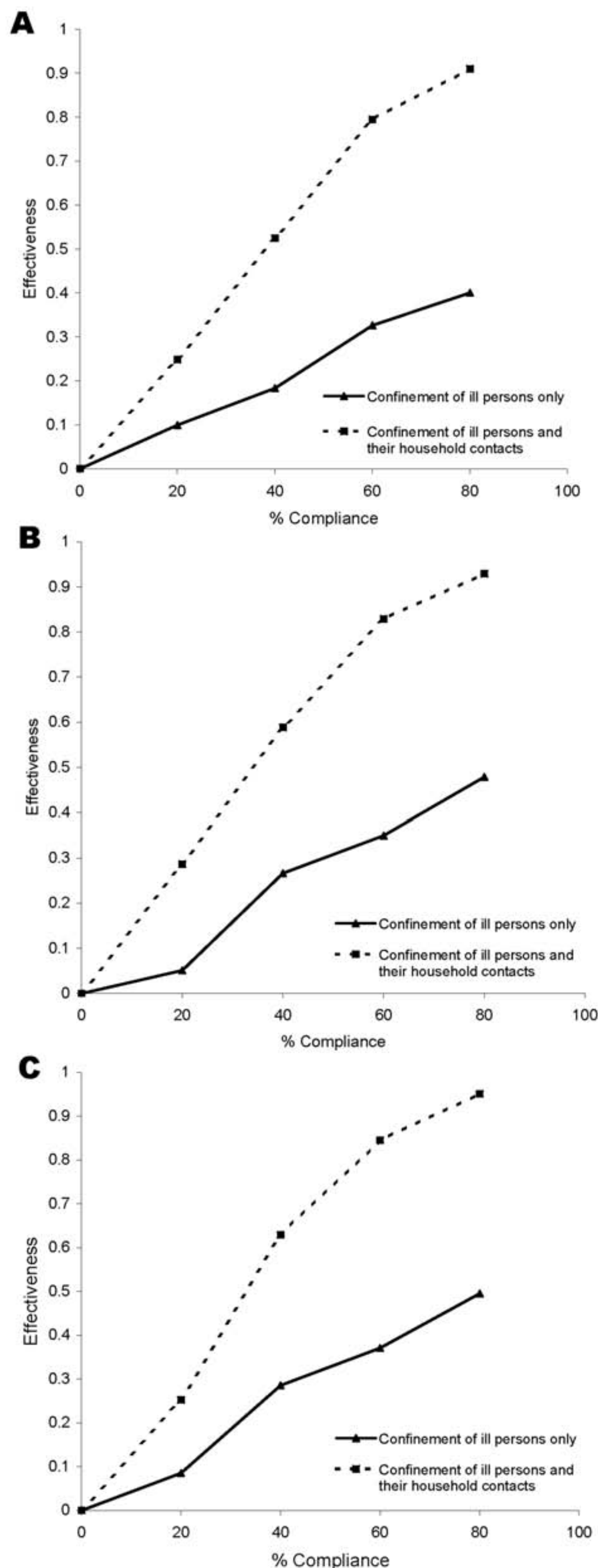

Figure 2. Estimated effectiveness of confinement to home 2 days after onset of respiratory symptoms on illness (A), hospitalization (B), and death (C) rates during a simulated pandemic.

School Closings

The most important parameters related to the effectiveness of school closings are those that underlie the contacts between children while they are in school. In our simulations we assumed that on a school day each child makes contact with 10 other schoolchildren, each contact lasting 120 minutes (see section D.1.a in the online Supplemental Materials Appendix). Some of these contacts may be concurrent. To examine the effect of changing each child's exposure to other schoolchildren on the effectiveness of school closures, we increased and decreased the baseline duration of 120 minutes by 50%. Table 3 shows the effectiveness of closing schools for 14 days for the 3 baseline values of duration of school contact. As we see, longer or shorter durations of contact while schools are open do not result in substantial changes in the effectiveness of school closings.

Confinement to Home

We varied the values of several parameters in the baseline model and examined the effects these changes had on estimates of the effectiveness of confinement of ill persons to their homes (Table 4). We assumed that 40% of ill persons without severe symptoms were confined to home within 2 days of symptom onset. When the fraction of infected persons who developed symptoms was increased from 0.67 to 0.93, then the illness rate without an intervention (i.e., at the baseline level) changed only from 0.333 to 0.319, while implementation of the intervention changed this rate from 0.272 to 0.242. Thus, the effectiveness of this intervention increased from 0.183 to 0.241. The alternative values we used in Table 2 modeled a more severe pandemic than the pandemic modeled with the baseline initial values.

Discussion

The continuing epizootic of influenza A (H5N1) among birds in Asia and Europe has raised concerns that the likelihood of an influenza pandemic may be increasing. Shortages in the supply of neuraminidase inhibitors, the antiviral agents most likely to be effective against a pandemic influenza strain, and the months needed from the isolation of a pandemic strain until the availability of vaccine suggest that reducing contact rates between infected and uninfected persons will represent one of the few sets of interventions that can be rapidly implemented. We used a stochastic simulation model to estimate the effectiveness of several interventions that could reduce contact rates on pandemic-related outcomes.

The Pandemic Influenza Strategic Plan and Public Health Guidance for State and Local Partners prepared by the US Department of Health and Human Services was released on November 2, 2005 (*7*). This plan discusses the

Table 2. Estimated effects of pandemic interventions in long-term care facilities (LTCFs) on illness, hospitalization, and death rates

Outcome rates	Rates for general population			Rates for LTCF residents		
	Illness	Hospitalization	Death	Illness	Hospitalization	Death
Reduction in contacts with ill residents,%						
25	0.02*	0.10	0.14	0.22	0.32	0.33
50	0.04	0.13	0.23	0.37	0.44	0.41
75	0.04	0.14	0.24	0.54	0.55	0.59
100	0.03	0.14	0.21	0.65	0.60	0.60
Reduction in contacts with visitors, %						
25	0.01	0.11	0.12	–0.02	0.03	–0.03
50	0.02	0.06	–0.02	0.03	–0.05	–0.05
75	0.04	0.15	0.20	0.00	0.05	–0.03
100	0.04	0.07	0.12	0.03	0.11	0.11

*Thus, a 25% reduction in contacts with ill residents of LTCFs was estimated to reduce the illness rate for the population by 2% and the illness rate for LTCFs by 22%.

use of individual-level (e.g., isolation and quarantine) and community-level (e.g., school closings) containment measures. Our study considered possible interventions of both kinds, including early identification and confinement of case-patients and their household contacts, limiting visits to LTCFs, and closing of schools.

Our findings suggest that closing schools would result in relatively small reductions in morbidity and mortality rates during a pandemic. For example, when schools were closed when ≥10% of children had influenza symptoms and remained closed for 14 days, the rates of illness, hospitalization, and death decreased from the baseline rates of 32.1%, 197/100,000, and 63/100,000 to 26.5%, 170/100,000, and 54/100,000, respectively. Thus, the effectiveness of school closings was ≈14%–18%. When we increased the threshold of illness incidence required for school closing to 20%, then these rates were 31.9%, 203/100,000, and 69/100,000, respectively. These mild decreases in the rates of illness and death after school closures are explained by the fact that in our models, children whose schools were closed were more likely to increase their contacts with other groups. The attack rate of 62% that we used for school-age children may be considered high. However, if the attack rate were reduced, school closings would have an even smaller effect. Our results do not contradict recent findings that vaccination of schoolchildren could be effective in controlling transmission during a seasonal influenza epidemic (*15*). Vaccination of children reduces their chances of infection and of transmitting infection to household and community contacts, whereas closing schools may not decrease the likelihood of infection substantially and could increase the probability that an infected child will infect household and community contacts (*14*).

The effect of school closings on overall illness rates in an influenza pandemic has been estimated in other recent simulation studies. Germann et al. (*16*) modeled the effect of a pandemic on the entire US population. They found that for $R_0 \geq 1.9$, closing of schools without any additional interventions had limited effectiveness. On the other hand, for $R_0 \leq 1.6$, school closings reduced the extent of illness. Carrat et al. (*17*), by using a simulation model for the spread of influenza in a community, found school closings to be effective. We believe that these inconsistencies in the reported effects of school closings depend on the details of the various simulation models, especially on the way the community is affected by school closing in terms of increased contact rates of schoolchildren when their school is closed.

Our simulations predict that it might be possible to decrease illness and death rates by as much as 50% by reducing the contact rates of all ill persons. However, achieving this level of effectiveness would require persuading 60% of those with symptoms to withdraw to their homes and confine themselves. Simulation studies by Longini et al. (*13*) and Ferguson et al. (*14*) found that quarantine, when used in conjunction with vaccines and antiviral agents, would be effective in containing an influenza pandemic in Southeast Asia. One should remember that the effectiveness of any behavioral/social intervention may vary across cultures.

Residents of LTCFs are likely to be at high risk for serious pandemic-related illness and death. We found that by limiting contacts of ill residents, illness and death may be reduced among other residents. These are notable findings, as this vulnerable population responds poorly to seasonal influenza vaccination, and they are unlikely to receive the limited quantities of pandemic vaccine when it first becomes available.

The effectiveness of any particular intervention designed to reduce contact rates depends on the initial values selected for the parameters affecting influenza transmission (e.g., contact durations, probability of withdrawal due to severe symptoms), and a limitation of our study is

that few data exist on which to base these values. Studies designed to obtain reliable estimates of these parameters during seasonal, interpandemic influenza outbreaks should be a high priority. However, the major findings of this study seem to be robust, given a range of realistic values for the parameters we used. The target attack rates we used to calibrate the contact parameters (provided in the Supplemental Materials Appendix) are high, but lowering these attack rates should not have a major effect on our findings, because both the pre- and postintervention incidence rates would decrease concomitantly.

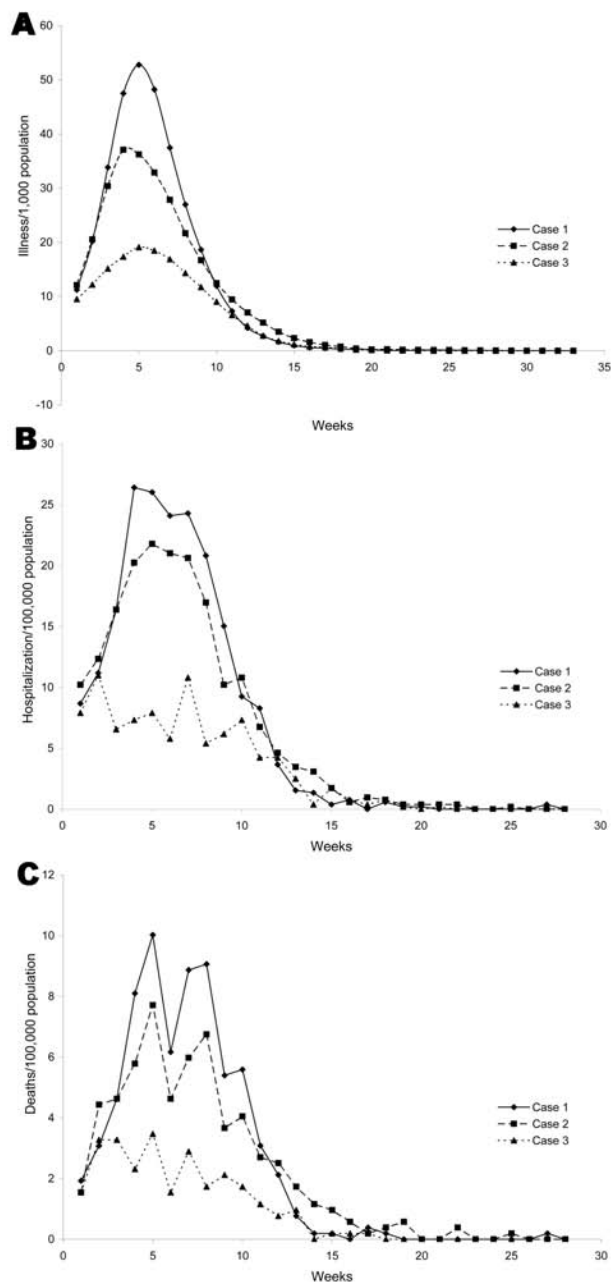

Figure 3. Dynamics of the influenza pandemic. Case 1: no interventions. Case 2: schools are closed for 14 days when prevalence reaches 10%. Case 3: ill persons and all their household contacts are confined to their homes after the second day of illness of the index case-patient, and the compliance rate is 40%. A) illness; B) hospitalizations, C) deaths.

We did not make formal estimates of the economic costs and benefits of the interventions we examined. However, some likely consequences of school closings may be considered, given current childcare practices. Obviously, the longer the duration of school closure, the more costly the consequences as working parents either have to take time off work to supervise children or pay for somebody else to care for them. If a large number of school days are lost, school districts might consider extending the school year, which would incur additional costs, although the conditions would be expected to vary greatly between school districts. These increased costs would have to be weighed against the limited predicted effectiveness of this intervention. Encouraging the voluntary withdrawal of ill persons appears to be a more effective strategy than school closings in reducing the impact of a pandemic, and it may represent a relatively inexpensive intervention. However, researchers have found that US workers routinely miss <1 day of work after reporting onset of influenzalike illness (*18*). Encouraging longer durations of work loss could decrease compliance with self-isolation and increase the economic cost per case avoided. Home quarantine of the immediate family members of an ill person would likely increase the costs per case averted. For example, during the quarantine efforts related to the severe acute respiratory syndrome outbreak in Toronto (*19*), many families found it too expensive to rigidly comply with a household-level quarantine of ≥7 days.

Our stochastic simulation model has several strengths. The model considers the length of time 2 persons are in contact, in addition to the total number of contacts. The model parameters we used are not related to the size of the simulated population, unlike previous models (*6*). We repeated the simulations conducted for this study with a population twice as large as the original population and the same input parameters. The resulting rates were almost unchanged, so the differences can be attributed to the random effects associated with these simulations. The weaknesses of our present model are that it requires many input parameters and that it does not include the effects of antiviral medications. Our model allows for estimating vaccine effects for susceptibility and infectiousness; however, this option was not used in the present study.

On February 1, 2007, the Centers for Disease Control and Prevention issued an Interim Pre-Pandemic Planning Guidance: Community Strategy for Pandemic Influenza Mitigation in the United States (*20*). This document recommends several nonpharmaceutical interventions

Table 3. Effect of baseline contact durations in school on effectiveness of closing schools for 14 days

School baseline contact duration, min	% Ill for school closing	% Effectiveness		
		Illness rate	Hospitalization rates	Death rates
120	10	17	14	14
180	10	17	16	20
60	10	12	5	6
120	15	6	12	17
180	15	8	8	12
60	15	3	1	–13
120	20	1	–3	–8
180	20	3	4	5
60	20	–1	0	–8

during a severe pandemic, including isolation of persons with confirmed or probable influenza, voluntary home quarantine of members of households with confirmed cases, dismissal of students from schools and school-based activities, and closure of childcare programs. During a pandemic with a severity index of 4 or 5 (defined as a case fatality rate of ≥1%), this new guidance recommends not only school dismissals of ≤12 weeks but also measures to protect children from being exposed or exposing others to the pandemic virus via reduction of their out-of-school social contacts and community mixing. In this article, we assessed the effectiveness of school closures of 1–3 weeks duration after school absenteeism rates reached high levels. We assumed that children dismissed from schools would increase their out-of-school contacts. These assumptions reduced the effectiveness of school closures in our model. In future work, we will explore the effectiveness of early dismissal of students from schools, together with changes in out-of-school contacts, and other interventions using our model.

In summary, if persons who suspect they are infected with pandemic influenza virus were to withdraw to their homes quickly, the rates of illness and death associated with a pandemic may be substantially reduced. The withdrawal of all household contacts may further reduce rates of illness and death, but this additional intervention is likely to be relatively costly and difficult to implement. Restricting the movement of ill LTCF residents will be beneficial in reducing their adverse health outcomes. Before early and rapid implementation of such interventions during a pandemic is feasible, the public will need to be educated about the early symptoms of influenza and measures developed to increase the social acceptability of self-isolation when ill.

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Table 4. Effectiveness of confinement of ill persons to their homes, with a 2-d delay and 40% compliance, for differing values of the initial parameters

Parameter	% Effectiveness		
	Illness rates	Hospitalization rates	Death rates
Rate of withdrawal due to severe symptoms (children/adults)			
0.75*/0.50*	0.18	0.27	0.29
0.55/0.30	0.34	0.40	0.41
Relative contact duration when withdrawn due to severe symptoms			
0.50*	0.18	0.27	0.29
0.70	0.14	0.21	0.19
Fraction of infected persons having symptoms			
0.67*	0.18	0.27	0.29
0.93	0.24	0.24	0.27
Relative infectiousness of non-ill persons			
0.50*	0.18	0.27	0.29
0.70	0.19	0.24	0.28

*Values used in the baseline simulation models.

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Spanish Influenza in Japanese Armed Forces, 1918–1920

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With the recent outbreaks of avian influenza A (H5N1), the risk for the next influenza pandemic has increased. For effective countermeasures against the next pandemic, investigation of past pandemics is necessary. We selected cases diagnosed as influenza from medical records and hospitalization registries of Japanese army hospitals during 1918–1920, the Spanish influenza era, and investigated clinical features and circumstances of outbreaks. Admission lists showed a sudden increase in the number of inpatients with influenza in November 1918 and showed the effect of the first wave of this pandemic in Tokyo. The death rate was high (6%–8%) even though patients were otherwise healthy male adults.

Because of the emergence of avian influenza A (H5N1) virus in Southeast Asia, potential evolution of a novel type of influenza in the near future is of great concern (*1,2*). If an outbreak of a novel form of influenza occurs, a major worldwide pandemic is predicted because humans would not have immunity against this virus. To take effective countermeasures against new pandemics, investigation of past pandemics is essential.

Four pandemics occurred in the 20th century: Spanish flu in 1918, Asian flu in 1957, Hong Kong flu in 1968, and Russian flu in 1977 (*3,4*). Spanish influenza was the largest pandemic, and Japan was seriously affected. Despite abundant public records related to Spanish influenza, few primary documents, such as medical records, remain in Japan. Recently, medical records from the early 20th century were found in the depository of the International Medical Center of Japan (IMCJ) Hospital, Tokyo. We used these records to investigate the clinical characteristics of Spanish influenza. To help prepare countermeasures, we investigated the outbreak situation, clinical findings, and outcomes of Spanish influenza in the Japanese military during 1918–1920.

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Patients and Methods

The documents were stored at the medical history depository of IMCJ Hospital, at the medical records and hospitalization registries of Tokyo First Army Hospital (a predecessor of IMCJ Hospital), and at the Fifth Japanese Army Garrison Hospital, Krasnoyarsk, Russia. Medical records in which influenza was diagnosed between January 1918 and December 1920 were selected. Because the influenza virus had not yet been discovered at that time, no serologic or virologic diagnostic methods for influenza infection were available, and no examinations such as chest radiographs were performed. Thus, the diagnosis flu (*kanbo* in Japanese) was defined as clinical influenza.

Three types of documents were investigated. The first type was the hospitalization registry of Tokyo First Army Hospital, in which records of 127 patients from January 1918 through November 1918 were included. Because these records were bound, it was assumed that no records were missing. The second type was the medical records of 132 patients at the Fifth Japanese Army Garrison Hospital in Russia from March 1919 through April 1920. These records were also bound, and it was again assumed that no records were missing. The third type was the medical records of 419 patients at Tokyo First Army Hospital from January 1918 through May 1920. These records were not associated with time and were partially discontinuous, which indicated that some records (dates) were missing.

Information on the hospitals (e.g., numbers of beds and physicians) was unclear. This research was reviewed and approved by the research review boards at IMCJ

Hospital. Statistical significance of between-group differences was analyzed by using the Mann-Whitney U test. A p value <0.05 was considered statistically significant.

Results

We first investigated hospitalization registries of Tokyo First Army Hospital from January to November 1918. These registries had the names and diagnoses of patients admitted to the hospital on a monthly basis. Numbers of patients admitted for respiratory infectious disease during this period are shown in the Figure. Cases diagnosed as pneumonia, acute bronchitis, and influenza were classified as respiratory infectious diseases. Although records of patients with tuberculosis were found, we excluded them from this study. In the 10-month period from January to October, the mean (± standard deviation) monthly numbers of patients with pneumonia, acute bronchitis, and influenza were 10.9 ± 6.5, 10.0 ± 3.6, and 1.8 ± 4.1, respectively (22.7 ± 9.6 for all 3 illnesses). Death rates from pneumonia, bronchitis, and influenza during this period were 3.4% (4/116), 0% (0/109), and 0% (0/18), respectively. The number of influenza patients suddenly increased to 109 in November, and 9 of them died (8%). Because our information about these 109 patients was found only in the hospitalization registries (the patients' medical records were not found), we could not identify their clinical symptoms. However, it can be assumed that the hospital experienced the first wave of Spanish influenza in November 1918. Because no hospitalization registry before this period was found, comparison with outbreaks in average years was not possible.

We then investigated the medical records of the Fifth Japanese Army Garrison Hospital, which may have been a Japanese military field hospital in Krasnoyarsk, Russia. These records covered 2 years (1919 and 1920), but most cases of clinical influenza were concentrated in May–November 1919. All 132 cases were in infantrymen 19–49 years of age (mean ± standard deviation 22.7 ± 4.1 years); 47% had been farmers before entering the military, and 72% had no recorded medical histories. The initial symptom was sudden fever in 116 patients (94%), headache in 101 (81%), chills in 92 (74%), cough in 86 (69%), general malaise in 60 (48%), appetite loss in 56 (45%), pharyngalgia in 45 (36%), arthralgia/myalgia in 33 (27%), and vomiting/diarrhea in 27 (22%). On admission, reddish pharynx was noted in 105 (85%), coated tongue in 95 (77%), thoracic rale in 71 (57%), facial flush in 24 (19%), and conjunctival congestion in 16 (13%). The period from onset to admission was 4.7 ± 3.9 days (range 0–24 days), the duration of hospitalization was 14.8 ± 12.0 days (range 3–79 days), and the death rate was 6.0% (8/132). A comparison of 124 patients who survived and 8 who died is shown in the Table. In the patients who died, body

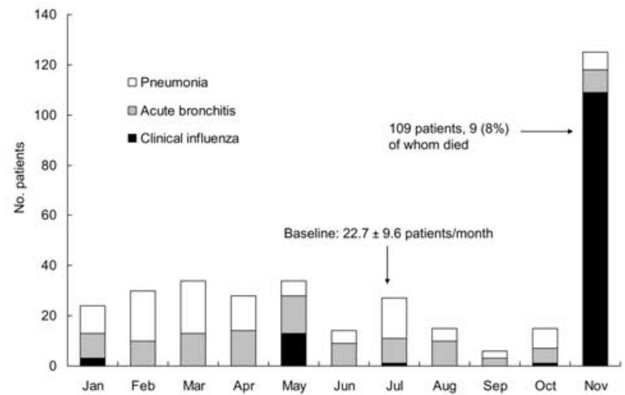

Figure. Number of patients hospitalized for respiratory infection, Tokyo First Army Hospital, 1918.

temperature and pulse rate were higher at the time of hospital admission, and thoracic rale and an "agonized facial expression" (a painful expression as reported by Japanese doctors) were observed in many patients. The death rate was higher in patients who had digestive symptoms, but the difference was not statistically significant. In fatal cases, the patients died an average of 13.9 days after symptom onset and 7.1 days after admission.

Fever patterns in 132 patients were classified as typical (fever resolved in <10 days after onset), prolonged (fever persisted for ≥10 days), biphasic (fever resolved and then recurred), and other (unclassifiable). A total of 77 (58%), 38 (29%), 7 (5%), and 10 (8%) patients, respectively, were classified as these types.

Discussion

Once a pandemic occurs, it may cause large-scale effects worldwide; various countries and organizations, including the World Health Organization, must prepare for such a situation (*5,6*). To take countermeasures against pandemics, past pandemics should be further investigated. Researchers are particularly interested in the biggest pandemic of the 20th century, Spanish influenza in 1918. Influenza A (H1N1) virus was the causative agent of Spanish influenza. However, this pandemic occurred almost 90 years ago, which makes investigation difficult.

Virus genes have been isolated from lung samples of patients who died of Spanish influenza. The nucleotide sequences of these genes have been determined, and the viral characteristics have been reported (*7,8*). However, the only method that can clarify the pathophysiology of Spanish influenza is investigation of the medical records at the time of the pandemic. Thus, the discovery of medical records and hospitalization registries of the Spanish influenza pandemic is useful. Although we studied only patients in Japan, studying patients in the Japanese Army

Table. Characteristics of patients who survived influenza with those who died of influenza, Fifth Japanese Army Garrison Hospital, 1919–1920*

Characteristic	Patients who survived (n = 124)	Patients who died (n = 8)	p value
Age, y	22.7	22.8	>0.9
Medical history	31/116	2/8	>0.9
Body temperature at time of hospitalization, °C	38.7	39.9	0.0005
Heart rate, beats/min	89	106	0.004
Rales	63/116	8/8	0.01
Reddening of throat	98/116	7/8	>0.9
Digestive symptoms	23/116	4/8	0.07
"Agonized facial expression"†	5/116	3/8	0.009
No. days from onset of illness to hospitalization	4.5	6.8	0.14
No. days hospitalized	14.8	7.1	0.04

*Mann-Whitney U test was used to test differences between the 2 groups. Fisher exact test was used to test differences in the ratio of the cross-calculation table. One case (at day 79) was excluded. This patient was admitted to the hospital with flulike symptoms but died of a bacterial infection.
†A painful expression as reported by Japanese doctors.

Garrison hospital in Russia may provide a clue to the conditions of the influenza outbreak in that region.

Many theories exist as to the source of Spanish influenza, but the first reported case in the United States likely occurred in March 1918 (*3*). After that, it spread worldwide through 1920. Of the 1.8–2.0 billion persons in the world at that time, 600 million were affected and 20–40 million died from this disease (*9*). In Japan, 23 million persons were affected, and 390,000 died. The first wave occurred in Japan in August 1918, and many cases were reported in Tokyo in mid-October (*10,11*). Hospitalization registries of the Tokyo First Army Hospital showed a sudden increase in admissions for influenza in November 1918; this outbreak may have been the beginning of the Spanish influenza pandemic in Japan.

Because the patients were all soldiers in the Japanese army, they were essentially healthy men. Medical records indicated that the soldiers did not go home for long periods and lived together in barracks without external contact. Once a virus infection occurred, it may have caused an outbreak among the soldiers within a short time. Initial symptoms were fever, headache, chills, and cough, and their frequency was not different from those of patients with seasonal or ordinary influenza. When cases in patients who survived were compared with those in patients who died (Table), high fever, tachycardia, thoracic rale, and an "agonized facial expression" were associated with poor outcomes. All treatments were antisymptomatic supportive therapy, and none of the drugs used was typical of modern medical care. Thus, most cases may not have been affected by treatments. The duration of hospitalization was shorter for patients who died, perhaps due to rapid exacerbation of symptoms and discharge within a short time.

Frequencies of hemoptysis and bloody sputum were reportedly high in patients with Spanish influenza (*12*), but high frequency of these signs was not observed in these patients, perhaps because of the limitation of our investigation to symptoms at the time of hospital admission. Bloody sputum was noted during hospitalization for some patients, but these episodes were excluded from the analysis because many descriptions of it during hospitalization were difficult to read. Because chest radiographs were not taken, we cannot discuss complications of pneumonia and its characteristics. However, chest auscultation indicated rales in 57% of the patients, which suggests that many patients also had pneumonia. Generally, complication by secondary bacterial pneumonia prolongs fever and leads to relapses (*13*).

To evaluate the presence of complications by bacterial pneumonia, we classified the fever patterns. Fever pattern was prolonged or biphasic in approximately one third of the patients, which suggests that many patients had secondary bacterial infection. The death rate was 6%–8%, and patients died an average of 2 weeks after onset of symptoms. The former Japanese Ministry of the Interior reported that the mean death rate from Spanish influenza in Japan was 1.21%–5.29% (*11*), but the death rate for our study patients was higher. The death rate from influenza is high for patients ≥65 years of age, but the death rate from Spanish influenza was also reported to be high for persons 20–30 years of age (*14,15*). The mean age of the population investigated was 22.7 years. Persons 20–30 years of age had the highest death rate and this may have been the reason for the overall higher death rate. The cause of a high death rate in young persons is unclear, but it may have been related to poor immunity because they had not previously been exposed to influenza virus. It may have also been related to poor conditions in military field hospitals at the end of World War I. Poor conditions in military field hospitals in foreign countries have also been reported (*16,17*).

Our study shows that when a novel influenza virus emerges, a large-scale outbreak can suddenly occur in large groups living together, such as military personnel. However, our data were incomplete because many documents were missing and radiographs were not available. Diagnoses were made solely on the basis of clinical symp-

toms, but this was unavoidable because no definitive virologic diagnostic techniques were available at that time.

We now have new tools against influenza, such as antiviral agents, vaccines, and rapid diagnostic methods, that were not available during the Spanish influenza pandemic. However, during the first wave of an outbreak of a new form of influenza, infection would be difficult to avoid and is likely to occur. To prepare for the next pandemic, a surveillance system for early detection of an outbreak (*18*), specific vaccines and rapid diagnostic test kits, and effective treatment strategies must be developed.

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RESEARCH

Global Distribution of Panton-Valentine Leukocidin–positive Methicillin-resistant *Staphylococcus aureus*, 2006

Anne Tristan,* Michele Bes,* Helene Meugnier,* Gerard Lina,* Bülent Bozdogan,† Patrice Courvalin,† Marie-Elisabeth Reverdy,* Mark C. Enright,‡ François Vandenesch,* and Jerome Etienne*

We determined the *agr* type, multilocus sequence type, protein A gene type (*spa* typing), toxin gene profile, and antimicrobial drug resistance profile of 469 isolates of Panton-Valentine leukocidin–positive community-acquired methicillin-resistant *Staphylococcus aureus* isolates (PVL-positive CA-MRSA). The isolates had been collected from around the world from 1999 through 2005 by the French National Reference Center for Staphylococci. We found that some continent-specific clones described in 2003, such as clone ST8, have now spread all over the world. Likewise, some PVL-positive CA-MRSA have spread to several countries on various continents. New clones have emerged (e.g., ST377) on new genetic backgrounds. PVL-positive CA-MRSA that were usually susceptible to most antistaphylococcal antimicrobial agents have acquired new resistance determinants (e.g., to gentamicin) in certain countries. The major trait shared by all these clones is a short staphylococcal chromosomal cassette *mec* element of type IV or V.

By definition, community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains infect patients with no risk factors for acquiring an MRSA strain of hospital origin. CA-MRSA infections usually affect previously healthy young patients (*1*). These infections are mostly of skin and soft tissue, but deep-seated infections such as necrotizing pneumonia and disseminated invasive osteomyelitis have been described (*2*). Many CA-MRSA isolates produce Panton-Valentine leukocidin (PVL) and harbor a type IV staphylococcal chromosomal cassette *mec* (SCC*mec*) element, but some isolates harboring the SCC*mec* V element have been reported (*3*). PVL-positive CA-MRSA clones have spread throughout the world (*4*).

In 2003, Vandenesch et al. described continent-specific PVL-positive CA-MRSA clones (mainly on an *agr3* background) and characterized them by their sequence type (ST) (*4*). The main European clone, ST80, was detected in France, Switzerland, the Netherlands, England, Belgium, and Germany (*5*,*6*), but also in northern Europe (e.g., Denmark), where MRSA strains are rare in hospitals (*7*). One of the most prevalent PVL-positive CA-MRSA clones in the United States (USA300) belongs to ST8 (*8*); other US clones include USA400 (ST1), USA1000 (ST59), and USA1100 (ST30) (*9*). ST30 is also a major clone in Asia and Oceania (*10*,*11*) and is referred to as the South West Pacific clone (*11*). In Singapore, an international travel hub, several clones belonging to ST80, ST30, and ST59 have been reported (*12*). The prevalence of PVL-positive CA-MRSA varies considerably from continent to continent. In the United States, MRSA were isolated from 50% of patients with skin and soft-tissue infections seen in emergency departments of 11 cities (97% of isolates belonged to clone USA300) (*13*). In Europe, the prevalence is lower, at ≈1–3% (*14*).

Since 1999, the French National Center for Staphylococci has characterized 469 PVL-positive CA-MRSA isolates collected throughout the world. The isolates were typed by multilocus sequence type (MLST), protein A gene type (*spa* typing), antimicrobial drug resistance profiling, and toxin and resistance gene analysis. We describe the evolution and spread of PVL-positive CA-MRSA clones since their initial description.

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Materials and Methods

Bacterial Isolates

From 1999 through 2005, the French National Reference Center for Staphylococci received 469 PVL-positive CA-MRSA isolates from 17 countries. These isolates were voluntarily sent to the center for PVL for gene detection and genomic characterization (clone designation). A single strain was selected per patient. Twenty-two isolates corresponded to an outbreak linked to a maternity unit that occurred in France in 2002–2003.

DNA Extraction and Identification of *agr* Alleles

The strains were grown on brain–heart infusion agar or in brain–heart infusion broth at 37°C overnight. Genomic DNA was extracted with a standard procedure (*15*). Amplification of *gyr*A was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. All PCR products were analyzed by electrophoresis on ethidium bromide–stained 1% agarose gels (Sigma, Lyon, France). The *agr* group (*agr* 1–4) was determined by PCR as previously described (*15*).

Detection of the *mec*A Gene and SCCmec Typing

The *mec*A gene (coding for methicillin resistance) was detected by PCR as previously described (*3*). The staphylococcal chromosomal cassette *mec* (SCC*mec* I-IV) was detected as described by Oliveira et al. (*16*), and SCC*mec* type V was detected as previously described (*3*). The following reference strains were used as controls: COL (SCC*mec* I), BK2464 (SCC*mec* II), HU106 (SCC*mec* III), and BK2529 (SCC*mec* IV).

Detection of Toxin Genes

Using PCR, we determined the presence of 22 specific staphylococcal virulence genes, as described previously (*15,17*). We detected sequences specific for staphylococcal enterotoxin genes (*sea-e, seh, sek, sem, seo*), as well as the toxic-shock syndrome toxin gene (*tst*), exfoliative toxin genes (*eta, etb, etd*), PVL genes (*luk*S-PV–*luk*F-PV), LukE–lukD leukocidin genes (*luk*E–*luk*D), the class F lukM leukocidin gene (*luk*M), hemolysin genes (gamma [*hlg*], gamma variant [*hlgv*] and β [*hlb*]), and epidermal cell differentiation inhibitor genes (*edinA/B/C*).

Antimicrobial Susceptibility Testing

MICs of penicillin, oxacillin, cefoxitin, kanamycin, tobramycin, gentamicin, erythromycin, clindamycin, tetracycline, pristinamycin, ofloxacin, fusidic acid, vancomycin, teicoplanin, fosfomycin, trimethoprim/sulfamethoxazole, rifampin, mupirocin, quinupristin/dalfopristin, and linezolid were determined by using the standard agar dilution technique, as recommended by the French Society for Microbiology. Structural genes for resistance to tetracycline, aminoglycosides, and macrolide-lincosamide-streptogramin (*18*) were identified by PCR. DNA was amplified in a model 2400 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) with *Taq* (Qbiogene, Inc., Carlsbad, CA, USA) or *Pfu* (Stratagene, La Jolla, CA, USA) DNA polymerase, as recommended by the manufacturers. PCR elongation times and temperatures were adjusted to the expected size of the PCR product and to the nucleotide sequences of the primers, respectively.

spa Typing

spa typing was performed on PVL-positive MRSA isolates as previously described (*19*). The x region of the *spa* gene was amplified by PCR. *spa* types were determined with the Ridom Staph Type software (Ridom GmbH, Würzburg, Germany), which automatically detects *spa* repeats and assigns a *spa* type according to Harmsen et al. (*20*) (http://spaserver.ridom.de). When the recently developed algorithm BURP (Based Upon Repeat Patterns) was applied, *spa* types were clustered into different groups with calculated cost between members of a group ≤6. *spa* types shorter than 3 repeats were excluded from analysis because no reliable deduction about ancestries can be made from these types. In addition to point mutation events, the new algorithm takes all other modifications that can occur (repeat or duplication or deletion) into account when the relatedness of different *spa* types is calculated. Because of speed constrains, a heuristic version of the EDSI-Alignment (Excisions, Duplications, Substitutions, Insertions), as described by Sammeth et al., was used (*21*). BURP *spa* clonal complexes (*spa*-CC) were automatically assigned by the Ridom Staph Type software (www.ridom.de/staphtype/).

MLST

MLST was performed on representative strains of each clonal group, as described elsewhere (*22*). The allelic profile of each strain was obtained by sequencing internal fragments of 7 housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi, yqiL*) and entering them on the MLST home page (http://saureus.mlst.net), where 7 numbers depicting the allelic profile were assigned that defined a ST (*22*). Similar STs were grouped into clonal complexes (CC).

Results

agr and STs

The 469 PVL-positive CA-MRSA isolates were *agr* type 1 (n = 46, 9.8%), *agr*2 (n = 9, 1.9%), or *agr*3 (n = 414, 88.3%); none was *agr*4 (Table 1). The 469 PVL-positive isolates belonged to 11 STs: the *agr*1 isolates were ST8, ST59, ST22, ST766, or ST377; the *agr*2 isolates were all

ST5; and the *agr*3 isolates were ST80, ST30, ST37, ST93, or ST1 (Table 1). None of the STs were shared by different *agr* types. The most frequent ST was ST80 (n = 357, 76.1%), which corresponded to the European clone.

spa Types and *spa* Clonal Complexes

The *spa* types were specific for *agr* type and ST. Minor variations of *spa* types (deletions or duplications of SSR units) were observed in several isolates within the same ST. For instance, 9 *spa* types were recognized among the 357 ST80 isolates, but t044 was the major *spa* type (n = 333, 93.3%); 8 of these *spa* types belonged to the same *spa* CC. A unique *spa* CC corresponded to each ST, except for ST1 isolates, which formed 3 different *spa* CC (Table 1).

Geographic Origin and Spread

A previous study (*4*) showed a limited number of clones and a limited geographic distribution. Schematically, ST80 was detected in Europe, ST8 and ST1 in the United States, and ST30 in Oceania. The results of our study suggest intercontinental exchanges of several clones (Table 1): 1) the ST8 clone (USA300) from the United States toward Europe; 2) the ST1 clone (USA400) from the United States toward Europe and Asia; 3) the ST59 clone (USA1000) from the United States toward Asia; 4) the ST80 clone from Europe toward Asia (*12*); and 5) the ST30 clone from Oceania toward Europe. Countries with numerous international exchanges (e.g., Singapore) have the highest clonal diversity.

New clones have been detected since 2003. One, ST22, has been found in Europe only. Another new clone, ST766, which belongs to the same CC (CC22) as ST22, was found in Singapore (*12*). Clone ST377 (with a type V SCC*mec*) was reported simultaneously in Europe and Australia (*3*). Clone ST5 was detected in Europe only. Clone ST93 (the Queensland clone), described in Australia before 2003, has not yet been detected in other countries (*11*).

Toxin Gene Content

The toxin gene distributions were compared to determine the genetic background of the different clones with minor variations. For instance, ST80 isolates were all positive for *etd*, *luk*S-PV, *luk*F-PV, and *edinA/B/C*; very few lacked *luk*DE or *hlgv* or harbored *hlB* (Table 2). Superantigenic toxin genes were detected in isolates that belonged to the different STs, except for ST377, ST80, and ST93 (Table 2).

Antimicrobial Drug Resistance

Isolates of each ST were grouped according to the number of antimicrobial drug resistance determinants they harbored. Initial PVL-positive CA-MRSA isolates were susceptible to most antimicrobial agents. For instance, 8 of the 25 ST8 isolates included in this study between 2003 through 2005 were resistant to penicillin and oxacillin alone, as were 17 of the 32 ST1 isolates and 18 of the 20 ST30 isolates (Online Appendix Table, available at www.cdc.gov/EID/content/13/4/594-appT.htm). ST80 isolates were initially resistant to penicillin, oxacillin, kanamycin, and tetracycline, and intermediate to fusidic acid. Since 2003, new antimicrobial resistance determinants have been acquired (e.g., gentamicin and ofloxacin). One ST8 isolate was resistant to penicillin, oxacillin, kanamycin, erythromycin, tetracycline, and ofloxacin; 1 ST1 isolate was resistant to penicillin, oxacillin, kanamycin, tobramycin, and gentamicin. A few ST80 isolates from Algeria were resistant to multiple antimicrobial agents. Most PVL-positive CA-MRSA strains with multiple antimicrobial resistant determinants were detected in Asia (Singapore, People's Republic of China) or Africa (Algeria).

Antimicrobial Resistance Genes

Antimicrobial resistance genes were sought in a subset of 153 ST80 isolates. The *aph3′-III* gene, which encodes high-level resistance to kanamycin and neomycin but also to amikacin and isepamycin, was detected in all 153 isolates (100%). The *tetK* efflux gene was detected in 125 (82%) of tetracycline-resistant isolates. The *ermC* gene, an erythromycin ribosome methylase, was detected in 61 (40%) of erythromycin-resistant isolates. The *far-1* gene was detected in 133 (87%) of fusidic acid–intermediate isolates.

SCC*mec* Types

SCC*mec* type was determined for 22 *agr*1 isolates (10 ST8, 1 ST59, 1 ST22, and 10 ST377); 5 *agr*2 isolates (ST5); 190 *agr*3 isolates (179 ST80, 9 ST30, 2 ST93, and 7 ST1). All the isolates were SCC*mec* type IV, except for the 10 ST377 isolates, which were SCC*mec* type V.

Discussion

This study has several findings. First, the continent-specific clones of PVL-positive CA-MRSA described in 2003 by Vandenesch et al. (*4*) have now spread to other continents. For instance, the ST1 clone USA400 is now detected in Europe and Asia. Some PVL-positive clones, such as ST1 and ST30, can now be considered pandemic, as they are detected in America, Europe, and Asia. Second, on a given continent, PVL-positive CA-MRSA have spread from country to country. For instance, in Europe, PVL-positive CA-MRSA were recently detected in Slovenia, Romania, and Croatia. Third, new PVL-positive CA-MRSA clones are emerging in strains with different

Table 1. Geographic distribution of PVL-positive CA-MRSA clones according to their *agr*-type, ST, and *spa* type*

agr type	ST (CC)	No. (%)	*spa* type (*spa* CC)	No. (%)	Location: Detected before 2003	Location: Newly detected (this work)	Other reports in literature
*agr*1		46 (9.8)					
	ST8 (8)	25 (54.3)			USA (*4*)	Netherlands, France, Spain, Switzerland, French Polynesia	N. Norway (*23*) Greece (*24*)
			t008 (s)	25 (100.0)			
	ST59	7 (15.2)			USA (*4*)	Netherlands, France, Singapore	Taïwan (*25*)
			t437 (8)	6 (85.7)			
			t216 (8)	1 (14.3)			
	ST22 (22)	3 (6.5)				Netherlands, Germany	
			t005 (4)	2 (66.7)			
			t310 (4)	1 (33.3)			
	ST766 (22)	1 (2.2)				Singapore	
			t1276 (4)	1 (100.0)			
	ST377	10 (21.7)				Netherlands, France, Greece, Switzerland, Australia	
			t355 (6)	9 (90.0)			
			t595 (6)	1 (10.0)			
*agr*2		9 (1.9)					
	ST5 (5)	9 (100.0)				France, Switzerland, Algeria	
			t311 (5)	5 (55.5)			
			t1277	3 (33.3)			
			t450	1 (11.1)			
*agr*3		414 (88.3)					
	ST80	357 (83.2)			France, Switzerland (*4*)	Algeria, Singapore, Romania, Germany, Belgium, Greece, Slovenia, Netherlands	Denmark (*7*), Sweden (*7*), notheren Norway (*23*), England (*26*), Finland (*27*), Libya (*28*), Croatia (*29*), Scotland (*7*), Greece (*24*)
			t044 (1)	333 (93.3)			
			t131 (1)	9 (2.5)			
			t376 (1)	8 (2.2)			
			t639 (1)	2 (0.6)			
			t237 (1)	1 (0.3)			
			t1199 (1)	1 (0.3)			
			t1201 (1)	1 (0.3)			
			t1206 (1)	1 (0.3)			
			t1200 (†)	1 (0.3)			
	ST30 (30)	20 (4.8)			New Zealand, Western Samoa (*4*)	Netherlands, Australia, Japan, Switzerland, Singapore, China, French Polynesia	Sweden (*30*), Brazil (*31*), Uruguay (*32*), England (*26*), Hong Kong (*10*)
			t019 (2)	17 (75.0)			
			t021 (2)	1 (5.0)			
			t318 (2)	1 (5.0)			
			t1273 (2)	1 (5.0)			
	ST37 (30)	1 (0.2)				Netherlands	
			t914 (2)	1 (100.0)			
	ST93	4 (1.0)			Australia (*4*)		
			t202 (s)	4 (100.0)			
	ST1	32 (7.7)			USA (*4*)	France, Singapore	Switzerland (*28*), Canada (*33*)
			t128 (3)	18 (56.2)			
			t125 (3)	3 (9.4)			
			t558 (3)	1 (3.1)			
			t175 (7)	8 (25.0)			
			t1274 (7)	1 (3.1)			
			t1272 (s)	1 (3.1)			

*PVL, Panton-Valentine leukodin; CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; *agr*, accessory gene regulator; ST, sequence type; CC, clonal complexes; *spa* CC, *spa* clonal complexes.
†Excluded because ≤3 repeats; s, singleton.

Table 2. Toxin gene content of PVL-positive community-acquired methicillin-resistant *Staphylococcus aureus* clones*

agr type	ST	No. (%)	Toxin genes always detected (100%)	Toxin genes sometimes detected (%)
agr1		46 (9.8)		
	ST8	25 (54.3)	*lukPV, lukDE*	*hlgv* (95.8), *sek* (91.7), *sed* (16.7), *seb* (4.2), *hlB* (4.2)
	ST59	7 (15.2)	*lukPV, hlgv*	*hlB* (87.5), *sek* (87.5), *seb* (62.5), *lukDE* (12.5)
	ST22	3 (6.5)	*sem, seo, lukPV, hlg*	
	ST766	1 (2.2)	*sem, seo, lukPV, hlg*	
	ST 377	10 (21.7)	*lukPV, edinA/B/C, hlB, hlg*	
agr2		9 (1.9)		
	ST5	9 (100)	*sem, seo, lukPV, lukED, hlgv*	*edinA/B/C* (55.5)
agr3		414 (88.3)		
	ST80	357 (83.2)	*etd, lukPV, edinA/B/C*	*lukDE* (99.7), *hlgv* (99.7), *hlB* (0.8)
	ST30	20 (4.8)	*sem, seo, lukPV, hlg*	*sek* (5.0), *tst* (5.0)
	ST37	1 (0.2)	*sec, sem, seo, tst, lukPV, hlg*	
	ST93	4 (1)	*lukPV*	
	ST1	32 (7.7)	*lukPV, seh, lukDE, hlgv*	*sea* (78.1), *sec* (68.7), *sek* (68.7), *seb* (25.0), *edinA/B/C* (3.1)

*PVL, Panton-Valentine leukocidin; ST, sequence type; *luk*PV, PVL genes; *luk*DE, LukE–lukD leukocidin genes; gamma (*hlg*), gamma variant (*hlgv*), and β (*hlb)* hemolysin genes; *sea* to *see, seh, sek, sem, seo,* staphylococcal enterotoxin type A to E, H, K, M, and O genes, respectively; *tst,* toxic shock toxin gene; *eta, etb, etd*: exfoliative toxin type A, B, and D genes, respectively; *edinA/B/C,* epidermal cell differentiation inhibitor; *agr,* accessory gene regulator.

genetic backgrounds. While most of the clones described in 2003 by Vandenesch et al. (*4*) had an *agr*3 background, the newly described clones are *agr*1 or *agr*2. Fourth, PVL-positive CA-MRSA, which were initially susceptible to most antistaphylococcal antimicrobial agents, have acquired new antimicrobial resistance determinants, to gentamicin and ofloxacin, for instance.

The global ST distribution of PVL-positive CA-MRSA isolates in this study depends, of course, on the sources of the isolates received by the French National Reference Center for Staphylococci and does not reflect the current epidemiology. Our collection represents a passive surveillance study and is related to the increased attention paid to CA-MRSA by certain regions. Nevertheless, our results agree with other reports which confirm that ST80 is mainly found in Europe (e.g., Denmark [*7*], Finland [*27*], and Greece [*24*]), but also in Libya (*28*); ST30 is pandemic (*34*).

PVL-negative, hospital-acquired MRSA belong to 5 major CCs (CC5, CC8, CC22, CC30, CC45). PVL-positive CA-MRSA of the same clonal classes were also detected in our study, with the exception of CC45, but the PVL + MRSA strains showed a broader CC diversity. For instance, none of the ST80 isolates belonged to CCs harboring hospital strains. PVL-positive CA-MRSA are gradually causing an increasing number of hospital-acquired infections in countries, such as the United States, where their prevalence is high (*35*). Kourbatova et al. reported that, during the period 2003–2004, five prosthetic joint infections were caused by USA300 strains (*36*).

The worldwide spread of PVL-positive CA-MRSA is likely related to international travel. ST80 isolates recovered in France were mainly detected in patients who were originally from Algeria, a country that reported a high rate of community- and hospital-acquired infections due to ST80 isolates in 2006 (*37*). Maier et al. recovered ST22 strains from Turkish migrants in Germany (*38*). Acquisition of new antimicrobial resistance determinants could be related to misuse of antimicrobial agents; the spread of multidrug-resistant strains could be facilitated by poor hygiene, regardless of country.

It is not known whether PVL-positive CA-MRSA clones arose through acquisition of the PVL phage by strains with a methicillin resistance background or, conversely, through acquisition of the SCC*mec* element by strains with a PVL-positive background. On analyzing the database of the French National Reference Center for Staphylococci, which contains >5,000 toxin gene profiles, we found isolates that were related to the PVL-positive MRSA clone ST80 but lacked either the PVL genes (5 isolates) or the *mec*A gene (7 isolates) (data not shown). These isolates, like the ST80 clone, were *agr*3, *etd*+, *edin*A/B/C+; 1 isolate (PVL– *mec*A+) was ST80, and another (PVL+ *mec*A–) was ST635 (a single-locus variant of ST80). These "atypical" isolates were discovered in Algeria, Switzerland, and France. We are unable to state whether they are ancestors or descendants of the most prevalent strains.

Deep-seated infections due to PVL-positive *S. aureus* can be extremely severe. For example, necrotizing pneumonia carries a mortality rate close to 75% (*39*). Whether the pathogenesis of these acute infections is related to the effect of PVL alone or in combination with other virulence factors such as superantigenic toxins is unclear. We found that some PVL-positive CA-MRSA clones (ST80) lacked any superantigenic toxin genes. Among the *S. aureus* virulence factors (not screened for here), ST30 strains harbor the *bbp* gene, which encodes bone sialoprotein (*34*). The SCC*mec* elements detected in our collection were type IV or V and corresponded to the smallest SCC*mec* element.

PVL-positive CA-MRSA are usually susceptible to most antistaphylococcal antimicrobial agents. Clone ST80 is usually resistant to tetracycline (mediated by the *tetK* gene), intermediate to fusidic acid (*far1* gene), and resistant to kanamycin (*aph3′-III* gene). We observed the emergence of rare isolates with multiple resistances to antimicrobial agents such as gentamicin and ofloxacin. From the therapeutic viewpoint, all the isolates were susceptible to trimethoprim-sulfamethoxazole, glycopeptides, and linezolid.

The involvement of PVL in CA-MRSA infections has not been proved in mouse sepsis and abscess models developed by Voyich et al.: isogenic PVL-negative strains of USA300 and 400 were as lethal as wild-type strains, and they caused comparable skin diseases (*40*). Other experiments have to be conducted to determine if PVL is secreted in such a model.

In summary, since 2003 we have observed an impressive worldwide spread of PVL-positive CA-MRSA clones initially described at the beginning of this decade, and we have also detected PVL-positive CA-MRSA strains of other lineages. To counter this emerging global threat to public health, systematic surveillance of both hospital and community isolates is required, together with measures designed to limit their spread.

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Genetic Stasis of Dominant West Nile Virus Genotype, Houston, Texas

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The accumulation and fixation of mutations in West Nile virus (WNV) led to the emergence of a dominant genotype throughout North America. Subsequent analysis of 44 isolates, including 19 new sequences, from Houston, Texas, suggests that WNV has reached relative genetic stasis at the local level in recent years.

Previous phylogenetic analyses of North American West Nile virus (WNV) isolates have shown genetically distinct variants that group in a temporally and geographically dependent manner (*1*). Recent studies have provided substantial evidence that a dominant genetic variant has emerged across much, if not all, of North America (*2–4*). The establishment of a dominant genotype across the continent and the displacement and possible extinction of earlier progenitor genotypes appear to have resulted from the accumulation and fixation of multiple nucleotide mutations throughout the WNV genome. Despite the occurrence of 13 conserved nucleotide mutations (out of 11,029 nt/genome) in isolates belonging to the dominant genotype, only 1 of these mutations, E-1442(U to C); E159(Val to Ala), resulted in an amino acid substitution (out of 3,433 amino acids/polyprotein). Consequently, a scarcity of nonsynonymous mutations in the dominant genotype compared with progenitor genotypes has made it difficult to evaluate and quantify any relative fitness advantages possessed by the dominant strains (*5,6*).

The rapid emergence of the dominant genotype across North America and subsequent displacement of other genotypes suggested an apparent fitness advantage conferred by mutations in the genome of the dominant variants. In fact, studies by Ebel et al. suggest that the dominant genotype is transmitted by *Culex pipiens* after fewer days of extrinsic incubation than are needed by the prototypical strain, WN-NY99, leading to a possible increase in the transmission efficiency of the dominant genotype (*6*). The proliferation of this genotype over such a relatively short period (≈3 years, from the summer of 1999 through the summer of 2002) and vast geographic scale has led us to consider whether the genetic divergence of the virus has continued at a similar rate. To further characterize the evolutionary patterns of WNV, we chose to focus on a readily available population of virus isolates that provide a representation of the current state of WNV evolution at a localized level.

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The Study

The 44 WNV isolates included in this study were obtained in the Houston, Texas, metropolitan area during the course of 5 years (2002–2006). All of the 25 earlier isolates and 19 newly sequenced isolates came from dead birds or mosquitoes and were isolated as previously described (*7*). The complete premembrane (prM) and envelope (E) protein genes (2004 nucleotides) were sequenced by reverse transcription–PCR (RT-PCR) with RNA extracted from cell culture supernatant of either the original isolation or after a single passage in Vero cells. RT-PCR protocols, primer sequences, and sequencing methods have been described elsewhere and are available on request (*1*). Nucleotide and deduced amino acid sequences of all isolates were aligned with the prototypical North American WNV, WN-NY99, by using AlignX in the VectorNTI software package (Informax, Frederick, MD, USA). The year, source, and corresponding GenBank accession number for each isolate are described in Table 1. A phylogenetic tree was generated by maximum likelihood analysis by using PAUP (*8*)(Figure).

Not surprisingly, all of the isolates analyzed in this study contained the 3 nt mutations in the prM and E protein genes that differentiate the dominant genotype from other genotypes (prM-660[C to U]; E-1442[U to C]; E-2466[C to U]), which places each of the newly sequenced isolates in the dominant genotype clade relative to WN-NY99. Although several isolates shared additional mutations, most made up a large, indistinct polytomy with little branching and subclade formation. Most isolates from 2002 had the shortest branch lengths from the node separating the dominant clade from WN-NY99, which supports the hypothesis that the 2002 isolates represented the early stages of the emergence of the dominant genotype. The isolates from 2003, 2004, and 2005 had, on average, longer branch lengths than those of 2002, but few had >3 nt mutations from the dominant clade defining node, which suggests relative stasis in the divergence of the virus.

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Of particular note was the presence of a subclade at the apical portion of the phylogenetic tree, composed of only isolates from 2005 and 2006. This distinct subclade was the result of a conserved silent nucleotide mutation at position E-2469(C to U), which had not been identified in any other publicly available WNV sequences (data not shown). The four 2005 isolates that were not included in this subclade (i.e., that do not have this mutation) were collected in the spring (March/April), while all other 2005 isolates were collected in early summer (June/July), suggesting that this mutation occurred during the 2005 WNV season but did not become fixed in the population until at least the beginning of the annual peak transmission period (June–September). Alternatively, this mutation may have already been present in a virus introduced into the Houston

Table 1. West Nile virus isolates, Houston, Texas, 2002–2006

Isolate	Year of isolation	Source	GenBank accession no.
Bird 113	2002	Bluejay	AY185906
Bird 114	2002	Bluejay	AY185907
Bird 119	2002	Bluejay	AY185908
Bird 123	2002	Hawk	AY185909
Bird 135	2002	American crow	AY185910
v1151	2002	*Culex quinquefasciatus*	AY185911
Bird 227	2002	Bluejay	AY185912
Bird 1519	2003	Bluejay	DQ158227
Bird 1574	2003	Bluejay	DQ158228
Bird1576	2003	Bluejay	DQ158229
Bird 1461	2003	Bluejay	AY712947
Bird 1153	2003	Mourning dove	AY712945
Bird 1171	2003	Great-tailed grackle	AY712946
Bird 1175	2003	Bluejay	DQ158220
Bird 1240	2003	Bluejay	DQ158221
Bird 9045	2003	Bluejay	DQ158223
Bird 9114	2003	Bluejay	DQ158222
v4095	2003	*C. quinquefasciatus*	DQ158224
v4369	2003	*C. quinquefasciatus*	AY712948
v4096	2003	*C. quinquefasciatus*	DQ158226
v4370	2003	*C. quinquefasciatus*	DQ158225
Bird 2541	2004	*C. quinquefasciatus*	DQ158234
Bird 2419	2004	Bluejay	DQ158233
Bird 3588	2004	Bluejay	DQ164206
Bird 3218	2004	Bluejay	DQ158235
M8447†	2004	*C. restuans*	EF205419
M8451†	2004	*C. restuans*	EF205420
M8977†	2004	*C. restuans*	EF205421
Bird 4511†	2005	American crow	EF205422
Bird 4486†	2005	American crow	EF205423
Bird 4276†	2005	Bluejay	EF205424
Bird 4487†	2005	American crow	EF205425
Bird 5001†	2005	Bluejay	EF205426
Bird 5014†	2005	Bluejay	EF205427
Bird 5055†	2005	House sparrow	EF205428
Bird 5058†	2005	Bluejay	EF205429
M11769†	2005	*C. quinquefasciatus*	EF205430
M12214†	2005	*C. quinquefasciatus*	EF205431
M12251†	2005	*C. quinquefasciatus*	EF205432
M12357†	2005	*C. quinquefasciatus*	EF205433
M1977†	2006	*C. quinquefasciatus*	EF205434
Bird 5784†	2006	Bluejay	EF205435
Bird 5810†	2006	Common grackle	EF205436
M2766†	2006	*C. quinquefasciatus*	EF205437

*Isolates IS98STD1 (GenBank accession no. AF481864) and WN-NY99 (GenBank accession no. AF196835) were used during phylogenetic analysis.
†Isolate was sequenced for this study.

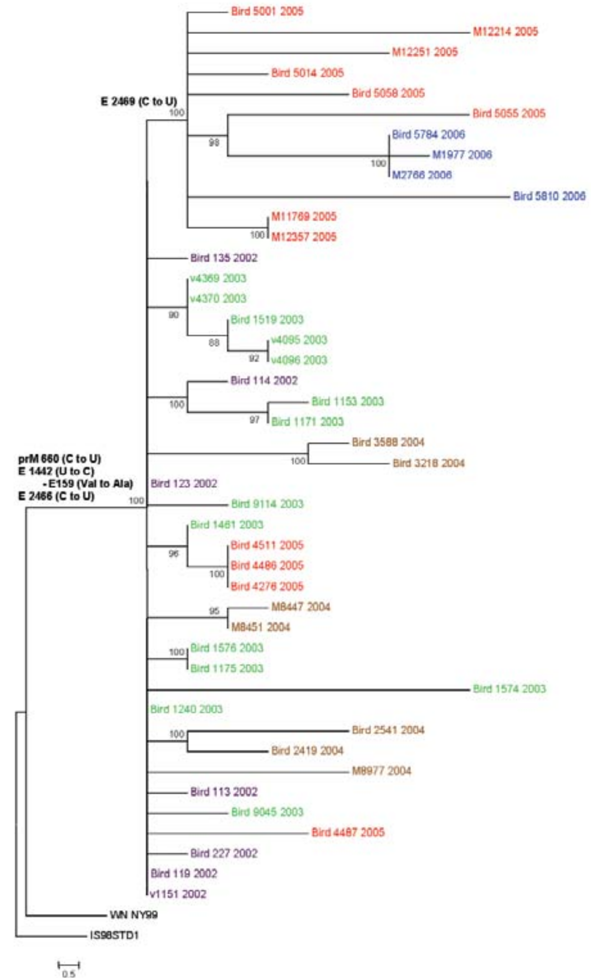

Figure. Phylogenetic tree generated by maximum likelihood analysis of a nucleotide alignment of the premembrane and E protein genes (2004 nucleotides) of previous and newly sequenced West Nile virus (WNV) isolates collected in the Houston metropolitan area from 2002 to 2006. The tree was rooted with the most closely related Old World WNV strain, Israel-1998. Maximum likelihood analysis was used to generate trees using PAUP (Version 4.0b11, Sinauer Associates, Sunderland, MA, USA) under the general time-reversible model and a γ distribution of substitution rates with statistical support and tree topology confirmation provided by 1,000 bootstrap replicates (bootstraps values shown at each node). Parsimony informative nucleotide mutations and deduced amino acid substitutions responsible for the observed clade topologies were added to the tree at relevant nodes. The year of isolation is color coded for each isolate on the tree (2002, purple; 2003, green; 2004, brown; 2005, red; 2006, blue), and the scale bar represents 0.5-nt changes.

area during early summer 2005. Regardless, the fixation of this mutation in isolates from 2005 and 2006 indicated its presence in the population at the start of intense WNV transmission in 2005, and it may have become fixed not as a result of positive selection or an increase in fitness but simply because it was present in those viruses that were subjected to an increased frequency of transmission during the early summer months.

To demonstrate the degree to which isolates have diverged from year to year, nucleotide sequences were grouped by year and the average pairwise distances between groups were calculated. Table 2 shows that the percent nucleotide divergence from WN-NY99 has generally increased over time (2002, 0.29%; 2006, 0.60%). Surprisingly, isolates from 2005 were on average slightly less divergent from WN-NY99 (0.45%) than were 2004 isolates (0.46%). Analysis of nucleotide sequence alignments showed that the 2005 isolates did not share any mutations with the 2004 isolates other than those denoting the dominant genotype (data not shown), which suggests that the 2005 isolates did not acquire mutations from viruses circulating during the previous transmission season. In addition, as shown in Table 2, the nucleotide divergence has marginally increased from year to year (2002–2003, 0.15%; 2003–2004, 0.32%; 2004–2005, 0.41%; 2005–2006, 0.48%). This finding indicates that, while WNV has continued to diverge, most mutations that occur each year are not passed on, and even viruses circulating in the same location continually diverge from 1 another if mutations are not fixed from year to year.

Table 2. Average pairwise percent nucleotide divergence between groups by year*

Group	ISR 1998	WN NY99	2002	2003	2004	2005	2006
ISR 1998							
WN NY99	0.20						
2002	0.29	0.29					
2003	0.36	0.36	0.15				
2004	0.46	0.46	0.24	0.32			
2005	0.45	0.45	0.24	0.31	0.41		
2006	0.60	0.60	0.38	0.46	0.56	0.48	

*ISR, Israel; WN, West Nile.

Conclusions

The relative stasis of the Houston metropolitan area WNV population and lack of newly emergent subclades containing 2002, 2003, and 2004 isolates suggest that few, if any, new genotypes of WNV from other regions of North America have been introduced locally and that the dominant genotype has been established and maintained in a local endemic transmission cycle. Additional sequence data from outside the Houston area collected during the same sampling period will be necessary to confirm or refute this hypothesis.

Of the 16 deduced amino acid substitutions that have occurred in the population studied (data not shown), only a single substitution (E159-Val to Ala) has become fixed, an indication that the dominant variant may be in a period of relative stasis. Only a single, silent nucleotide mutation has become fixed in the population since 2002, which indicates the infrequency of such molecular events and may reflect restrictions imposed on the genome; alternatively, it may indicate a lack of positive selective pressures acting on the virus population. During such intervals, if no fitness advantage is conferred by a particular substitution, random mutations may continue to accumulate at low frequencies, eventually giving rise to new subclades as a result of fixation. In this case, fixation may be due to the increased rate of transmission of a particular virus population during intensified transmission periods (i.e., after an increase in mosquito density), rather than in response to selective pressures or increased viral fitness. Thus, the forces driving the evolution of WNV may differ from location to location, and the newly described stasis of the Houston WNV population described in this study may or may not reflect similar trends in the continuing evolution of the virus in other regions of North America. Additionally, it is notable that there has been a lack of genetic divergence in an area lying on a major migratory bird flyway. The epidemiologic consequences of stasis within the Houston WNV population may have important implications for the endemicity of WNV disease in the Houston area and other similar locations.

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Autochthonous Transmission of *Trypanosoma cruzi*, Louisiana

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Autochthonous transmission of the Chagas disease parasite, *Trypanosoma cruzi*, was detected in a patient in rural New Orleans, Louisiana. The patient had positive test results from 2 serologic tests and hemoculture. Fifty-six percent of 18 *Triatoma sanguisuga* collected from the house of the patient were positive for *T. cruzi* by PCR.

Chagas disease is endemic in Latin America; 13 million people are infected with the causative agent, the protozoan parasite *Trypanosoma cruzi*, and 200,000 new cases are reported annually (*1*). Although Chagas disease occurs mostly as heart disease, megasyndrome (enlargement of the visceral organs) is also seen in patients in South America. Transmission is usually by contamination of a person with parasite-laden feces of a triatomine bug (family *Reduviidae*, subfamily *Triatominae*, commonly known as kissing bugs), which deposits feces on the skin while feeding. The parasite can then enter through the bite wound, mucous membranes, or conjunctiva. Transmission by blood transfusion, organ transplant, and congenital and oral routes can also occur.

Only 5 autochthonous cases of infection with the Chagas disease parasite have been reported in the United States: 3 in infants in Texas (*2,3*), 1 in an infant in Tennessee (*4*), and 1 in a 56-year-old woman in California (*5*). The most important triatomine species in the United States for Chagas disease transmission are *Triatoma sanguisuga*, whose broad range extends across the southeast and reaches Maryland and Texas, and *T. gerstaekeri*, found in Texas and New Mexico (*6*). There is an active sylvan cycle in the United States; *T. cruzi* has been identified directly or by serologic analysis in ≥18 species of mammals (*7*), including raccoons, opossums, armadillos, foxes, skunks, dogs, wood rats, squirrels, and nonhuman primates (housed in outdoor research facilities). In Louisiana, *T. cruzi* infection has been identified in 28.8% and 1.1% of armadillos (*8,9*), 37.5% of opossums (*9*), 4.7% of rural dogs (*10*) and rarely in nonhuman primates (*11*, P.L. Dorn et al., unpub. data). The lack of human cases is usually attributed to not having a suitable habitat for the bugs in most US homes, a preference for animal hosts, and delayed defecation of triatomines found in the United States compared with those found in Latin America (*12*).

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The Study

In June 2006, a 74-year-old woman residing in a house in rural New Orleans was bothered by a considerable number (>50) of insect bites. The woman observed many bugs in the house and showed them to a fumigator, who identified them as triatomines. An internet search showed the potential for transmission of Chagas disease, and the woman sought help from a local health sciences center.

Serum samples from both residents of the house were tested for antibodies to *T. cruzi* at the Centers for Disease Control and Prevention (CDC) by an indirect fluorescent antibody (IFA) test. Samples were also tested at Loyola University (New Orleans, LA, USA) and then at CDC by using an experimental dipstick assay (*Trypanosoma* Detect; InBios International Inc., Seattle, WA, USA). The woman resident was positive for antibodies to *T. cruzi* by IFA at dilutions of 1:128 (≈4 weeks after being bitten) and 1:64 (≈10 weeks after being bitten) and by dipstick assay. She was positive for trypanosomes by hemoculture testing with ≈10 mL blood and coculture in macrophages (*13*) ≈4 months after being bitten. Trypanosomes consistent with *T. cruzi* were observed in culture beginning on day 46 of culture, and amplification of a *T. cruzi*–specific 24Sα rRNA gene target confirmed that the isolate was *T. cruzi*. The other resident was negative by both serologic tests.

The index resident had a history of 5 trips to areas endemic for Chagas disease: Zacatecas, Mexico (1970); Cozumel, Mexico (1990); Belize (1991); Guatemala (1998); and Costa Rica (1998), each of <2 weeks duration, with stays in improved tourist hotels (less likely to harbor triatomines) except for the Belize trip, which included an ≈1-week stay in a palm thatch–roofed cabin. She had not used intravenous drugs or had a blood transfusion or organ transplant, and she is not the daughter of Latin American immigrants. Except for fatigue, the index patient had no symptoms and had an active lifestyle. Cardiac evaluation that included an electrocardiogram showed normal results, and she decided not to take medication.

Her residence of 29 years was located on 7.66 acres of bottomland hardwood forest, with many gaps that provided ready access for insects. A house inspection showed

fecal streaks characteristic of triatomines on walls, which were identical to what the patient reported seeing on her nightgown. Twenty dead adult triatomines were collected in the house (after fumigation) and in another building on the property that contained a bed. No nymphs or eggs were found, which suggests that the house had not been colonized. One live second-stage nymph was collected in a nearby armadillo burrow ≈50 m from the house. All triatomines collected were identified as *T. sanguisuga* according to the key of Lent and Wygodzinsky (*6*) (Figure).

Because all triatomines except the nymph were dead, PCR was used to determine *T. cruzi* infection status (*14*). The last 2 segments of the abdomen were removed from each insect, placed in 200 µL 1× PCR buffer (Applied Biosystems, Foster City, CA, USA), boiled for 15 min, and centrifuged. A total of 5 µL of supernatant was amplified in a 50-µL reaction (3.5 mmol/L $MgCl_2$ and 2 U Taq DNA polymerase). The primers used anneal to the *T. cruzi* minicircle DNA and were TC3: 5′-TTGAACGCCCCTCC-CAAAAC-3′ and TC4: 5′-GATTGGGGTTGGTGTA ATATA-3′. The cycling parameters were an initial denaturation step at 94°C for 3 min; 35 cycles at 94°C, 55°C, and 72°C, each for 1 min; and a 10-min extension at 72°C (programmable thermal controller; MJ Research, Watertown, MA, USA). Twenty percent of the PCR product was subjected to electrophoresis on a 1.8% agarose gel and visualized by UV transillumination after staining with ethidium bromide. A positive control of 5 µL of *T. cruzi* parasites boiled in 1× PCR buffer and a negative control without the DNA template were included with every PCR. Samples that failed to amplify were spiked with 5 µL of *T. cruzi* parasites boiled in 1× PCR buffer and reamplified to ensure that the lack of product was not caused by inhibition of the PCR. More than half of the triatomines were positive for *T. cruzi* (56%, 10/18; 3 failed to amplify), with more positive females (73%, 8/11) than males (50%, 3/6).

Figure. Male *Triatoma sanguisuga*.

Plasma from the resident dog and 7 other dogs living ≈1 mile away all tested negative by IFA at CDC.

Conclusions

The assertion that the patient contacted *T. cruzi* in Louisiana is strongly supported by limited travel history to disease-endemic areas and stays mostly in improved housing (risk for Chagas disease transmission is associated with longer residence in disease-endemic areas), lack of other risk factors, and large numbers of infected *T. sanguisuga* in the house. No periorbital swelling was reported. However, the streaks on her nightgown consistent with triatomine feces indicate exposure, and the parasite could have been introduced into any of her numerous bite wounds.

The residents had not previously noticed large numbers of *T. sanguisuga* in the house. However, Hurricane Katrina had hit the area 9 months earlier and increases in domestic infestation with triatomines have been previously reported after a hurricane (*15*). Anecdotally, the armadillo population increased substantially in the months after Hurricane Katrina, and one can speculate that these hosts supported a larger bug population, who later sought other bloodmeal sources as the armadillo population returned to prestorm levels. Follow-up studies of local *T. sanguisuga* ecology and animal reservoirs are planned.

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Endpoints for Lymphatic Filariasis Programs

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In 2000, annual mass administration of diethlycarbamazine and albendazole began in Leogane Commune, Haiti, to interrupt transmission of lymphatic filariasis (LF). After 5 years of treatment, microfilaremia, antigenemia, and mosquito infection rates were significantly reduced, but LF transmission was not interrupted. These finding have implications for other LF elimination programs.

Globally, more than 1 billion persons are at risk for lymphatic filariasis (LF), a mosquito-transmitted parasitic infection that causes lymphedema and hydrocele. Transmission of LF can be interrupted by annual mass treatment with drugs that target microfilariae, the stage of the parasite that circulates in the blood (*1*,*2*). Programs in Egypt, Samoa, Zanzibar, and other settings have recently completed 5 rounds of mass drug administration (MDA) (*3*,*4*), a proposed endpoint for treatment, and are now confronted with a critical question: can MDA be stopped without fear of recrudescence of LF infection?

The LF demonstration project in Leogane, Haiti, was designed as an operational research project to monitor the impact of MDA on LF infection in a high-prevalence setting. The intervention was annual MDA with diethylcarbamazine (DEC) and albendazole. We discuss how sentinel site data were used to determine whether to continue MDA after 5 rounds and how our experience may be relevant to other LF programs.

Leogane is located 30 km west of Port-au-Prince. Before the first MDA in 2000, 4 sentinel sites in Leogane commune were selected for annual follow-up of microfilaremia and antigenemia (*5*). Monitoring of filarial infection prevalence in the vector, *Culex quinquefasciatus,* began in these sites 3 months before the first MDA, using CDC gravid traps (Model 1712, J.W. Hock Co., Gainesville, FL, USA) (*6*); testing continued on a semimonthly basis. Infected mosquitoes were defined as those carrying microfilariae or larvae (L1–L3); L3 were the infectious larval stage. Protocols for collecting data from sentinel sites were approved by the Centers for Disease Control and Prevention Institutional Review Board and the Ethics Committee at Hôpital Ste. Croix.

In October 2000 and every October thereafter, DEC (6 mg/kg) and albendazole (400 mg; GlaxoSmithKline, Brentford, UK) were co-administered at stationary posts to persons >2 years of age, excluding pregnant women and persons too ill to receive the drugs. Adverse events were monitored each year by recording the number of persons who returned to distribution posts with complaints. Cluster surveys were conducted after the first and third MDA to assess coverage and the effect of health messages on compliance (*7*,*8*).

Reported coverage in 2000–2004 for MDAs 1–5 was 69%, 50%, 84%, 89%, and 104%, respectively (Table). Decreased coverage in 2001 may have been related to a relatively high incidence of adverse events caused by death of microfilariae and adult worms during the first MDA (*9*). The increase in reported coverage in 2004 may have been due to an influx of displaced persons from areas of Haiti affected by civil strife. Survey-based coverage in 2000 and 2002 was 71% and 79%, respectively (*7*,*8*). Adverse events diminished with each year of treatment, from 23.1% of persons treated during 2000 to 3% during 2004 ($p<0.0001$).

Baseline microfilaremia prevalence rates were 0.8%, 7%, 12%, and 16% in the sentinel sites of Mapou, Barrier-Jeudi, Masson-Mathieu, and Leogane, respectively (Figure 1). Microfilaremia prevalence decreased significantly in each of the sentinel sites (Mapou, $p = 0.0291$; each of the other sites, $p<0.0001$). Antigenemia prevalence declined less dramatically, by 18.6%, 34.6%, 74.2%, and 54.7% in Mapou, Barrier Jeudi, Masson-Mathieu, and Leogane, respectively ($p<0.0001$ in all sites except Mapou).

Baseline mosquito infection rates 3 months before the first MDA were 0.5%, 2.9%, 3.5%, and 4.0% in Mapou, Masson-Mathieu, Leogane, and Barrier-Jeudi, respectively. After MDA 1, infection prevalence decreased significantly only in Masson-Mathieu ($p = 0.004$); however, after 2 rounds of MDA, infection was reduced significantly at all sites ($p<0.007$) except Mapou (Figure 2). After MDA 4, infection prevalence was 0% during some months at all sentinel sites, although infected mosquitoes were detected sporadically at all sites but Mapou. The prevalence of infective mosquitoes was lower than the prevalence of infected mosquitoes ($p<0.05$), but parallel declines were observed after MDA (data not shown).

These data were collected to monitor progress and to provide a basis for programmatic decisions. In January 2005, 3 months after MDA 5, a meeting was convened in Leogane with program and ministry staff to discuss the

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Table. Drug coverage for Leogane Commune, Haiti*

	2000	2001	2002	2003	2004
No. treated†	74,000	55,000	94,000	102,000	122,000
Reported coverage‡	69%	50%	84%	89%	104%
Surveyed coverage§	71%	NA	79%	NA	NA
Adverse events¶	23%	16%	9%	8%	3%

*NA, not available
†Based on tally counts from distribution posts.
‡Using 108,000 as the total population of Leogane determined by cluster survey in 2000 (7), adjusted for annual population growth. International Data Base (IDB): available from http://www.census.gov/ipc/www/idbprint.html
§Published data (*7*,*8*).
¶Based on the number of persons who reported to distribution posts with reports adverse events as a percentage of the reported coverage. See reference (*9*) for a more detailed discussion of adverse events associated with the first round of mass drug administration.

need for further MDA. Sustained reductions in LF infection in both humans and mosquitoes demonstrated the substantial effects of the intervention through 4 MDA rounds. However, persistent antigenemia and sporadic parasitemia were detected at all sentinel sites. Project leaders adopted a conservative approach and planned for MDA 6 on the basis of the evidence of continued transmission in the sentinel sites.

Several factors supported this decision, including detection of infections in both humans and mosquitoes and concerns about systematic noncompliance (*8*), the potential for inflated coverage estimates due to population migration, the nonrepresentativeness of sentinel sites for estimating MDA impact, and the insensitivity of blood smears for monitoring microfilaremia. Since the cost of an additional MDA was not prohibitive, project staff decided that the evidence of continued transmission, the health benefits of mass treatment to the community, and the risk of stopping treatment prematurely justified a sixth round of MDA in October 2005. Results from Leogane and other programs have demonstrated that 5 rounds of MDA may not be sufficient to interrupt transmission when baseline antigenemia is high, whereas in low-prevalence areas <5 rounds appears to be adequate (*3*). Mathematical models as well as program experience suggest that the number of MDAs required depends on baseline intensity of infection, assuming adequate coverage (*10*,*11*).

Adequate monitoring data are important for making decisions regarding continuation of mass treatment. Microfilaremia and immunochromatographic card test (ICT) testing are the gold standards for measuring the impact of MDA; however, nocturnal blood collection required for microfilaria testing is inconvenient, the high cost of the ICT is a concern (≈$2.65), and the sensitivity of both tests decreases as LF intensity and prevalence decline.

Entomologic monitoring provides an alternative method of measuring the impact of MDA on transmission. Although it circumvents the human cost of repeated blood collection and provides a direct, real-time measure of potential transmission, continuous mosquito collection and dissection were more costly and labor-intensive than other monitoring methods that we used. Conducting intermittent rather than continuous collections may be an alternative approach.

The limitations of these monitoring tools highlight the need for more sensitive, standardized tools to help programs define MDA endpoints and to conduct surveillance. Antibody responses may develop before patent infection and serve as a cumulative measure of filarial exposure and a proxy for transmission (*12*). In Egypt, antibody surveys of children beginning school were used to monitor for incident exposure, indicative of ongoing transmission (*3*). Additional studies are needed to validate antibody tests and to analyze the relationship between antibody prevalence and transmission intensity.

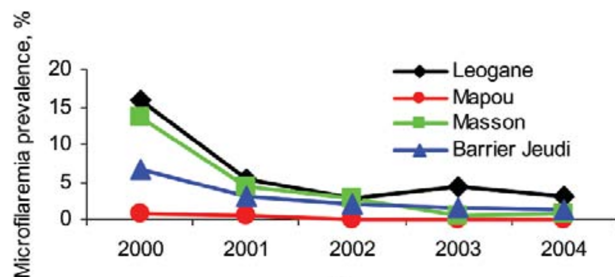

Figure 1. Microfilaremia prevalence determined by screening thick blood films before and 6–9 months after annual mass drug administration in sentinel sites in Leogane Commune, Haiti. Blood samples (20 μL) were collected from 7–9 PM.

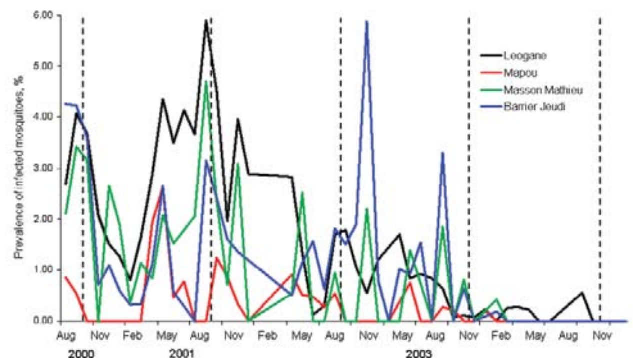

Figure 2. Prevalence of infection (microfilaremia, L1, L2, and L3) in dissected mosquitoes collected by using gravid traps in sentinel sites in Leogane Commune, Haiti. Data are aggregated on a monthly basis. Dashed lines represent annual mass drug administration intervention.

In summary, MDA-based LF programs, including that in Leogane, lead to dramatic declines in filarial infection in humans and mosquitoes after several annual rounds of MDA (*2*,*3*,*13–15*). The outcomes of the Leogane project and others that have completed 5 rounds of MDA strongly suggest that the duration of treatment is related to the baseline transmission intensity and infection prevalence. Several issues—population migration, systematic noncompliance, and sentinel site bias—have emerged as variables that complicate decision making. Investigating their effect on infection and transmission in an operational context is critical.

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Genetic Characterization of HPAI (H5N1) Viruses from Poultry and Wild Vultures, Burkina Faso

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Genetic analysis of highly pathogenic avian influenza (H5N1) viruses from poultry and hooded vultures in Burkina Faso shows that these viruses belong to 1 of 3 sublineages initially found in Nigeria and later in other African countries. Hooded vultures could potentially be vectors or sentinels of influenza subtype H5N1, as are cats and swans elsewhere.

On February 7, 2006, the first African outbreak of highly pathogenic avian influenza (HPAI) (H5N1) virus was reported from a farm in Kaduna State, northern Nigeria. Since then, 7 other African countries, including Niger, Egypt, Cameroon, Burkina Faso, Côte d'Ivoire, Sudan, and Djibouti, have officially reported HPAI (H5N1) in poultry farms to the World Organization for Animal Health. On April 3, influenza A (subtype H5N1) was first confirmed in Burkina Faso. We genetically analyzed influenza A (H5N1) viruses from Burkina Faso poultry and the first gene sequences obtained from African wild birds, hooded vultures (*Necrosyrtes monachus*).

In March and April, cloacal and tracheal swabs from 3 adult hooded vultures were collected in Ouagadougou, Burkina Faso. The first of these birds of prey had dyspnea and neurologic signs; the second showed asthenia and locomotion problems. While only samples from these 3 vultures were available for sequencing, an additional 45 hooded vultures were found dead or sick throughout Ouagadougou from February to June 2006; symptoms in these birds included diarrhea, respiratory disorders, prostration, apathy asthenia, and ruffled feathers. Seventeen of these were confirmed influenza positive by rapid test (Influenza A&B test kit, BinaxNOW, Binax Inc., Portland, ME, USA). All birds of prey were collected as part of a passive surveillance program, and only birds sick enough to attract attention (and to be caught without special equipment) were sampled. In addition, swabs were collected from 1 domestic guinea fowl in Ouagadougou and from 4 adult backyard chickens from flocks with high numbers of deaths in Bobo-Dioulasso, Tenado, and Sokoroni, located ≤150 km from each other. The swabs of all birds were positive for HPAI (H5N1) virus, as evidenced by generic influenza A M-gene reverse transcription–PCR (*1*) and specific H5 PCR, as recommended by the European Union (http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_237/l_23720060831en00010027.pdf, 04/08/2006).

The hemagglutinin (HA) sequences of influenza A (H5N1) viruses from Burkina Faso clustered with recent western Asian, Russian, European, and African strains and are clearly distinct from southeastern Asian lineages (data not shown). Phylogenetic comparison of the HA1 genes from Burkina Faso with all African influenza A (H5N1) HA sequences available from GenBank showed that the Burkina Faso strains cluster together and with A/chicken/Ivory Coast/1787/2006 (Figure 1). Nucleotide differences between all 8 Burkina Faso HA1 sequences were relatively small (0.1% [1 nt] to 0.9% [10 nt]). HA1 sequences of viruses from 3 hooded vultures (A/hooded vulture/Burkina Faso/1–2-5346–10/2006) showed 0.2%–0.5% (3–6 nt) differences. A poultry virus from Côte d'Ivoire (A/chicken/Ivory Coast/1787/2006) was more similar to Burkina Faso strains than to other African strains, with a mean percentage nucleotide difference for HA1 of 0.5% (2–10 nt). HA1 sequences from the southwestern Nigerian farm (coded SO) (*2*) showed >1% nt differences when compared with the Burkina Faso strains. The maximum percentage nucleotide difference within African influenza A (H5N1) HA1 sequences ranged up to 1.8% (between A/chicken/Egypt/5611NAMRU3-AN/2006 and A/chicken/Sudan/1784/2006). Mean percentage nucleotide differences within Burkina Faso and within Nigeria (sequences from southwestern SO and BA farms and northern Nigeria combined) were similar, reaching 0.52% and 0.54%, respectively. Viruses detected in Niger, Sudan, and Egypt were more homogeneous, with ≤0.24% nucleotide diversity. In Nigeria, both the diversity and the phylogenetic pattern suggested at least 3 independently introduced influenza A (H5N1) lineages in the country (*2*). The branching of the Burkina Faso sequences (Figure 1) suggests that they emerged from a common ancestor, probably shared with other African avian viruses (A/chicken/Ivory Coast/1787/2006, A/chicken/Nigeria/641/2006, and

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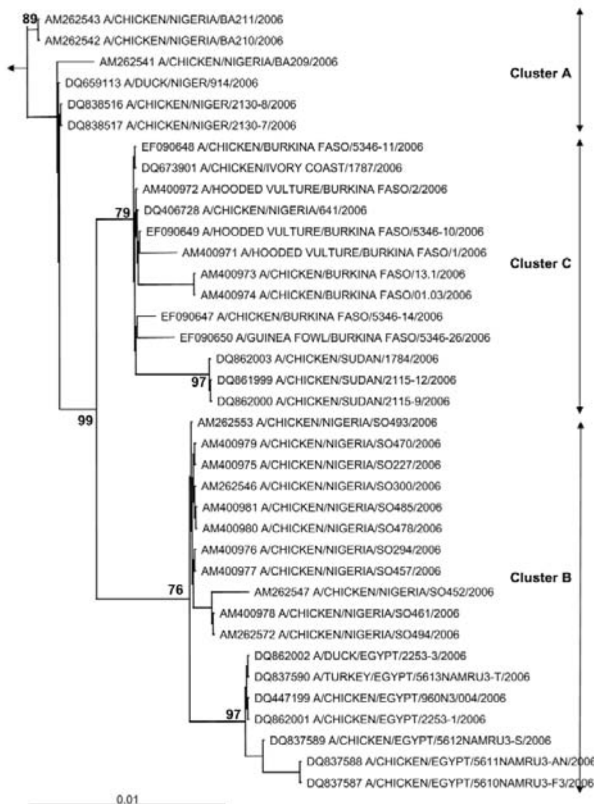

Figure 1. Phylogenetic tree for the hemagglutinin (HA) gene of African influenza A (H5N1) strains. The maximum likelihood method was used with 100 bootstraps and 3 jumbles (DNA-ML, PHYLIP version 3.6) to construct a tree for HA1 nucleotide sequences. Bootstrap values of major nodes are shown. The arrow points to the outgroup strain, A/goose/Guangdong/96. As detailed in the text, cluster C regroups highly pathogenic avian influenza (H5N1) strains from Burkina Faso, northern Nigeria, Sudan, and Côte d'Ivoire; cluster A regroups strains from a southwestern Nigerian farm (coded BA) and Niger; and cluster B regroups strains from a southwestern Nigerian farm (coded SO) and Egypt. The scale bar represents ≈1% of nucleotide changes between close relatives.

strains from Sudan). Strains from Burkina Faso, northern Nigeria, Sudan, and Côte d'Ivoire constitute the putative African cluster C. The data presented in Figure 1 do not support the occurrence of multiple lineages of influenza A (H5N1) viruses in Burkina Faso. However, phylogenetic analysis further indicates that the African HPAI (H5N1) strains form 2 additional clusters. Cluster A contains the strains found in the southwestern Nigerian BA farm and includes all strains found in Niger. Cluster B includes the strains from the southwestern Nigerian SO farm and includes all Egyptian strains. The 3 sublineages correspond to those described previously in Nigeria (*2*) that now show a clear geographic distribution in Africa (Figure 2).

The amino acid sequence of the HA cleavage site of Burkina Faso strains, PQGERRRKKRG, was identical to that of the recent HPAI strains from western Asia, Russia, Europe, and Africa. The 4 strains had a ^{222}Q-^{224}G pattern (H5 numbering [*3*]), compatible with a preferential binding of the virus to α2,3 sialic acid (*4*), mostly found in avian strains. All other amino acid residues thought to be relevant for receptor binding (positions 91, 130–134, 149, 151, 179, 186, 190–191, and 220–225 [*3*]) were identical to those found in all western Asian, Russian, European, and African influenza A (H5N1) sequences as well as in HK/156/97. Therefore, we concluded that the Burkina Faso and other African strains do not contain mutations that facilitate binding to human α2,6 sialic acid receptors.

Although migratory birds in Egypt and a sparrowhawk (*Accipter nisus*) in Côte d'Ivoire were reported to be infected by H5N1 viruses (http://www.oie.int), this study is the first, to our knowledge, to describe the molecular characterization of HPAI (H5N1) viruses detected in African wild birds. We show that the H5N1 strains from northern Nigeria, Burkina Faso, Côte d'Ivoire, and Sudan probably evolved from a common ancestor. The vector of this transmission has not been identified. Virus transmission from domestic to wild birds has rarely been observed (*5,6*), but it appears to be a likely scenario since vultures feed on dead poultry and since during the same time (March 2006) an outbreak of HPAI (H5N1) occurred in an intensive farming system (http://www.oie.int/) in Ouagadougou, where infected vultures were also found. Moreover, the virus strains obtained from chickens and vultures in Ouagadougou are phylogenetically similar, as previously described. Hundreds of these birds can be observed around sites such as abattoirs or market places (*7*). Their feeding behavior could facilitate transmission within domestic poultry, and they could be involved in

Figure 2. Northern equatorial Africa. Only countries where highly pathogenic avian influenza A (H5N1) sequences from avian species are available are named. Strain similarities are indicated as follows: black for countries with cluster A strains, dark gray for countries with cluster B strains, and light gray for countries with cluster C strains .

transmission from farm to farm, consistent with conventional mechanisms of spread through human activity. Moreover, these birds are in close contact with many other scavengers (e.g., hyenas, lions) with similar feeding habits. Hooded vultures also come in close proximity with humans since they are gregarious around human settlements and are used in traditional medicine, which adds to a small but potential risk for virus transmission to humans. Since these vultures often stay close to their local urban or semiurban feeding grounds, they may play a role primarily as short-range vectors, probably through mechanical transmission. In West Africa, some populations may also move north and south with the rains (*7*). Further studies are clearly required to better understand the potential role of vultures in the transmission of HPAI (H5N1) virus. Although vultures are clearly susceptible to this virus, their signs and symptoms could not be unequivocally attributed to subtype H5N1 alone. If these viruses cause severe disease in hooded vultures, these ubiquitous scavenging birds may simply be dead-end hosts. As they scavenge on many dead species, they may also function as conspicuous sentinels in Africa, similar to the role of raptors or swans in Europe or cats in Indonesia (*8*).

Proper disposal of infected carcasses must be carefully enforced on affected farms to avoid primary infections of carrion feeders. The role of hooded vultures and other scavenger birds as vectors of HPAI (H5N1) to other wild birds, poultry, and mammals, including humans, as well as their potential role as sentinels, requires further investigation in Africa.

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Bluetongue in Belgium, 2006

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Bluetongue has emerged recently in Belgium. A bluetongue virus strain was isolated and characterized as serotype 8. Two new real-time reverse transcription–quantitative PCRs (RT-qPCRs) that amplified 2 different segments of bluetongue virus detected this exotic strain. These 2 RT-qPCRs detected infection earlier than a competitive ELISA for antibody detection.

Bluetongue is a noncontagious disease caused by an orbivirus of the family *Reoviridae*. The bluetongue virus (BTV) serogroup consists of 24 serotypes. BTV is transmitted by arthropods of the genus *Culicoides* and its distribution worldwide is restricted to regions that contain competent vectors (*1*). An outbreak of bluetongue was reported and confirmed in the Netherlands on August 17, 2006 (*2*). Belgium reported its first cases of bluetongue 1 day later, and Germany and France reported outbreaks on August 21, 2006, and August 31, 2006, respectively (*2*,*3*). We report detection and characterization of a BTV strain and an overview of laboratory test results 4 weeks after the onset of the outbreak.

The Study

Twenty-one animals (16 cattle and 5 sheep) showing clinical signs suggestive of bluetongue were sampled by the Federal Agency for the Safety of the Food Chain on August 18, 2006, at 11 farms in northeastern Belgium. Two serologic tests that detect antibodies against the major serogroup antigen VP7 (bluetongue virus antibody competitive ELISA [cELISA]; Veterinary Medical Research and Development Inc., Pullman, WA, USA and competitive vp7 bluetongue kit; IDVET, Montpellier, France) identified 21 virus-positive animals. Two newly developed and validated reverse transcription–quantitative PCRs (RT-qPCRs) that detected BTV strains representing the 24 serotypes (*4*) were then conducted to determine whether these seropositive animals also had viral RNA. The first assay (RT-qPCR_S1), which amplified a 357-nt fragment in segment 1, detected virus in erythrocytes of the 21 seropositive animals (mean cycle threshold [Ct] value 29.0). The second assay (RT-qPCR_S5), which amplified a 94-nt fragment in segment 5, detected virus in the same 21 seropositive animals (mean Ct value 26.5). The 2 serologic tests and the 2 molecular assays detected BTV in 21 animals from 11 Belgian farms within 14 hours.

Virus isolation was conducted on August 18, 2006, by injection of blood from infected sheep into 11-day-old embryonated chicken eggs, followed by passage on BHK-21 cells (ATCC-CCL10) as previously described (*5*). The specificity of the cytopathic effect observed 48 hours after passage on BHK-21 cells was confirmed by RT-qPCR and electron microscopy (Figure 1) after fixation and negative staining as previously described (*6*). Two virus neutralization tests were conducted on 2 virus strains isolated by the Belgian and the French reference laboratories at 2 Belgian farms 30 km apart. The 2 BTV isolates were completely neutralized with reference serum for serotype 8. Each strain was also partially neutralized by reference serum against serotype 18, which confirmed cross-neutralization between serotypes 8 and 18 (*7*).

From August 19, 2006, to September 14, 2006, the study farms were screened for animals with clinical signs of bluetongue. Blood samples were tested by serologic tests or RT-qPCR. For cattle, 97 (68%) of 142 samples had antibodies to BTV and 32 (78%) of 41 samples contained viral RNA (Table 1). However, for sheep, only 23 (29%) of 79 samples had antibodies to BTV and 15 (45%) of 33 samples contained viral RNA. Other diseases that cause similar signs might explain this lower frequency in sheep.

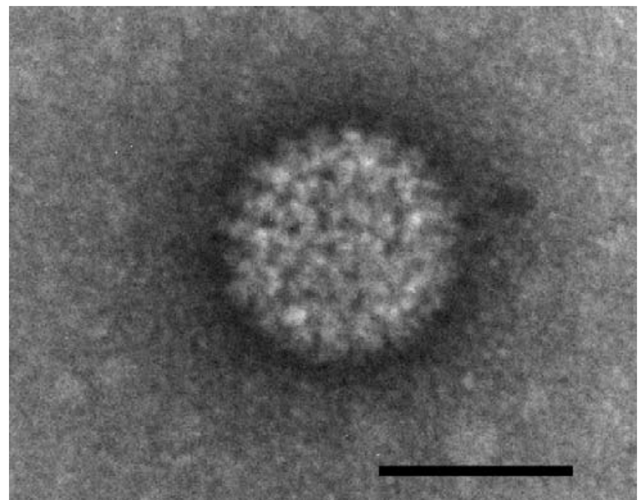

Figure 1. Negatively stained bluetongue virus–like particle that caused a cytopathic effect in BHK-21 cells. Scale bar = 50 nm.

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Table 1. Bluetongue virus infection in cattle and sheep with bluetonguelike clinical signs by IDVET cELISA and RT-qPCR, Belgium, 2006*

Test	Cattle	Sheep
cELISA		
No. negative	45	56
No. positive	97	23
% Confirmed cases	68	29
RT-qPCR		
No. negative	9	18
No. positive	32	15
% Confirmed cases	78	45

*cELISA, competitive ELISA;RT-qPCR, reverse transcription–quantitative PCR.

Contagious ecthyma was diagnosed by using PCR and electron microscopy for several sheep that showed bluetonguelike signs but did not have antibodies to BTV or viral RNA.

Agreement between cELISA and RT-qPCR results was analyzed for 124 animals (Table 2). One sample negative by RT-qPCR_S1 and RT-qPCR_S5 was positive by cELISA. Although this result might reflect lack of specificity of the cELISA, elimination of BTV RNA from the animal several weeks after being infected cannot be ruled out. A false-negative result in the RT-qPCR is unlikely because 1) 2 different RT-qPCRs that amplified 2 different segments were used, 2) the quality of the RNA was confirmed by a third RT-qPCR that quantified mRNA of β-actin, and 3) both RT-qPCRs are highly sensitive (they can detect 0.01 infectious doses of virus) (*4*). False-positive results were not observed with the IDVET cELISA when we analyzed 650 negative serum samples from artificial insemination centers and field samples collected from Belgian livestock in 2004 and 2005. Thus, the specificity of the cELISA is >99.8%. Seven animals with bluetonguelike clinical signs were positive according to each RT-qPCR but negative according to the cELISA (Table 2). These results support the finding that RT-qPCR can be used to detect viral RNA in infected animals before antibodies are detectable. The clinical signs indicative of a recent infection support this finding.

On September 14, 2006 (4 weeks after the first identification of BTV in Belgium) as many as 84 Belgian farms had at least 1 BTV-infected animal. The maximal distance between herds in this study was ≈110 km (Figure 2A). Most outbreaks were confirmed in the area where the disease was initially detected (area I, Figure 2). Most (64%) infected animals showed a high virus load with individual Ct values <30. Of the remaining animals, 30% had moderate virus loads (Ct values 30–35) and 6% had Ct values >35. The high Ct values for the latter animals might have remained undetected had pooled blood samples been analyzed. Thus, results of pooled samples need to be validated before being used for diagnosis. Distribution of Ct values differed slightly, depending on the origin of the animals. None of the animals from zones III and IV showed a Ct value <30, whereas all animals from zone II showed low Ct values, which are indicative of high virus loads. Lower virus loads and acute clinical signs in animals from zones III and IV might indicate onset of infection. However, we cannot rule out decreased infection in these animals because they also had positive serologic results that indicated infection for at least 4–5 days. Further epidemiologic studies are required before conclusions can be drawn on the evolution of these epidemics.

Table 2. Agreement between results of IDVET cELISA and RT-qPCR_S5 for bluetongue virus infection, Belgium, 2006*

cELISA result	RT-qPCR result†	
	Negative	Positive
Negative	75	7
Positive	1	41

*cELISA, competititve ELISA; RT-qPCR, reverse transcription–quantitative PCR.
†Samples with different results in cELISA and RT-qPCR_S5 were retested with RT-qPCR_S1 (*4*). This last test always confirmed the result of the RT-qPCR_S5.

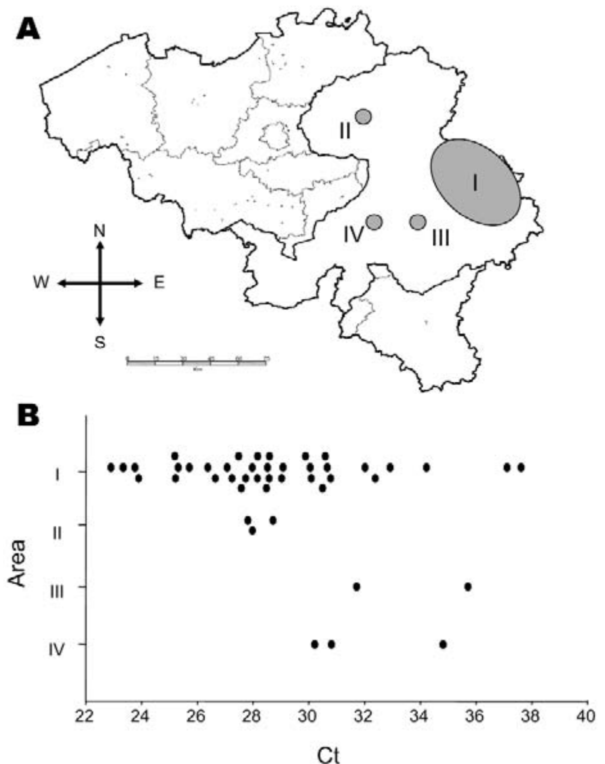

Figure 2. A) Distribution of outbreaks of bluetongue (shaded areas) reported in Belgium from August 18 through September 14, 2006. Area I is where the disease was initially detected. B) Cycle threshold (Ct) values observed in different zones as a result of conducting reverse transcription–quantitative PCR_S5 on individual blood samples.

Conclusions

Bluetongue has emerged in some countries of northern Europe. BTV has been detected and its isolation and characterization have considerably progressed in the first weeks of the epidemics. Results of virus neutralization tests for 2 Belgian isolates and molecular characterization of the Dutch BTV strain by the community reference laboratory (*8*) indicate that BTV serotype 8 is present in Belgium and the Netherlands. Although this observation suggests 1 serotype circulating in northern Europe after a common virus introduction, it must be confirmed by detailed epidemiologic studies. The mechanism of introduction of BTV strain serotype 8 is unknown. Northward spread of bluetongue in Europe has been correlated with climate warming (*9*). However, BTV serotype 8 has not been found in the Mediterranean basin.

One characteristic of the current epidemics of bluetongue is the severity of clinical signs reported in cattle (*10*). The present results also demonstrate that clinical signs observed in cattle are more specific than those observed in sheep. Confusing clinical signs in sheep underline the need for developing diagnostic tests to discriminate between bluetongue and other confounding diseases such as contagious ecthyma, border disease, and foot-and-mouth disease. Our results also indicate the usefulness of RT-qPCR, which detected viral RNA in recently infected animals with clinical signs of bluetongue but no detectable antibodies to BTV. The RT-qPCR and ELISA are independent but complementary tests because they detect viral RNA and virus-specific antibodies, respectively. These tests indicated that an outbreak of bluetongue was occurring in Belgium. Despite high sensitivity of RT-qPCR (*4*), our results suggest that using this test with pooled samples might not detect animals with low viral loads. This possibility should be explored and validated by testing individual and pooled samples. RT-PCR–positive results in animals that are no longer infectious (*11*) should also be considered before deciding whether pooled samples are acceptable.

Dr Toussaint is a research scientist in the Department of Virology, Veterinary and Agrochemical Research Centre, Brussels, Belgium. His research interests include development, evaluation, and optimization of DNA vaccines against bovine herpesvirus 1, and new tools for detection of bluetongue virus and foot-and-mouth disease virus.

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Influenza Vaccine Effectiveness among US Military Basic Trainees, 2005–06 Season

Jennifer K. Strickler,* Anthony W. Hawksworth,* Christopher Myers,* Marina Irvine,* Margaret A.K. Ryan,* and Kevin L. Russell*

Virtually all US military basic trainees receive seasonal influenza vaccine. Surveillance data collected from December 2005 through March 2006 were evaluated to estimate effectiveness of the influenza vaccine at 6 US military basic training centers. Vaccine effectiveness against laboratory-confirmed influenza was 92% (95% confidence interval 85%–96%).

Public health concerns over the potential for a devastating influenza pandemic in the near future are well known. Surveillance efforts have increased throughout the world, and much time and money have been directed toward preparedness for such a pandemic. Given that vaccination rates vary greatly among the nonmilitary population and that influenza diagnostics are sporadically available, annual influenza vaccine effectiveness studies based on laboratory-confirmed diagnoses are rare. However, evidence of locally circulating strains evading the vaccine-induced protection could be critical for early recognition and intervention. In addition, the emergence of pandemic strains within military populations has been noted. The first documented influenza outbreak in the spring of 1918, before the great influenza pandemic of 1918–19, was among recruits at Fort Riley, Kansas (*1*). In 1976, a unique strain of influenza (H1N1) caused an outbreak at Fort Dix, New Jersey, causing 1 death, and creating concern over spread of this nonvaccine strain (*2*). Highly vaccinated military populations, under close surveillance, provide the opportunity for annual calculation of influenza vaccine effectiveness, thereby benefiting global pandemic preparedness.

The Study

The Naval Health Research Center (NHRC) began conducting tri-service surveillance for febrile respiratory illness at military training centers in 1996; by 1999, this surveillance network had expanded to include 8 of the largest military basic training centers in the United States (*3*). This surveillance includes the systematic collection of throat swab specimens and clinical data (including but not limited to gender, date of birth, symptoms, influenza vaccination status, type of vaccine received, and date of vaccination) from consenting US military trainees meeting the case definition for febrile respiratory illness (oral temperature ≥100.5°F [38.0°C] and a cough or sore throat). Samples are stored locally at each site at –70°C until they are forwarded to the Naval Respiratory Disease Laboratory at NHRC for viral culture and molecular diagnostic processing. Research personnel at participating surveillance sites report the weekly number of trainees who sought care for febrile respiratory illness and total trainee populations for their respective sites, and rates for such illnesses are calculated.

During the 2003–04 influenza season, we recognized the opportunity of using data from this ongoing active surveillance to estimate influenza vaccine effectiveness in protecting against both laboratory-confirmed influenza and febrile respiratory illness of any cause among US military basic trainees. Despite concerns that vaccine effectiveness during the 2003–04 season would be low because of the poor match between the components of the vaccine and the circulating strain (*4*), the vaccine provided good protection (94.4%) against laboratory-confirmed influenza that season (*5*). Annual vaccine effectiveness calculations are important as we heighten our preparedness for pandemic influenza strains; therefore, we performed similar calculations for the 2004–05 and 2005–06 seasons.

During the late fall and winter seasons, all active-duty military forces are required to receive the influenza vaccine, and this policy is strictly enforced in training camps. Upon arrival, all incoming trainees receive mandatory influenza vaccination, either the trivalent inactivated influenza vaccine by injection (FluZone, Sanofi Pasteur, Lyon, France) or intranasal cold-adapted, live, attenuated influenza vaccine (CA-LAIV) spray (FluMist, MedImmune, Gaithersburg, MD, USA).

For this analysis, vaccine protection was assumed to begin 14 days postvaccination. Therefore, in an 8-week training program, 25% of trainees were considered "unvaccinated" at any given time, assuming immunity takes 14 days to develop. Likewise, 33% of trainees in a 6-week training program were considered unprotected by the vaccine at any time. These assumptions allow estimates of denominator data for "vaccinated" and "unvaccinated" person-weeks in calculations of vaccine effectiveness.

From January through March 2006 all new trainees arriving for basic training received the influenza vaccine; all recruits already present had been vaccinated. The observation period for this analysis included January 1–March 31, 2006. However, 2 sites, Naval Service

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Training Command, Great Lakes, and Marine Corps Recruit Depot, San Diego, had completed vaccination by December 2005. Therefore, December was included in the observation period for those sites as well. Total person-weeks in recruit training during the observation period were obtained directly from the participating training centers. Vaccine effectiveness was calculated for both laboratory-confirmed influenza and any cause of febrile respiratory illness as follows: 100 × (1 – relative risk = 1 – [rate in vaccinated group]/[rate in unvaccinated group]).

During the observation period, 6 of 8 surveillance sites had influenza activity and were included in this analysis. In 479,181 person-weeks of observation, 4,052 cases of febrile respiratory illness were reported from these 6 sites, and 722 patients were enrolled into the surveillance study (includes throat swab specimen, case data, and consent). Seventy (9.7%) specimens tested positive for influenza, by either culture or molecular techniques.

Rates of laboratory-confirmed influenza were higher among unvaccinated trainees at all sites except Fort Benning, Georgia, which had only 3 cases (Figure). Overall, influenza vaccine effectiveness among US military trainees was 92% (confidence interval [CI] 85.4–95.6%) during the 2005–06 season (Table). Vaccine effectiveness against laboratory-confirmed influenza was high (range 86%–94%) in each of the past 3 seasons. Vaccine effectiveness against non–laboratory-confirmed febrile respiratory illness was lower, ranging from –10% in 2005–06 to 52% in 2004–05.

Conclusions

This analysis suggests that the 2005–06 influenza vaccine was highly effective in protecting US military basic trainees against laboratory-confirmed influenza. Furthermore, these data suggest that both the trivalent inactivated vaccine injection and the CA-LAIV intranasal spray were equally effective, because the Marine Corps Recruit Depot in San Diego vaccinated its trainees with CA-LAIV almost exclusively, and vaccine effectiveness at that site was 95% (vaccine effectiveness at all other sites combined = 90%).

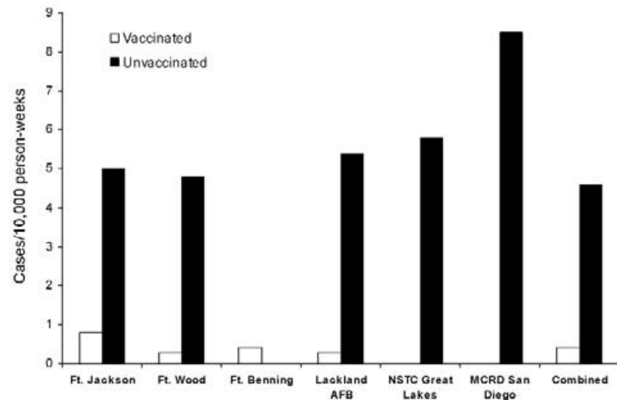

Figure. Incidence of laboratory-confirmed influenza by vaccination status. AFB, Air Force Base; NSTC, Naval Service Training Command; MCRD, Marine Corps Recruit Depot.

These estimates of effectiveness were supported by results of additional analyses that would be expected to bias the outcome toward the null hypothesis. For example, a 7-day lag period before immune response was considered in an alternative analysis, and it yielded similar results: the calculated vaccine effectiveness changed only slightly, from 92% to 90%. We also analyzed vaccine effectiveness, assuming that 10% fewer trainees were vaccinated at any given point, yet the calculated vaccine effectiveness was only reduced to 87%.

In contrast to the consistently high effectiveness of the vaccines against laboratory-confirmed influenza, the effectiveness against febrile respiratory illness of any cause was much lower and varied with each season (13.9% in 2003–04, 52.1% in 2004–05, and –10% in 2005–06). This lower effectiveness in 2005–06 is most likely due to the generally high proportion of adenovirus infection seen in this population (*6*), and the lesser effectiveness is further exacerbated by the tendency for adenoviral infections to occur beyond the second week of training. The lower vaccine effectiveness seen against febrile respiratory illness of any cause gives credence to

Table. Vaccine effectiveness against laboratory-confirmed influenza among US military basic trainees, 2005–06*†

Site	Vaccinated person-weeks	Unvaccinated person-weeks	Cases in vaccinated trainees	Cases in unvaccinated trainees	Vaccine effectiveness (%)	95% CI
Fort Jackson, SC	77,874	25,958	7	13	82.1	
Fort Wood, MO	67,513	22,504	2	11	93.9	
Fort Benning, GA	68,652	22,884	3	0	–	
Lackland AFB, TX	37,435	18,690	1	10	95.0	
NSTC Great Lakes, IL	67,763	22,588	0	13	100.0	
MCRD San Diego, CA	35,490	11,830	0	10	100.0	
Total	354,727	124,454	13	57	92.0	(85.4%, 95.6%)

*CI, confidence interval; SC, South Carolina; MO, Missouri; GA, Georgia; AFB, Air Force base; TX, Texas; NSTC, Naval Service Training Command; IL, Illinois; MCRD, Marine Corps Recruit Depot; CA, California.
†Assuming 14 d before vaccine is protective.

the estimates of high vaccine effectiveness against laboratory-confirmed influenza. If a measurement bias existed, both estimates would be affected.

As a highly vaccinated population, military personnel, and basic trainees in particular, can provide critical information regarding the effectiveness of each year's influenza vaccine formulations. Because of the annual variations of both the vaccine formulations and the circulating strains, influenza vaccine effectiveness should be evaluated annually. With the ever-rising concerns of an imminent influenza pandemic, reliable and rigorous influenza surveillance is paramount. Our existing surveillance network will allow us to repeat the methods used in this analysis each year, thus providing valuable estimates of influenza vaccine effectiveness to the public health community.

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Ms Strickler has coordinated epidemiologic studies for the Department of Defense Center for Deployment Health Research at Naval Health Research Center since 2000. Her research interests focus on respiratory illness among military populations.

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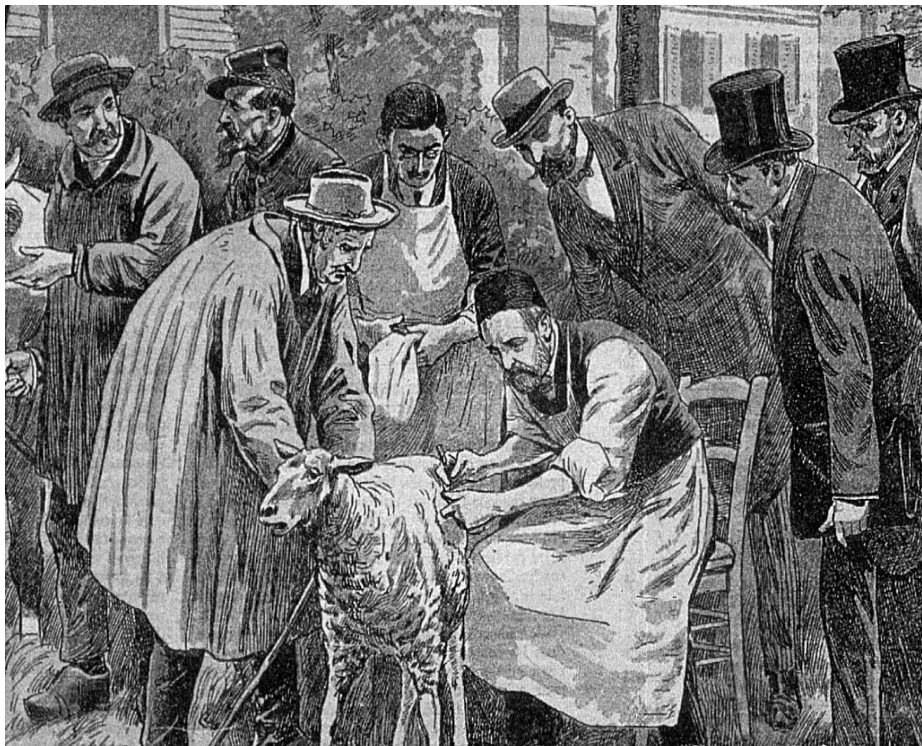

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Human Sapovirus in Clams, Japan

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Human sapovirus was detected in 4 of 57 clam packages by reverse transcription–PCR and sequence analysis. This represents the first finding of sapovirus contamination in food. Closely matching sequences have been detected in stool specimens from patients with gastroenteritis in Japan, which indicates a possible food-to-human transmission link.

Sapoviruses and noroviruses are etiologic agents of human gastroenteritis. Human noroviruses are the most important cause of outbreaks of gastroenteritis worldwide and can be transmitted by a variety of routes, including food (*1*). Sapovirus infections are mostly associated with sporadic gastroenteritis in young children; however, foodborne transmission routes are yet to be determined. The most widely used method of detection is reverse transcription–PCR (RT-PCR), which has a high sensitivity and can also be used for genetic analysis. Sapovirus strains can be divided into 5 genogroups; GI–GV infect humans; sapovirus GIII infects porcine species. Phylogenetic studies have also designated sapovirus clusters or genotypes to further describe strains.

The Study

The purpose of this study was to detect sapovirus in the clam *Corbicula japonica* (bivalve mollusk) and describe the genetic diversity of the strains. A total of 57 clam packages (30–60 clams per package) were collected from supermarkets or fish markets from 6 different areas in western Japan from December 8, 2005, to September 6, 2006. The samples were shucked, and the digestive diverticulum was removed by dissection and weighed. One gram of digestive diverticulum (10–15 clam/package) was homogenized with an Omini-mixer (Sorvall Inc., Newtown, CT, USA) in 10 mL phosphate-buffered saline. After centrifugation at 10,000× *g* for 30 min at 4°C, the supernatant was centrifuged at 100,000× *g* for 2 h (SW41 Rotor, Beckman Instruments, Inc., Fullerton, CA, USA). The pellet was resuspended in 140 µL distilled water and stored at –80°C until use.

RNA extraction and nested RT-PCR were performed as described (*2*). Briefly, for the first PCR, F13, F14, R13, and R14 primers were used; for the nested PCR, F22 and R2 primers were used. All RT-PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. RT-PCR products were excised from the gel and purified by the QIAquick gel extraction kit (QIAGEN, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1, Applied Biosystems, Warrington, England) and determined with the ABI 3130 sequencer (ABI, Boston, MA, USA). Nucleotide sequences were aligned with ClustalX, and the distances were calculated by Kimura's 2-parameter method, as described elsewhere (*2*). Nucleotide sequence data determined in this study have been deposited in GenBank under accession nos. EF104251–EF104254.

Four (7%) of 57 clam packages were contaminated with sapovirus (termed Shijimi1, Shijimi2, Shijimi3, and Shijimi4). Genetic analysis of the partial capsid gene showed that these 4 sequences shared >98% nucleotide similarity and >97% amino acid identity. Phylogenetic analysis grouped these 4 sequences in the same genotype, i.e., GI/1 (Figure). Similar sequences were found on the database (Figure). Strains from this cluster likely represent the dominant genotype worldwide (*3*). Three of 4 sapovirus-positive clam packages were collected from different areas and at different times (Figure). The clam packages that were contaminated with Shijimi1 and Shijimi3 were collected from the same area, but 6 weeks apart, which indicates an ongoing sapovirus contamination or resistance in the natural environment. The seasonality of sapovirus infection in Japan is unknown; however, as with norovirus, sapovirus infections may also peak during winter, although further epidemiologic and environmental studies are needed.

In a recent study, we detected sapovirus strains in 7 of 69 water samples, which included untreated wastewater, treated wastewater, and a river in Japan (*4*). Three of 7 sapovirus sequences detected in the water samples belonged to GI/1 and shared >97% nucleotide similarity with the sapovirus sequences detected in the clam packages. Additionally, sapovirus sequences belonging to GI/1 and sharing >99% nucleotide similarity, for example, Chiba/010598F strain (Figure), have been detected in stool specimens from children with sporadic gastroenteritis in Japan (*5*,*6*). The closely matching sapovirus sequences detected in the water, clams, and patients suggest that sapovirus contamination in the natural environment can

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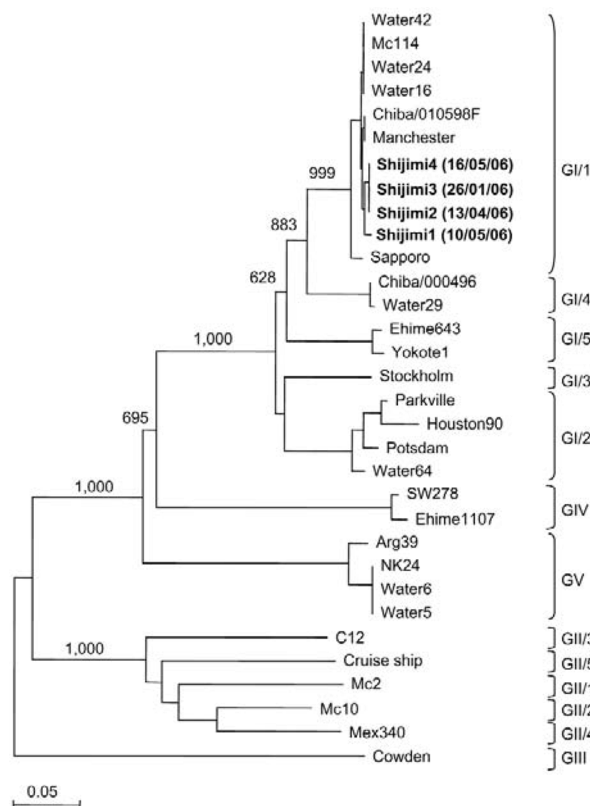

Figure. Phylogenetic analysis of sapovirus capsid sequences (≈300 nt) showing the different genogroups and clusters. Numbers on each branch indicate bootstrap values for the genotype. Bootstrap values of ≥950 were considered statistically significant for the grouping. The scale represents nucleotide substitutions per site. GenBank accession nos.: Mex340, AF435812; Parkville, U73124; Cowden, AF182760; Potsdam, AF294739; Sapporo, U65427; Stockholm, AF194182; SW278, DQ125333; water samples, DQ915088–DQ915094; and Yokote, AB253740. **Boldface** represents sequences detected in this study.

lead to foodborne infections in humans, although direct evidence is lacking. More important, a recent study found animal sapovirus in oysters and suggested that coinfection with human and animal sapovirus strains could result in genomic recombination and the emergence of new strains (*7*). At the same time, we recently described the first human sapovirus intergenogroup recombinant strain (*8*). Phylogenetic analysis of the nonstructural region (i.e., genome start to capsid start) grouped this sapovirus strain in GII, while the structural region (i.e., capsid start to genome end) grouped this strain in GIV.

A large number of studies have detected norovirus in oysters. In 2 recent studies, norovirus was detected in oysters (*Crassosterea gigas*) harvested from geographically isolated areas in Japan (*9*,*10*). We also screened the same oyster samples for sapovirus; however, all of the samples were negative for sapovirus. That sapovirus was detected in the clam samples, but not in the oyster samples, is of interest. In the past several years, increasing evidence has emerged that human noroviruses bind to histo-blood group antigens (HBGAs) (*11*). These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, and in oyster digestive tissues. A number of studies have found that different norovirus strains exhibit different binding patterns to HBGAs and oyster digestive tissues (*12*,*13*). In a recent study, we found that sapovirus GI and GV strains showed no such binding activity to HBGAs (*14*). These results suggest that human norovirus and sapovirus strains have different binding receptors or that human sapovirus may not concentrate in detectable levels in oysters.

Conclusions

Foodborne diseases are a major problem worldwide. We report what is, to the best of our knowledge, the first account of sapovirus contamination in food destined for human consumption. The report may represent a possible food-to-human transmission link, although direct evidence is lacking. In Japan, clams are usually boiled before they are consumed in soups. However, boiling to open the clam may not inactivate the virus (*15*); in addition, some areas in Japan do not boil clams before eating them. Further studies are needed to determine if boiling inactivates sapovirus and if the contaminated clams are indeed infectious. In conclusion, these novel results highlight the importance of sapovirus, in particular the GI/1 strains. A new awareness of sapovirus transmission routes is necessary and may help reduce sapovirus infections.

Dr Hansman is a scientist at the National Institute of Infectious Diseases, Japan. He studies viruses that cause gastroenteritis in humans, namely sapovirus and norovirus. His research interests include epidemiology, virus expression, and cross-reactivity.

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Cryptosporidiosis Decline after Regulation, England and Wales, 1989–2005

Iain R. Lake,* Gordon Nichols,†
Graham Bentham,* Florence C.D. Harrison,*
Paul R. Hunter,* and R. Sari Kovats‡

Since new drinking water regulations were implemented in England and Wales in 2000, cryptosporidiosis has been significantly reduced in the first half of the year but not in the second. We estimate an annual reduction in disease of 905 reported cases and ≈6,700 total cases.

Cryptosporidiosis is a common cause of gastroenteritis worldwide. In England and Wales, ≈4,500 cases are reported each year (*1*). In the 1990s, several cryptosporidiosis outbreaks in England and Wales were associated with public drinking water supplies; in 2000, new drinking water regulations were implemented to address this problem. Risk assessments were required at all water treatment plants, and those that did not meet the standards were required to monitor regularly for *Cryptosporidium* spp. Consequently, water companies closed some plants, upgraded others, and paid close attention to the maintenance and operation of their works (*2*). Since these regulations were implemented, a reduction in reported cases of cryptosporidiosis, especially the disappearance of the spring peak, has been reported in northwestern England (*3*). The aim of our research was to quantify the public health impact of the regulations by assessing whether they have led to statistically significant reductions in cryptosporidiosis.

The Study

All cases of cryptosporidiosis in England and Wales reported to national surveillance from 1989 through mid-2005 were analyzed; those associated with recent foreign travel were excluded. The average weekly number of cryptosporidiosis cases preregulation (1989–1999) were plotted against the same data postregulation (2000–2005) (Figure). Since the regulations were implemented, fewer cryptosporidiosis cases have occurred in the first half of the year but more in the second. However, as the standard deviation bars on the figure indicate, the number of cases fluctuated from year to year both before and after the regulations. This trend makes it difficult to ascertain whether the changes after regulation are part of the natural interannual variability or represent real changes in incidence. It also makes it difficult to quantify the public health impact of the regulations.

Climatic variability and community spread from imported travel cases are suggested as the main sources of this interannual variability (*4*,*5*). Precipitation may wash *Cryptosporidium* organisms from land into public water supplies, and warm, dry weather may increase the number of countryside visits. Both of these could result in exposure to *Cryptosporidium* organisms. Consequently, we developed a predictive model of weekly cryptosporidiosis cases using weekly incidence data (1989–1999) and national data on temperature, rainfall, river discharges, and reported number of travel-associated cases. Separate models were produced for different periods of the year. Ordinary least-squares regression was used for analyses.

The results indicated that between mid-March and the end of June cryptosporidiosis cases were positively associated with river discharges that occurred 2 weeks previously. From July through early September, cryptosporidiosis was positively associated with warm, dry weather in the previous 2 months. No associations between cryptosporidiosis and weather existed at other times. Travel cases were not significant in any of the models. The detailed methods and results of this analysis are available from the author. The results are consistent with previous research (*4*,*5*).

Comparable data on temperature, rainfall, and river discharges were obtained for the postregulation period (2000–2005) and entered into the predictive model. This estimated the number of cases that would have been expected, for each week, from 2000 through 2005. To provide an overview of these predictions, the estimates were summed to produce totals for each half of the year, for every year after regulation.

The results are presented in the Table alongside the 95% confidence interval of the prediction, the actual numbers of cases reported, and the difference between the actual and predicted cases. In the first half of the year, cryptosporidiosis was significantly reduced ($p<0.05$) every year since 2000. For this finding to be attributable to the regulations, other factors important in cryptosporidiosis etiology should have remained constant during this period. Cryptosporidiosis has been associated with recreational swimming and person-to-person contact and, to our knowledge, the levels of these have remained unchanged.

The greatest reduction in cases occurred in the first

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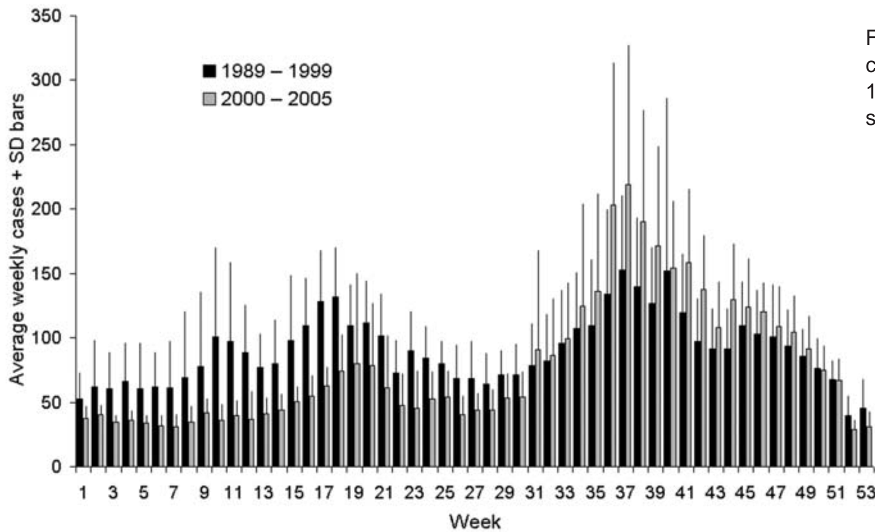

Figure. Weekly cryptosporidiosis cases, England and Wales, 1989–1999 and 2000–2005. SD, standard deviation.

half of 2001, a period that coincides with the foot-and-mouth disease epidemic. This epidemic led to the slaughter of >6 million livestock and restricted public access to agricultural land (*6*). The large reduction in cases in 2001 has been attributed to this epidemic (*7*,*8*), but our results indicate that cases were already depressed in the first half of 2000, and these reductions continued into 2002. Therefore, the large reduction observed in the first half of 2001 is also likely to be due to the new drinking water regulations.

Another reason for lower cryptosporidiosis incidence since 2000 could be lower levels of *Cryptosporidium* spp. in livestock after the foot-and-mouth epidemic (*9*). However, a recent study has discounted this (*3*), and factors associated with the 2001 epidemic cannot explain the reductions in cases observed in 2000. We conclude, therefore, that improved water treatment associated with the new drinking water regulations has led to cryptosporidiosis reductions during the first half of the year.

In the second half of the year, the pattern is less straightforward. The numbers of cases are significantly ($p<0.05$) lower than predicted in 2001, 2002, and 2004, but significantly higher ($p<0.05$) in 2000 and 2003. One explanation for the excess cases in the second half of 2000 and 2003 is that they may represent unreported travel-associated cases or community transmission from these cases. The Table demonstrates that many foreign travel–associated cases occurred in both these periods (>300 in 2000 and 2003 compared with <200 for other years), and these are poorly recorded in national surveillance (*10*). This inconsistency in the pattern between years, combined with the potential link between excess cases and travel-associated cases, led us to conclude that the overall increase in incidence in the second half of the year is not likely to be related to the regulations.

Conclusions

By averaging the differences between the observed and predicted cryptosporidiosis cases across the years, we can estimate the public health benefits of the regulations. The average excludes 2001 because of the confounding effect of the foot-and-mouth epidemic. Since 2000, an annual average reduction of 615 reported cases has occurred. This reduction comprises a large decrease in the

Table. Predicted and observed cryptosporidiosis England and Wales, 2000 to mid-2005

Period		Predicted cases	95% Confidence interval	Observed cases	Predicted minus observed cases	Reported travel-associated cases
First half of year (weeks 1–26)	2000	2,431	2,161–2,699	1,890	541	85
	2001	2,510	2,242–2,776	925	1,585	54
	2002	2,200	1,932–2,467	1,103	1,097	47
	2003	2,107	1,840–2,373	1,150	957	41
	2004	2,159	1,892–2,425	1,316	843	42
	2005	2,021	1,744–2,297	931	1,090	30
Second half of year (weeks 27–52)	2000	2,438	2,140–2,734	3,477	–1,039	322
	2001	2,927	2,627–3,226	2,461	466	194
	2002	2,294	1,996–2,591	1,795	499	65
	2003	2,713	2,416–3,010	4,287	–1,574	366
	2004	2,552	2,241–2,863	2,198	354	69

first half of the year and a small increase in the second half. If we assume that the increase in cases in the second half of the year is not associated with drinking water, the benefit of the intervention is 905 reported cases per year (the average reduction in the first half of the year).

Not all cases of cryptosporidiosis in the community are reported to national surveillance, and the ratio of reported to community cases is estimated to be 7.4 (*11*). This multiplier has uncertainties because it is based upon a single study. If this multiplier is applied to our estimate of 905 cases, it implies 6,770 fewer cases of cryptosporidiosis in the community each year. Two recent reports have suggested that even this multiplier may be an underestimate (*12*,*13*).

We have presented evidence that new drinking water regulations implemented in England and Wales during 2000 led to significantly fewer cryptosporidiosis cases in the first half of the year with no significant change in the second half of the year. We estimate a reduction in reported cases of 905 per year or ≈6,770 cases in the community each year. These findings indicate that regulations such as those implemented in England and Wales can have a significant public health benefit in reducing cases of cryptosporidiosis.

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Dr Lake is a lecturer in environmental sciences at the University of East Anglia. His research interests include environment and human health, climate change, and health and geographic information systems.

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New Water

Sharon Chmielarz

All those years–almost a hundred–
the farm had hard water.
Hard orange. Buckets lined in orange.
Sink and tub and toilet, too,
once they got running water.
And now, in less than a lifetime,
just by changing the well's location,
in the same yard, mind you,
the water's soft, clear, delicious to drink.
All those years to shake your head over.
Look how sweet life has become;
you can see it in the couple who live here,
their calmness as they sit at their table,
the beauty as they offer you new water to drink.

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Avian Influenza Viruses in Water Birds, Africa

Nicolas Gaidet,* Tim Dodman,† Alexandre Caron,* Gilles Balança,* Stephanie Desvaux,* Flavie Goutard,* Giovanni Cattoli,‡ François Lamarque,§ Ward Hagemeijer,† and François Monicat*

We report the first large-scale surveillance of avian influenza viruses in water birds conducted in Africa. This study shows evidence of avian influenza viruses in wild birds, both Eurasian and Afro-tropical species, in several major wetlands of Africa.

Wild water birds are considered to be the major natural reservoir for avian influenza viruses (AIV) (*1*). Large numbers of Eurasian breeding water birds overwinter in the sub-Saharan region of the African continent (*2*), where the survival of AIV is considered to be restricted by the tropical environment (*3*). Although the first reported isolation of AIV from wild birds (A/Tern/S.A./61 [H5N3]) was in Africa (*4*), a knowledge gap exists in the ecology of AIV in tropical regions (*1,5*). Whether AIV circulate in waterbird communities in Africa and whether tropical ecosystems can play a role in the perpetuation of AIV among waterfowl remain unknown. We report results from large-scale surveillance of water birds in 12 countries in Africa (Figure).

The Study

This surveillance program was implemented in early 2006 within the framework of the Food and Agriculture Organization (FAO)'s Technical Cooperation Programs of Emergency Assistance for Early Detection and Prevention of Avian Influenza. Field sampling operations were coordinated by Centre de cooperation Internationale en Recherche Agronomique pour le Développement and by Wetlands International, in partnership with wildlife and veterinary national services, international organizations[1], local ornithologic nongovernment organizations, as well as national hunting associations and safari operators. Study species were selected among bird families recognized as major AIV reservoirs (notably among the orders Anseriformes and Charadriiformes), in both Eurasian and Afro-tropical bird communities. Study sites important for congregatory water birds were selected in accordance with national surveillance programs and field logistic constraints and included sites where palearctic and Afro-tropical birds mix.

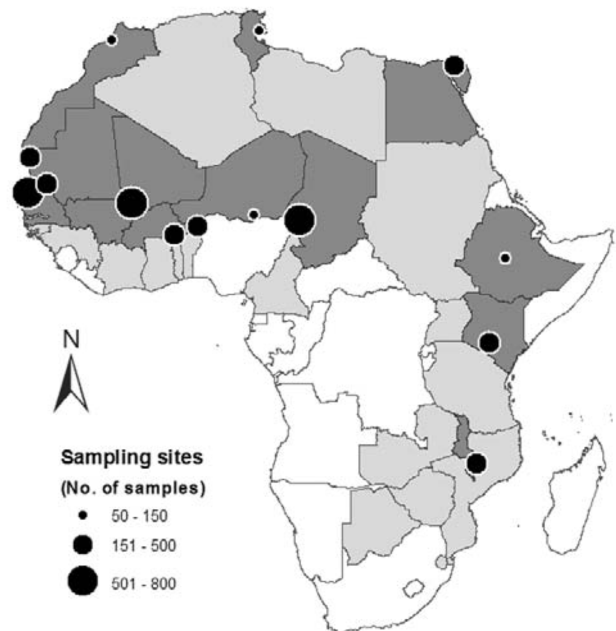

Figure. Locations of sampling sites (or clusters of sites) in surveyed African countries (dark gray) initially participating in the Food and Agriculture Organization's Technical Cooperation Programs (light and dark gray). All samples were collected from mid-January to early March 2006 (but until May in Tunisia).

From mid-January to early March 2006 (and early May in Tunisia), we collected cloacal swab samples from captured birds and from freshly killed birds provided by hunters. Samples of fresh droppings were also collected at roosting areas for gulls, terns, and some ducks. In Ethiopia, which has hunting restrictions, and in countries in which emergency surveillance operations were implemented after notification of influenza A (H5N1) outbreaks in Nigeria (Burkina Faso, Niger), special permits were obtained to shoot birds for sample collection (n = 732).

Materials used and storing procedures were standardized among field teams. The transport medium consisted

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[1]African Waterbird Ringing Scheme (AFRING), Oiseaux Migrateurs du Paléarctique Occidental (OMPO), Office National de la Chasse et de la Faune Sauvage (ONCFS), Dutch Centre for Field Ornithology or Stichting Openbaar Voortgezet Onderwijs Noord (SOVON), and Wildfowl and Wetlands Trust (WWT).

of an isotonic phosphate-buffered saline, pH 7.0–7.4, containing antimicrobial agents (penicillin 10,000 U/mL, streptomycin 10 mg/mL, amphotericin B 25 µg/mL, and gentamycin 250 µg/mL) supplemented with 10% glycerol. Samples were stored in liquid nitrogen containers or on ice and then stored at <–70°C after a few hours (generally <4 h, maximum of 24 h). They were shipped in dry ice in cryopacks until processed.

Samples were analyzed at the Istituto Zooprofilattico Sperimentale delle Venezie (Italy), except for samples from Egypt that were analyzed at the US Naval Medical Research Unit-3 (Egypt), samples from Kenya and Malawi which were analyzed at the Agricultural Research Council Onderstepoort Veterinary Institute (RSA), and samples from Tunisia which were analyzed at the Southeast Poultry Research Laboratory (USA). The samples were all screened by real-time reverse transcription (RT)–PCR specific for type A influenza viruses (*6*), and positive samples were tested by RT-PCR specific for H5 subtype. All type A–positive samples were subsequently processed for virus isolation by using standard methods (inoculation into the allantoic cavity of 9- to 10-day-old embryonated specific-pathogen–free eggs, EU directive 92/40). Isolates were characterized by hemagglutination and neuraminidase-inhibition tests by using specific hyperimmune chicken antisera to the reference strains of influenza virus (*7*). Molecular pathogenicity of H5 subtype–positive samples was determined by sequencing the hemagglutinin gene segment (BigDye Terminator v3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA, USA).

A total of 4,553 birds (Table 1), consisting mostly of Afro-tropical and Eurasian ducks (32% and 31% of sam-

Table 1. Prevalence of avian Influenza virus in wild birds*

Bird group	Species tested	No.	PCR positive, no. (%)	Positive country
African ducks	9 species (total, including 4 named below)	1,455	41 (2.8)	
	Dendrocygna viduata	1,181	38 (3.2)	TD, ET, ML, MR, NE, SN
	Sarkidiornis melanotos	117	3 (2.6)	ML, NE
	D. bicolor	88	0	
	Plectropterus gambensis	32	0	
Eurasian ducks	10 species	1,409	93 (6.6)	
	Anas querquedula	1,335	87 (6.5)	TD, ML, MR, NE, SN
	A. acuta	24	2 (8.3)	ML
	A. crecca	24	3 (12.5)	MA
	A. clypeata	6	1 (16.7)	MA
Eurasian waders	13 species	409	6 (1.5)	
	Philomachus pugnax	115	2 (1.7)	ML
	Tringa glareola	74	0	
	Calidris minuta	60	0	
	C. ferruginea	45	2 (4.4)	TN
	Himantopus himantopus	45	0	
	Gallinago gallinago	30	0	
	T. erythropus	23	2 (8.7)	ML
Rails	8 species	438	3 (0.7)	
	Porphyrio alleni	187	0	
	Amaurornis flavirostris	88	0	
	Fulica cristata	80	0	
	Gallinula chloropus	31	2 (6.5)	ML
	Porphyrio porphyrio	10	1 (10)	ML
Gulls	3 species	366	14 (3.8)	
	Larus genei	156	13 (8.3)	SN
	L. fuscus	129	1 (0.8)	MR
	L. melanocephalus	81	0	
Terns	7 species	159	2 (1.3)	
	Sterna sp.†	150	2 (1.3)	MR
Cormorants	2 species	148	0	
	Phalacrocorax carbo	130	0	
Other	36 species	196	0	
Total	87 species	4,553	159 (3.5)	

*Detected by reverse transcription–PCR (RT-PCR), for all RT-PCR–positive species and in species with >30 individuals sampled. Lower numbers in individual species are included in the total for each bird group. Countries where RT-PCR–positive samples were obtained are indicated (TD, Chad; ET, Ethiopia; ML, Mali; MR, Mauritania; NE, Niger; SN, Senegal; MA, Morocco; TN, Tunisia).
†Unidentified fresh dropping samples from a multispecies flock of *Sterna caspia*, *S. maxima,* and *S. sandvicensis*.

ples, respectively), were tested. The overall protion of AIV detected was 3.5% (n = 159 RT-PCR–positive samples, including both cloacal swabs and fresh droppings). Low-pathogenicity AIV were detected in 14 species of ducks, waders, gulls, terns, and rails, including both Eurasian and Afro-tropical species (Table 1). Positive samples were obtained from 8 countries (Chad, Ethiopia, Mali, Mauritania, Morocco, Niger, Senegal, and Tunisia). In the 2 most frequently sampled species, Eurasian ducks (garganey [*Anas querquedula*], n = 1,329) and Afro-tropical duck (white-faced whistling ducks [*Dendrocygna viduata*], n = 1,157), AIV were detected from most surveyed countries but with a highly variable prevalence (Table 2). Neither influenza A (H5N1) viruses nor any highly pathogenic AIV were detected. A total of 11 samples were positive for H5 subtype, mostly from garganey ducks (H5 prevalence of 0.7%). Finally, 5 low-pathogenicity AIV were isolated: 3 distinct isolates that originated from garganey ducks sampled in the Inner Niger Delta in Mali (H5N3, H11N9, H12N5) and 2 isolates that originated from white-faced whistling ducks sampled in Ethiopia (H8N4) and Senegal (H1N1).

Conclusions

The African continent, in particular its sub-Saharan region, constitutes a seasonal shelter for a large number of Eurasian water birds, including an estimated 5.4 million ducks that gather in western and eastern Africa during the northern winter (*8*). In their overwintering sites, these birds congregate and mix with a wide variety of Afro-tropical water birds, some of them with large populations widespread over Africa.

AIV have been isolated in wild ducks on wintering grounds in both Europe and North America (*9,10*). Results from this surveillance program established that AIV are also present in wild birds in Africa during the northern winter. Low-pathogenicity AIV were detected and isolated in several species from several major wetlands of northern, western, and eastern Africa, which indicates that environmental conditions in Afro-tropical ecosystems are favorable for the persistence and transmission of AIV.

Table 2. Reverse transcription PCR–based detection of influenza A virus in 2 wild duck species sampled in different countries

Species	Country	No. samples tested	No. PCR positive (%)
Garganey (*Anas querquedula*)	Chad	381	11 (2.9)
	Kenya	104	0
	Mali	411	22 (5.4)
	Mauritania	225	33 (14.7)
	Niger	87	4 (4.6)
	Senegal	121	17 (14.0)
White-faced whistling duck (*Dendrocygna viduata*)	Burkina Faso	167	0
	Chad	232	1 (0.4)
	Ethiopia	76	10 (13.2)
	Malawi	59	0
	Mali	36	1 (2.8)
	Mauritania	183	7 (3.8)
	Niger	232	8 (3.4)
	Senegal	172	11 (6.4)

We detected and isolated AIV in Eurasian and Afro-tropical species. This finding shows that AIV circulate in migratory water birds originating from Eurasia and in African species that remain in the continent throughout the year. Moreover, the detection of viruses in some Eurasian wader species during wintering (in January in Mali) and during migration (in May in Tunisia) contrasts with the apparent absence of AIV reported from previous studies of waders in Europe (*5*,*11*). Since waders form the most abundant group of African-Eurasian migratory water birds (*12*), these shorebirds may play a role in maintaining some AIV in waterbird communities at wintering and stopover sites.

The detection of AIV in Eurasian ducks in several of their major overwintering sites in West Africa (e.g., the Inner Niger Delta, the Senegal River Delta, and Lake Chad) supports the hypothesis that AIV can persist in wild duck populations year-round through a continuous circulation in a proportion of birds (*1*). Variability in the prevalence observed might be related to differences in local logistical constrains but also to differences between African regions in their waterbird assemblage and connectivity with European breeding grounds. The different isolates obtained from garganey from the Inner Niger Delta also indicate that various subtypes are circulating at the same time in a population, a finding that agrees with patterns observed in Europe and North America (*11*,*13*).

Various AIV subtypes were isolated from apparently healthy garganey and white-faced whistling ducks, which indicates that both Eurasian and Afro-tropical ducks may serve as reservoirs of AIV. These results not only suggest that some Eurasian ducks could carry AIV on their northward spring migration but also raise the possibility that AIV could persist in the tropical region and be disseminated over Africa through intra-African migratory ducks. The presence of AIV at African wintering and stopover sites, where birds from various geographic origins congregate and mix, provides opportunities for transmission of AIV between different populations and spread of AIV over extensive areas in both Eurasia and Africa.

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who processed the samples (see a detailed list of partners in Gaidet and Dodman [*14*]). We also thank Akiko Kamata, Felix Njeumi, Arnaud LeMenach, Astrid Tripodi, and Vincent Martin for assistance in preparing and coordinating these campaigns; Catherine Cetre-Sossah for technical assistance; Camille Danes for her help in database management and illustration; and Vittorio Guberti for advice in the preparation of field operations.

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Dr Gaidet is an ecologist in the Animal Production and Veterinary Department at the French Agricultural Research Centre for International Development. His primary research interests include the host ecology of avian influenza and West Nile viruses.

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MRSA Transmission between Cows and Humans

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We isolated methicillin-resistant *Staphylococcus aureus* (MRSA) from cows with subclinical mastitis and from a person who worked with these animals. The bovine and human strains were indistinguishable by phenotyping and genotyping methods and were of a low frequency *spa* type. To our knowledge, this finding indicates the first documented case of direct transmission of MRSA between cows and humans.

Since the introduction of β-lactamase–stable antimicrobial drugs into clinical use, methicillin-resistant *Staphylococcus aureus* (MRSA) strains have emerged worldwide as important nosocomial pathogens; their prevalence in the community is increasing substantially. Although *S. aureus* is known to be one of the most common causes of bovine mastitis and other severe animal diseases such as septicemia and wound, bone, and joint infections, MRSA strains have been rarely isolated from animals. MRSA strains have been isolated from cows with mastitis, horses and dogs with lesions, and dogs and cats that were carriers (*1*). Transmission of MRSA between humans and animals (e.g., dogs, horses, pigs) has been reported (*2–4*), but transmission between cows and humans has not, to our knowledge. We describe a first putative case of transmission of MRSA between cows and a person.

From January 2002 through December 2004, 595 milk samples were collected from cows with subclinical mastitis on a farm in Hungary and sent for bacteriologic analysis to the Bacteriological Department of the Hungarian Central Veterinary Institute. Samples were streaked onto a Columbia agar plate (Merck, Darmstadt, Germany) containing 5% sheep blood and 0.01% esculin and a Baird-Parker (BP) agar plate (Oxoid Ltd., Basingstoke, England). After incubation at 37°C for 24 h, the colonies were tentatively identified according to morphologic features, pigment production, Gram stain results, catalase test results, type of hemolysis, and characteristic growth on BP agar. The isolates initially characterized as staphylococci were tested for coagulase production (in tubes) and with Slidex Staph Plus test (bioMérieux, Marcy l'Etoile, France) to confirm their identification as *S. aureus*. From this farm, 375 *S. aureus* strains were isolated. The strains were tested for antimicrobial drug susceptibility, production of β-lactamases, and presence of *mec*A by PCR (*5*). The first MRSA strain was isolated in spring 2002; during the next 15 months, 26 additional MRSA strains were isolated from this dairy herd.

In December 2002, tonsil swabs were collected once from 12 workers on this farm who were in close contact with the cows (veterinarian, milkmen, and attendants) and who gave informed consent. (The study was approved by the Ethical Committee of the National Center for Epidemiology, Budapest, Hungary.) Culturing and identification of *S. aureus* were carried out by the above-described method. *S. aureus* was isolated from 3 samples. One of these isolates was resistant to methicillin by disk diffusion and E-test, and the presence of the *mec*A gene was confirmed by PCR.

All 28 MRSA strains (27 bovine and 1 human) produced PBP2a, according to latex agglutination test (Oxoid Ltd.). Eight of 27 randomly chosen bovine strains and the human strain containing the *mec*A gene, as well as 4 bovine and 2 human *mec*A–negative isolates, were phage typed with MRSA phages (*6*) at the Institute of National Public Health and Medical Officers Service in Hajdú-Bihar County. The phages were used in 2 concentrations: routine test dilution (RTD), and 100× RTD. None of the MRSA or methicillin-susceptible *S. aureus* (MSSA) strains were lysed by phages in RTD. All MRSA strains showed a similar lysis pattern with the 100× RTD MRSA phages (Table).

Susceptibilities to 7 antimicrobial agents were assessed by a disk diffusion method that used the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) breakpoints for 7 *mec*A–positive bovine strains and the human *mec*A–positive strain. All tested strains showed the same susceptibility pattern (resistant to ampicillin, cephalexin, tetracycline, and erythromycin and susceptible to enrofloxacin, gentamicin, and trimethoprim/sulfamethoxazole). Pulsed-field gel electrophoresis was performed on the 7 *mec*A–positive bovine strains, 1 human *mec*A–positive strain, 4 bovine MSSA strains, and 2 human MSSA strains, as described by McDougal et al. (*7*). The patterns of the *Sma*I–digested DNA of the strains are presented in the Figure. All bovine *mec*A–positive strains and the human *mec*A–positive strain (human 3) showed indistinguishable *Sma*I patterns. All *mec*A–negative isolates showed a pulsed-field gel electrophoresis pattern different from that of the MRSA strains.

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Table. Susceptibility of human and animal *Staphylococcus aureus* strains to phages and the presence of *mec*A by PCR*

Strain no.†	Reference no.	Identification of samples	*mec*A	MRSA 100× RTD‡
1	13535	795 LF	+	616/617/623/626/630
2	16480	588 RF	+	616/617/623/626/630
3	24069/2	490 RF	+	616/617/623/626/630
4	24069/4	723 RH	+	616/617/623/626/630
5	24069/13	1572 LF	+	616/617/623/626/630
6	30195	632 RF	+	616/617/623/626/630
7	23457	1379 LH	+	616/617/623/626/630
	29509†	540 RH	+	616/617/623/626/630
8	Human/3		+	616/617/623/626/630
9	24069/9	381 LF	–	
10	24069/10	429 LH	–	
11	24069/11	519 LH	–	
12	24069/15	2551 RH	–	
13	Human/4		–	
14	Human/2		–	

*Strains isolated from cows with subclinical mastitis, Hungary, January 2002–December 2004.
†See Figure for DNA analysis. Strain no. 29509 was not included in the DNA analysis; thus, no strain no. was assigned.
‡Reaction pattern with 100× routine test dilution (RTD) methicillin-resistant *Staphyloccocus aureus* (MRSA) phages (7). +, positive in *mec*A PCR; –, negative in *mec*A PCR.

For further identification, 1 human and 4 bovine MRSA isolates (29509, 24069/2, 24069/4, 30195; Table) underwent *spa* typing (*8*). All these isolates showed *spa*-type t127. Because the strains were indistinguishable by all methods, multilocus sequence typing (MLST) (*9*) and typing of the staphylococcal cassette chromosome (SSC*mec*) (10) were performed on just 1 strain (29509). This strain showed MLST sequence type (ST) 1 and SCC*mec* type IVa. The strain did not carry the Panton-Valentine leukocidin (PVL) toxin genes *lukF–lukS* as tested by PCR (*11*).

In this study, both phenotyping and genotyping showed that the MRSA isolates of bovine origin and the single human isolate were indistinguishable. The database of the European network for *spa* typing (www.seqnet.org) shows that MLST ST 1/*spa*-type t127 has a relative global frequency of 0.7%; according to this database, this type has not previously been reported in Hungary. A study of 135 human MRSA isolates collected in Hungary during 2001–2004 showed human epidemic clones of types other than ST 1 and *spa*-type t127 (*12*). We conclude that the bovine and human MRSA strains described in our study are epidemiologically related, which indicates transmission from either cow to human or human to cow. This strain is negative for the PVL genes, which differentiates it from community-associated MRSA ST 1, which is positive for PVL genes (*11*).

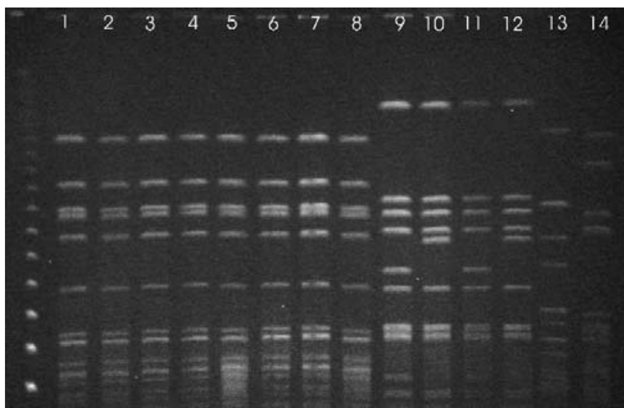

Figure. Pulsed-field gel electrophoresis patterns of *Sma*I-digested DNA of bovine and human strains of *Staphyloccocus aureus* isolated from cows with subclinical mastitis, Hungary, January 2002–December 2004. Strains are numbered as shown in Table.

Several cows had positive test results for MRSA, which indicated that MRSA was spread within the farm. On the farm, cases of clinical mastitis were treated with intramammary infusions containing penicillin, aminopenicillins, or cephalosporins. Each cow also received drying-off therapy with cloxacillin or cephalosporins. The use of antimicrobial drugs may have contributed to the emergence of MRSA in this dairy farm.

MSSA strains with ST 1 and *spa*-type t127 have been reported from human sources (*13*). MSSA strains may be induced to pick up the *mec*A gene from coagulase-negative staphylococci. Alternatively, mastitis may be caused by human MRSA strains or bovine MRSA strains already present in small numbers and selected for by the frequent use of long-acting antimicrobial preparations, especially β-lactams. *S. aureus* usually shows limited host specificity, and transfer between different host species may occur (*14*). The transmission of milk-associated *S. aureus* strains between cows and humans was suggested by Lee (*15*), whose study showed MRSA in milk samples with comparable antibiotypes as those in humans, but the transfer to humans was not proven. The risk for spread of MRSA from bovine sources into the human population is low. Generally, persons are not at risk as long as raw milk is not consumed. However, persons in close contact with MRSA-infected cattle, including veterinarians, farmers, milkers, and persons working at slaughterhouses, may become colonized from the bovine source.

We conclude that several cases of subclinical mastitis in cows on a farm in Hungary were caused by MRSA and that these strains were indistinguishable from MRSA isolated from a carrier working in close contact with the cows. This suggests the transmission of these isolates between humans and cows, although the direction of transfer (cow to human or human to cow) could not be proven.

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Dr Juhász-Kaszanyitzky is a veterinary microbiologist with special interest in antimicrobial resistance. She developed the

Hungarian national antibiotic resistance monitoring system, based on samples from healthy slaughter animals, and since 2001 has coordinated this system.

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Human *Babesia microti* Incidence and *Ixodes scapularis* Distribution, Rhode Island, 1998–2004

Sarah E. Rodgers*[1] and Thomas N. Mather*

Distribution of nymphal *Ixodes scapularis* in Rhode Island was used as a logistical regressor for predicting presence of human babesiosis. Although the incidence of babesiosis is increasing in southern Rhode Island, large areas of the state are free of babesiosis risk.

In recent years, cases of human babesiosis have increased across the northeastern United States, especially in coastal areas like southern Rhode Island. In the northeastern United States, human babesiosis is a tick-transmitted, malarialike infection caused by *Babesia microti* Franca parasites (*1*). The *B. microti* parasite shares the same principal rodent reservoir (white-footed mouse, *Peromyscus leucopus*) and tick vector (*Ixodes scapularis*) as the Lyme disease spirochete, *Borrelia burgdorferi*. Although the transmission processes of *B. burgdorferi* and *B. microti* are similar, *B. burgdorferi* is acquired nearly twice as often as *B. microti* (*2*). This less efficient transmission of *B. microti* seemingly requires more tick bites to maintain similar zoonotic prevalence. In a previous study, we suggested that a threshold of >20 nymphal ticks collected per hour was necessary to maintain zoonotic endemicity of *B. microti* in white-footed mouse populations (*3*). In this study, we used *I. scapularis* abundance to indicate the spatial distribution of risk for human babesiosis. We focused on babesiosis transmitted by ticks, not other sources such as blood transfusions (*4*).

The Study

Human babesiosis is not a nationally reportable disease; however, in Rhode Island all clinically diagnosed cases have been recorded by the Rhode Island Department of Health since 1994, when 2 cases were reported. A decade later, in 2004, 48 cases were reported. We analyzed all cases reported to the Rhode Island Department of Health from 1998 through 2004; the number of cases before 1998 was insufficient for analysis. Of 189 babesiosis patients, mean age was 59 years (SD 20.42), and 57% were >60 years of age. The case rate in Rhode Island more than doubled each year from 1998 through 2000, after which the rate of increase slowed. Geographic (latitude, longitude) coordinates of each patient's home address were determined by using EZlocate (www.geocode.com). Cases were aggregated into Rhode Island's 233 census tracts by using ArcMap (Environmental Systems Research Institute, Inc., Redlands, CA, USA). Spatial distribution of babesiosis incidence per 100,000 persons per year is shown in Figure 1. Most tracts, particularly those in urban areas (smaller census tracts), contain no babesiosis cases.

An extensive surveillance program for *I. scapularis* nymphal ticks has been in operation continuously since 1993. According to a random stratified design, sampling has occurred at least at the same 60 locations throughout Rhode Island (*5*). Forested areas, suitable as tick habitats, were drag-sampled twice each year; results were recorded as nymphs collected per hour. *I. scapularis* samples were taken each year from late May through the end of July, a time of peak nymphal activity; more detail on the sampling method can be found elsewhere (*5,6*). This type of surveillance is labor intensive; however, we did not routinely test ticks or rodents for *B. microti* infection. Rates for nymphs collected per hour were first interpolated by ordinary kriging (Gaussian process regression) to create a continuous tick-encounter surface. Variograms (to show spatial correlation of observations) were calculated from each year's tick data and used to provide data for kriging (*7*). Subsequently, this surface was averaged for each census tract area.

Associated with each census tract are nymphal tick abundance (nymphs collected per hour) and disease presence or absence. Simple logistic regression was performed by using SAS (SAS Institute, Cary, NC, USA). Nymphal tick abundance was used as the predictor for the presence of disease; regression parameters are given in the Table. Each logistic regression curve (not shown) allowed us to ascertain an acceptable threshold of risk for nymphal tick collection per hour, below which it was unlikely (<20% probability) that a census tract would contain any cases of human babesiosis for that year. Risk is a continuous measure, and this cut-off represents the authors' value judgment. The minimum threshold required to cause ≥20% risk of having 1 babesiosis case in any particular census tract was, for example, 135 nymphs per hour in 1998 but only 19 nymphs per hour in 2004. Nymphal threshold figures were used to create maps from the continuous nymphs-per-hour data (Figure 2). These effectively classified Rhode Island into 2 distinct zones; 1 that appears to be safe from

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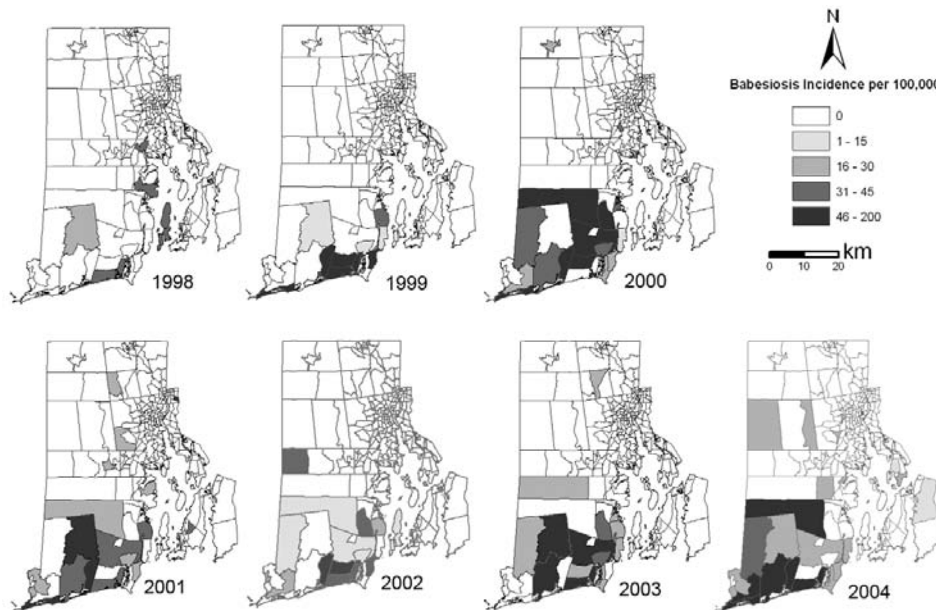

Figure 1. Human babesiosis incidence per census tract, Rhode Island, USA, 1998–2004. Data from Rhode Island Department of Health.

risk for tick-transmitted human babesiosis and 1 where residents and visitors are at risk. In general, the trend was a lowering of the nymphal tick abundance threshold associated with babesiosis over the course of the 7-year study.

Conclusions

These results support earlier research findings that *B. microti* infections in mice are closely linked to abundance of nymphal ticks; a threshold of ≥20 nymphs per hour is most likely to support enzootic parasite maintenance and potentially result in human disease (*3*). Clearly, areas of highest risk for babesiosis transmission are in southern Rhode Island, where the highest incidence of nymphal ticks and zoonotic babesiosis infections in rodent reservoirs are located. However, we observed a marked increase in human babesiosis incidence in Rhode Island from 1998 through 2004. This increase likely results in part from the expanding area in Rhode Island where numbers of nymphal ticks reach or exceed the proposed zoonotic endemicity threshold; the proportion of Rhode Island at risk increased from 6% in 1998 (6 cases) to ≈9% in 2004 (48 cases). The large increase in area at risk for babesiosis that occurred in 2002 (51%) did not unduly increase the number of cases during that year. Several years of abundant nymphal ticks may be needed before *Babesia microti* parasites are introduced and established in previously parasite-free mouse populations. Nevertheless, according to 2000 census figures, an average of ≈13% of Rhode Island's population resides in areas where they could acquire human babesiosis.

Occasionally, babesiosis cases were recorded from northern Rhode Island, where tick abundance is below the proposed transmission risk threshold. These cases are likely to have resulted from travel to areas with dense tick populations or receipt of transfusion (*4*) from donors who lived in *Babesia*-enzootic areas. These cases cannot be accounted for in our research, which is based on the assumption that disease was acquired peridomestically.

A proportion of the increase in human babesiosis incidence during 1998–2004 may be the result of greater awareness of this disease, more correct diagnoses, and more case reporting. To some extent, incidence of babesiosis does seem to be increasing in relation to that of Lyme disease but does not appear to be, as has been suggested, approaching that of Lyme disease in areas of Rhode Island (*8*). Most cases in our sample were in elderly persons (≥60 years of age), in contrast with research by Krause et al., which found that age-to-incidence rate ratio was similar for younger persons (<60 years of age) (*8*). When incidence rates for babesiosis and Lyme disease among patients ≥60 years of age were directly compared, the proportions of patients with Lyme disease who also had babesiosis were 2.4% in 1998, 7.7% in 1999, 18.8% in 2000, 18.8% in 2001, 16.7% in 2002, 12.6% in 2003, and

Table. Coefficients of simple logistic regression of *Ixodes scapularis* nymphs collected per hour, Rhode Island, USA

Year	Intercept	Slope	No. nymphs/h*
1998	–4.610	0.024	135
1999	–5.671	0.078	55
2000	–3.474	0.040	52
2001	–3.859	0.024	102
2002	–4.106	0.093	30
2003	–3.529	0.079	27
2004	–2.566	0.064	19

*Minimum no. nymphs that must be collected per hour to create a medium-high risk for babesiosis (20% probability of ≥1 case of human babesiosis per census tract).

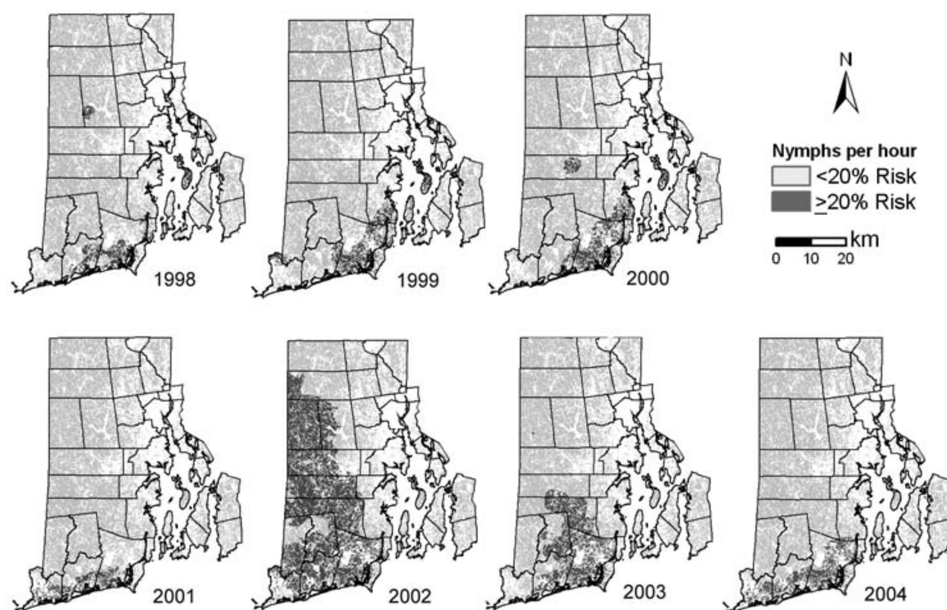

Figure 2. Risk for human case of babesiosis, Rhode Island, USA, derived from simple logistic regression analysis of census tracts with and without babesiosis cases (Figure 1), predicted by average *Ixodes scapularis* nymphs collected per hour per census tract. The cut-off level for the 2 classes was decided by the number of nymphs collected per hour needed to create a 20% probability of a babesiosis case occurring in a census tract. The continuous nymphal tick abundance surface was modified to subtract areas unsuitable for tick habitat because they contain water, unforested agriculture, or urban land.

not calculated in 2004 because of different method of recording Lyme disease. This differential is most likely explained by the smaller geographic distribution of *B. microti*–infected ticks compared with *B. burgdorferi*–infected ticks; however, the lower rate of *B. microti* infection in ticks is probably a contributing factor. This lower rate means that more tick bites per person are needed to produce infection.

The years leading up to, and immediately following, the observed reported increase in human babesiosis incidence may have been a period of *B. microti* introduction and enzootic establishment requiring a higher tick risk threshold, which rapidly decreased after establishment. In the absence of statewide reduction of ticks, local expansions of infected rodent populations are likely to continue and further extend the babesiosis risk zone in Rhode Island.

Acknowledgments

We thank the Rhode Island Department of Health for providing data for human babesiosis cases and the many field technicians for helping with tick sampling and making this study possible.

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Dr Mather is a medical entomologist and the director of the Center for Vector-Borne Disease at the University of Rhode Island. His research interests include tick-bite prevention methods and tickborne disease vaccine development.

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Human Bocavirus, a Respiratory and Enteric Virus

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In Spain, human bocavirus (HBoV) was detected in 48 (9.1%) of 527 children with gastroenteritis at similar frequency as for children with respiratory illness (40/520, 7.7%). Fecal excretion adds new concern about the transmission of HBoV. To our knowledge, this report is the first to document HBoV in human feces.

Human bocavirus (HBoV), a recently described new virus species belonging to the *Parvoviridae* family, was identified as a human pathogen in September 2005 (*1*). Since then, this parvovirus has been found in children with respiratory tract illness in practically all areas of the world in which it has been investigated (*2–5*), an indication of its wide dissemination.

The Study

To determine the prevalence and clinical characteristics of HBoV, we investigated the presence of this virus in children with respiratory tract infection in our region (Gipúzkoa, Basque Country, Spain). Among the first patients in whom HBoV was detected in nasopharyngeal aspirates, we found two 12-month-old children with diarrhea in addition to respiratory symptoms. Because animal parvoviruses are frequently associated with enteritis in young animals (*6*), we investigated the presence of HBoV in the diarrheal feces of both children. HBoV was detected in both samples, and no other intestinal pathogens were identified. To rule out the possibility that this result could have been due to fecal contamination resulting from swallowing respiratory secretions, and to determine whether the gastrointestinal tract is affected by this new respiratory virus, we studied its presence in patient feces in 527 episodes of acute gastroenteritis, unrelated to respiratory infection, in children <3 years of age, mainly from nonhospital centers (ambulatory clinics). Our analyses were conducted from December 2005 through March 2006.

Viral DNA and RNA were obtained from nasopharyngeal aspirates and stool specimens with an automatic extractor BioRobot M48 (QIAGEN, Hilden, Germany) by using the MagAttract Virus Mini M48 kit (QIAGEN). cDNA was obtained by using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) and random primers. Aliquots of the DNA and cDNA were frozen at –40°C until PCR for HBoV detection was performed. Respiratory samples were investigated for respiratory syncytial virus, influenza viruses A and B, parainfluenza virus types 1–4, and adenovirus by cell culture and PCR. Rhinovirus, coronavirus (NL63 coronavirus included), and metapneumovirus were studied by PCR alone. Fecal specimens were examined for *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter* spp., and enteroinvasive *Escherichia coli* O157 by standard culture methods. Rotavirus was investigated by enzyme immunoassay and norovirus by reverse transcriptase PCR. HBoV detection was performed by PCR with primers derived from the *NP1* gene (*1*). Positive samples were retested and confirmed as positive by using a second PCR assay with primers derived from another location in the HBoV genome (*VP1* gene) (*7*). Amplified *NP1* and *VP1* gene fragments (354 bp and 403 bp, respectively) were sequenced and analyzed by using the BLAST software (www.ncbi.nlm.nih.gov/BLAST). Each PCR run included a negative control (water) that was treated as the clinical sample throughout, and PCR was performed with the usual precautions to avoid contamination. Strain *Spain001* (GenBank accession no. EF186830) was included as positive control in each PCR run.

Conclusions

Of the 527 stool samples analyzed from December 2005 through March 2006, HBoV was detected in 48 (9.1%). From a second group of 520 children <3 years of age who came to the pediatric emergency unit of our hospital with an episode of acute respiratory infection during the same period, a similar frequency of HBoV detection was obtained (40/520, 7.7%) when nasopharyngeal aspirates were tested. Analysis of *NP1* and *VP1* partial gene sequences obtained from all fecal and respiratory HBoV-positive samples showed a similarity of >95% with previously published HBoV sequences.

Of 40 HBoV-positive respiratory samples, 25 (62.5%) showed coinfection with other viruses (respiratory syncytial virus in 13, rhinovirus in 3, influenza A in 3, coronavirus OC43 in 2, adenovirus in 1, influenza B in 1, respiratory syncytial virus and coronavirus OC43 in 1, and influenza A and rhinovirus in 1). Of the 48 HBoV-positive fecal samples, 28 (58.3%) showed coinfection with another intestinal pathogen (*Salmonella enteritidis* in 1, *Campylobacter jejuni* in 5, rotavirus in 14, norovirus in 7, and *C. jejuni* and norovirus in 1).

In this study, simultaneous detection of HBoV and other agents was frequent for respiratory or enteric specimens. The incidence of coinfection in respiratory illness

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was similar to that observed in studies that were not limited to specimens that had already tested negative for other microorganisms and in which a wide number of agents were investigated (*4*). Adenoviruses have been associated with infection of the colon and the gut and are a cause of severe gastroenteritis in nonindustrialized countries. In this study, coinfection of adenovirus and HBoV was detected in 1 respiratory specimen but these viruses together were not detected in any fecal sample.

HBoV and parvovirus B19 are the only 2 species of the *Parvoviridae* family that have been associated with disease in humans. To date, HBoV has only been detected in samples from the respiratory tract and has been associated with both upper and lower respiratory tract disease in infants and young children. The results of our study show that HBoV is also present in the gastrointestinal tract in children with gastroenteritis with or without symptoms of respiratory infection. The fecal excretion adds new concern about the transmission of HBoV.

To our knowledge, this report is the first to document HBoV in human feces. The high frequency of HBoV detection in the feces of children with gastroenteritis and the absence of any other intestinal pathogen suggest that this new virus species is an enteric, as well as a respiratory, pathogen. Further investigations to confirm this preliminary hypothesis and gain greater knowledge of the association between HBoV and enteric disease are required.

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Bocavirus

[bō-kə-vī-rəs]

Genus in the family *Parvoviridae*. Previously identified members of this genus are pathogens of bovines and canines. A parvovirus of human origin was recently discovered and called human **bo**cavirus because it is closely related to bovine parvovirus and **ca**nine minute virus. Human bocavirus is associated with respiratory tract infections, particularly in infants and young children.

Sources: Allander T, Tammi MT, Eriksson M, Bjerkner A, Tivelung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891-6.

Instructions for Infectious Disease Authors

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.

Antimicrobial Drug Resistance of *Salmonella* Isolates from Meat and Humans, Denmark

Marianne N. Skov,* Jens Strodl Andersen,† Søren Aabo,† Steen Ethelberg,‡ Frank M. Aarestrup,† Anders Hay Sørensen,† Gitte Sørensen,† Karl Pedersen,† Steen Nordentoft,† Katharina E.P. Olsen,‡ Peter Gerner-Smidt,‡ and Dorte L. Baggesen†

We compared 8,144 *Salmonella* isolates collected from meat imported to or produced in Denmark, as well as from Danish patients. Isolates from imported meat showed a higher rate of antimicrobial drug resistance, including multidrug resistance, than did isolates from domestic meat. Isolates from humans showed resistance rates lower than those found in imported meat but higher than in domestic meat. These findings indicate that programs for controlling resistant *Salmonella* spp. are a global issue.

Salmonella spp. are among the most common causes of human bacterial gastroenteritis worldwide, and food animals are important reservoirs of the bacteria (*1*). In recent years, an increase in the occurrence of antimicrobial drug–resistant *Salmonella* spp. has been observed in several countries (*2–5*). Fatality rates are higher for patients with infections caused by drug-resistant *Salmonella* spp., and these patients are more likely to require hospitalization and to be hospitalized for longer periods than are patients with infections caused by antimicrobial drug–susceptible *Salmonella* spp. (*6,7*).

Antimicrobial drug resistance of *Salmonella* spp. isolated from food animals in Denmark has so far been relatively low (*8*). However, an estimated 30% of all poultry, 10% of all pork, and 50% of all beef sold in Denmark is imported (*9*). Imported meat is therefore an important potential source of human infection with drug-resistant *Salmonella* spp. We compared antimicrobial drug resistance of *Salmonella* isolates from both imported meat and meat produced within Denmark (domestic meat), as well as from outpatients with diarrhea.

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Salmonella isolates from humans and meat were obtained from July 1998 through June 2002. Isolates from domestic poultry, pork, and beef were obtained through the national *Salmonella* control program (*10*), and isolates from imported poultry, pork, and beef were obtained from the Denmark import control and from the regional food control units. Human salmonellosis is a notifiable disease in Denmark, and all human *Salmonella* spp. isolates are collected at the Statens Serum Institute. The serovars included were restricted to *S*. Typhimurium, *S*. Hadar, *S*. Dublin, *S*. Saintpaul, *S*. Enteritidis, *S*. Virchow, and *S*. Newport because these were the serovars of which a sufficient number of isolates had been tested for antimicrobial drug susceptibility. Data on 4,081 *Salmonella* isolates from humans were included in the study.

Identification, serotyping, phage typing, and susceptibility testing were done as described (*8,11,12*). Susceptibility to the following antimicrobial agents was determined: ampicillin, ceftiofur, chloramphenicol, ciprofloxacin, co-amoxiclav, colistin, florphenicol, gentamicin, nalidixic acid, neomycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim.

Statistical analyses were performed using S-PLUS version 6.2 (Insightful Corp., Seattle, WA, USA). The trend in the occurrence of resistant isolates over time, the occurrence of multidrug-resistant isolates over time, and the occurrence of nalidixic acid–resistant isolates were investigated by fitting a logistic regression model with origin (domestic/imported), time (year), product type (beef, pork, poultry), and all 2-way interactions as explanatory variables. The regression models were reduced by using a likelihood ratio test. Significance in all 2-by-2 tables (only tables with minimum 30 domestic and 30 imported samples) was tested by a Pearson χ^2 test with continuity correction; if the number in any cell in the contingency table was <5, Fisher exact test was applied. All tests were done on a 5% significance level ($p<0.05$). No correction for multiple testing was done. An isolate was considered multidrug resistant if the isolate was resistant to ≥4 antimicrobial agents.

Salmonella spp. were isolated from 1,078 (11.8%) of 9,135 samples from imported poultry, pork, and beef and 2,985 (1.4%) of 213,214 samples from domestic poultry, pork, and beef. Among the isolates from domestic meat, the serovars *S*. Typhimurium, *S*. Infantis, and *S*. Derby were the 3 most frequently isolated; in imported meat, the 3 most frequently isolated serovars were *S*. Heidelberg, *S*. Typhimurium, and *S*. Hadar (Table 1). In isolates from domestic meat originating from pigs and poultry, *S*. Typhimurium was the most frequently isolated serovar; in beef isolates, *S*. Dublin was most common. Among isolates from imported meat, *S*. Typhimurium was the most frequently isolated serovar from pork and beef, while

Table 1. Number and proportion of susceptible (S), resistant (R), multidrug-resistant (M), and nalidixic acid–resistant (Nal) *Salmonella* spp. isolates within different serotypes isolated from meat and humans, Denmark, July 1998–July 2002*

	Domestic meat					Imported meat					Humans†				
Serotype	No. tested	S,%	R,%	M,%	Nal	No. tested	S,%	R,%	M,%	Nal	No. tested	S,%	R,%	M,%	Nal
Typhimurium‡	1,508	73	21	6	1	138	34	24	42§	9§	1,886	61	20	19	3
Infantis‡	184	94	4	2	2	50	84	10	6	8§					
Derby‡	163	55	44	1	1	34	32	59	9§	3					
Heidelberg	6	67	33	0	17	157	49	13	38	4					
Hadar‡	38	74	26	0	11	113	1	53§	46§	81§	189	26	71	3	58
Enteritidis‡	91	90	9	1	4	50	84	16	0	10	1,706	92	7	0	4
Indiana‡	94	95	4	1	0	40	45	43§	13§	3					
Newport	2	0	100	0	100	78	28	51	21	60	59	88	7	5	5
Kottbus	26	81	19	0	15	49	6	90	4	92					
Dublin	71	99	1	0	1	4	100	0	0	0	88	95	5	0	2
Anatum	50	100	0	0	0	12	75	8	17	0					
Saintpaul	9	11	0	89	22	39	31	8	62	15	58	72	9	19	7
Regent	47	0	100	0	100	1	0	100	0	0					
Virchow	3	100	0	0	0	39	44	36	21	49	95	35	56	9	62
Bredeney	3	100	0	0	0	38	34	0	66	11					
Other‡	690	71	24	5	5	256	56	24	20	17					
Total	2,985	74	22	4	4	1,078	42	30	28	26	4,081	73	17	9	7

*Only serotypes with ≥40 isolates are included.
†Only subsets of selected serovars are routinely tested for susceptibility to antimicrobial agents.
‡Indicates serotypes with >30 samples from Danish produced meat and >30 samples from imported meat, which were statistically tested.
§Indicates clinical significance.

S. Heidelberg was the most frequently isolated serovar from poultry.

A significantly higher (χ^2, $p<0.001$) proportion of the *Salmonella* spp. isolates from imported meat (58%) were resistant to ≥1 antimicrobial agents compared with isolates from domestic meat (26%) (Table 1). A significant difference (χ^2, $p<0.001$) was also observed between the proportions of multidrug-resistant isolates from domestic (4%) compared with imported (28%) poultry, pork, and beef.

The regression results (Table 2) showed a significant increase in the proportion of resistant ($p<0.001$) and multidrug-resistant ($p = 0.015$) isolates over time and an increase in odds per year of 27% (corresponding to an increase in probability of 5% per year) and 14% (corresponding to an increase in probability of 3% per year), respectively (Figure 1). Furthermore, the probability for isolating a resistant and a multidrug-resistant isolate from imported meat compared with domestic meat was significant, with an odds ratio of ≈5. The probability of isolating a resistant isolate differed between product types; pork had the highest probability, followed by poultry and beef.

A high proportion of resistant and multidrug-resistant isolates was found among *S*. Hadar, *S*. Newport, *S*. Typhimurium, and *S*. Heidelberg in imported meat

Table 2. Results from the reduced logistic regression models*

Variable	OR (95% CI)	Estimate (95% CI)	SE (Est.)	p value
Resistance vs. nonresistance				
Intercept	0.164 (0.129 to 0.207)	–1.81 (–2.05 to –1.57)	0.121	
Origin	5.08 (4.19 to 6.18)	1.62 (1.43 to 1.82)	0.0988	<0.00001
Year	1.27 (1.19 to 1.35)	0.235 (0.174 to 0.297)	0.0313	<0.00001
Cattle vs. poultry	0.400 (0.230 to 0.662)	–0.917 (–1.47 to –0.413)	0.268	<0.00001
Pork vs poultry	1.26 (1.06 to 1.51)	0.233 (0.0553 to 0.414)	0.0916	
Multidrug resistance vs. resistance				
Intercept	0.141 (0.0976 to 0.201)	–1.96 (–2.33 to –1.60)	0.185	
Origin	4.98 (3.87 to 6.44)	1.61 (1.35 to 1.86)	0.129	<0.00001
Year	1.14 (1.03 to 1.27)	0.133 (0.0259 to 0.240)	0.0547	0.0148
Nalidixic acid resistance vs. non–nalidixic acid resistance				
Intercept	0.0611 (0.0333 to 0.107)	–2.80 (–3.40 to –2.24)	0.296	
Origin	6.54 (3.45 to 12.8)	1.88 (1.24 to 2.55)	0.334	
Year	1.41 (1.18 to 1.69)	0.342 (0.167 to 0.526)	0.0914	
Origin and year	0.732 (0.587 to 0.909)	–0.311 (–0.532 to –0.0956)	0.111	0.00448
Cattle vs. poultry	0.0404 (0.00229 to 0.182)	–3.21 (–6.08 to –1.70)	1.01	<0.00001
Pork vs. poultry	0.0668 (0.0425 to 0.101)	–2.71 (–3.16 to –2.29)	0.220	

*OR, odds ratio; CI, confidence interval; SE, standard error; Est., estimated.

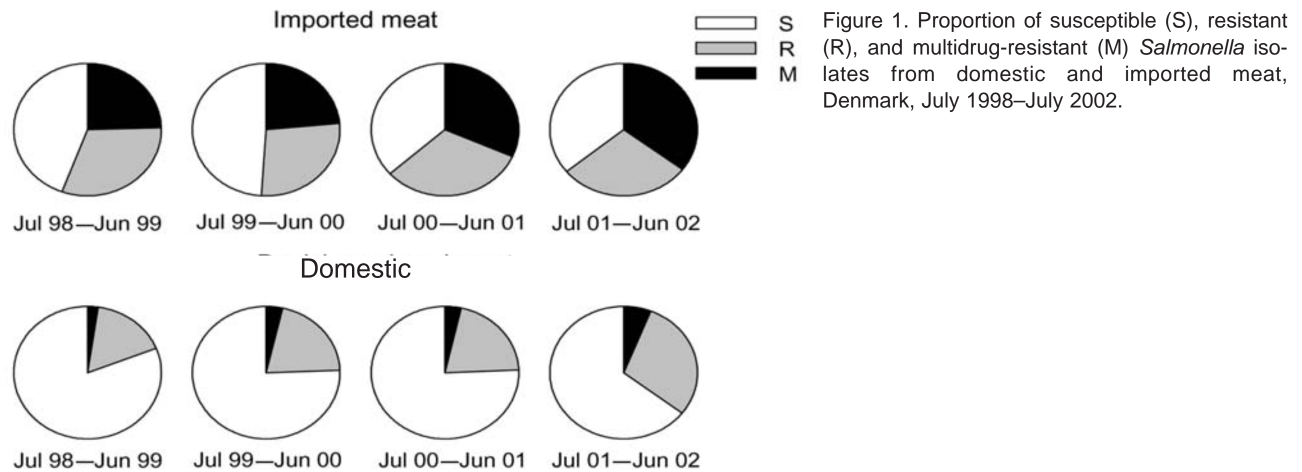

Figure 1. Proportion of susceptible (S), resistant (R), and multidrug-resistant (M) *Salmonella* isolates from domestic and imported meat, Denmark, July 1998–July 2002.

(Table 1). Among *S.* Typhimurium, antimicrobial drug resistance was particularly prominent in the phage types DT104, DT170, DT193, DT120, DT208, DT107, U302, and DT135 (Table 3). Multidrug-resistant DT104, DT120, and DT193 were found in both domestic and imported poultry, pork, and beef, whereas multidrug-resistant DT107, DT170, and DT208 were more common in domestic meat, and multidrug-resistant U302 was more common in imported meat (Table 3).

Resistance to nalidixic acid was higher among isolates from imported meat (26%) compared with isolates from domestic meat (4%) (χ^2, p<0.001, odds ratio = 6.54, Table 3), with an increase over time in the proportion of domestic nalidixic acid–resistant isolates (p = 0.004, data not shown). Furthermore, the probability of isolating a nalidixic acid–resistant isolate differed between product types; poultry (domestic 14%, imported 30%) had the highest probability, followed by pork (domestic 1%, imported 3.2%) and beef (domestic 1%, imported 0%). Nalidixic acid resistance among *Salmonella* spp. from imported products was highest among *S.* Hadar, *S.* Newport, *S.* Kottbus, and *S.* Virchow (Table 1).

For *S.* Typhimurium, *S.* Hadar, and *S.* Virchow, the proportion of resistant and multidrug-resistant isolates was much higher among isolates from humans than among isolates from domestic meat (Table 1, Figure 2). For *S.* Dublin and *S.* Enteritidis, the proportion of resistant and multidrug-resistant isolate did not differ between the meat sources and the human isolates, whereas for *S.* Saintpaul and *S.* Newport the rates of resistance and multidrug resistance were lower for isolates from humans than from both domestic and imported meat.

S. Hadar, *S.* Virchow, *S.* Newport, and *S.* Heidelberg were frequently found in imported products but rarely found in domestic products. Isolates that belong to these serovars are common causes of human salmonellosis in Denmark (*13*). Overall, a significantly higher number of resistant and multidrug-resistant *Salmonella* isolates were found among isolates from imported poultry, pork, and beef compared with domestic products. This finding implies that consumers in Denmark are more likely to be exposed to drug-resistant *Salmonella* spp. when eating imported compared with domestic meat. An increase in the occurrence of resistance over time was also observed among isolates from

Table 3. Number and proportion of susceptible (S), resistant (R), and multidrug-resistant (M) meat isolates within *Salmonella* Typhimurium phage types, Denmark, July 1998–July 2002*

Serovar/phage type	Domestic meat				Imported meat				Total no.
	M, %	R, %	S, %	Total no.	M, %	R, %	S, %	Total no.	
All *S.* Typhimurium isolates	6	21	73	1,508	42	24	34	138	1,646
DT104	70	13	17	23	88	7	5	43	66
DT170	3	68	29	97	0	0	0	0	97
DT193	13	37	51	63	50	17	33	6	69
DT120	16	29	55	38	57	43	0	7	45
DT208	57	40	3	30	0	57	43	7	37
DT107	5	55	41	22	0	0	0	0	22
U302	0	33	67	6	38	31	31	13	19
DT135	6	56	38	16	0	100	0	2	18
Other *S.* Typhimurium	5	21	74	1,213	21	32	47	60	1,273
Salmonella other than Typhimurium	3	22	75	1,477	26	31	43	940	2,417
Total	4	22	74	2,985	28	30	42	1,078	4,063

*Not all *S.* Typhimurium isolates from humans were phage typed.

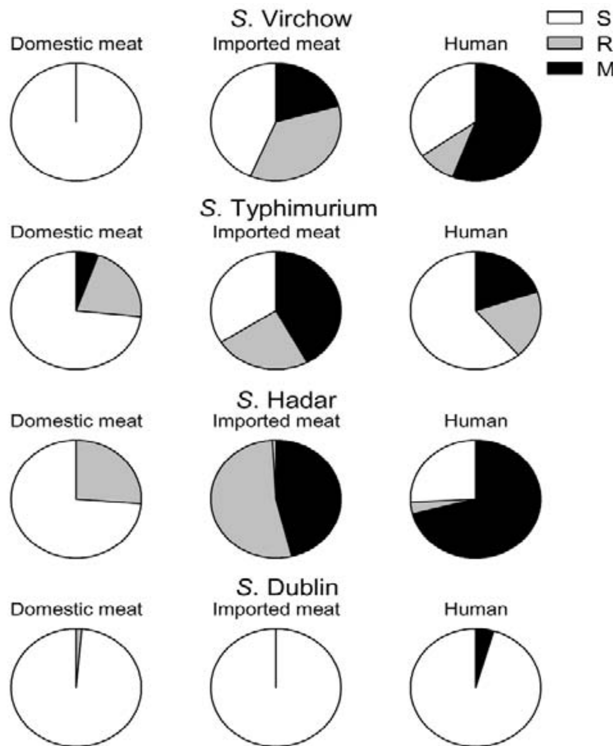

Figure 2. Proportion of susceptible (S), resistant (R), and multidrug-resistant (M) isolates among different *Salmonella* serotypes in isolates from domestic meat, imported meat, and humans, Denmark, July 1998–July 2002.

both domestic and imported meat; this is in agreement with observations worldwide (*2–5*). Antimicrobial agents might not be essential for treatment of gastroenteritis caused by *Salmonella* spp., but they are essential for treatment of patients with invasive infections. In particular, the frequent occurrence of resistance to quinolones is a matter of concern because these compounds are often used for first treatment of serious human infections, before the results of susceptibility testing are available.

International trade of food products is expected to increase in the future. Thus, endeavors to improve food safety must take into account the importance of resistant *Salmonella* spp. in imported food products and, through international agreements, limit contamination with antimicrobial drug–resistant *Salmonella* spp. at the primary production site.

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16S rRNA Methylase–producing, Gram-negative Pathogens, Japan

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To investigate the exact isolation frequency of 16S rRNA methylase–producing, gram-negative pathogenic bacteria, we tested 87,626 clinical isolates from 169 hospitals. Twenty-six strains from 16 hospitals harbored 16S rRNA methylase genes, which suggests sparse but diffuse spread of pan-aminoglycoside–resistant microbes in Japan.

Broad-spectrum β-lactams and fluoroquinolones have been widely prescribed in the treatment of gram-negative bacterial infections; as a result, resistance to these antimicrobial agents has developed in some species. Although these agents are not immune to an increasing number of resistance mechanisms, they remain relatively potent and continue to be essential antimicrobial drugs for treating life-threatening bacterial infections.

Although the production of aminoglycoside-modifying enzymes is the most common mechanism of resistance in aminoglycosides, the emergence of pan-aminoglycoside–resistant, 16S rRNA methylase–producing, gram-negative bacteria has been increasingly reported in recent years. Five types of plasmid-mediated 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, and RmtD) have so far been identified in east Asia, Europe, and South America (*1–7*). RmtA was first identified in 2001 in Japan (*3*) and has so far been identified exclusively in *Pseudomonas aeruginosa* (*8*). RmtC was subsequently identified only in *Proteus mirabilis* (*4*). RmtB has been found among various gram-negative bacterial species, including *Serratia marcescens*, *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca,* isolated in Japan, South Korea, and Taiwan (*2*,*5*,*6*,*9*). Another new 16S rRNA methylase was initially identified in *C. freundii* in Poland, submitted to European Molecular Biology Laboratory (EMBL)/GenBank in 2002 (accession no. AF550415), and later characterized and assigned as ArmA in *K. pneumoniae*, *E. coli*, *Enterobacter cloacae*, *Salmonella enterica*, and *Shigella flexneri* in France, Bulgaria, and Spain (*10*,*11*). Moreover, ArmA was also identified in *E. coli*, *K. pneumoniae*, *E. cloacae*, *C. freundii* and *S. marcescens* in South Korea, Japan, and Taiwan (*5*,*8*,*9*). This enzyme has also been identified in a glucose nonfermentative *Acinetobacter* sp. in South Korea and Japan (*6*,*8*). Quite recently, RmtD was newly identified in the SPM-1–producing *P. aeruginosa* strain PA0905, which was isolated in Brazil (*7*). In Japan, arbekacin, a semisynthetic aminoglycoside, has been approved for treatment of methicillin-resistant *Staphylococcus aureus* infections, and this agent is also very efficacious for gram-negative bacteria. However, 16S rRNA methylase–producing microbes can adapt to this agent, and its prescription may well be a selective pressure on the kind of microbes in the clinical environment. Thus, this investigation was conducted to determine the exact isolation frequency of 16S rRNA methylase–producing, gram-negative pathogenic bacteria in Japanese medical facilities and assess the possibility of the future prevalence of these hazardous microbes.

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The Study

From September 1 to October 31, 2004, 169 medical facilities with in-house microbiology laboratories participated in this investigation. Clinical specimens were collected from inpatients and outpatients with suspected infections. Bacterial isolates that belonged to the family *Enterobacteriaceae* or were nonfermentors of glucose, for example, *P. aeruginosa* and *Acinetobacter* spp., were included in this study. A total of 87,626 clinical isolates were analyzed. The results are shown in Table 1.

Twenty-nine strains (17 *P. aeruginosa*, 4 *A. baumannii*, 3 *E. coli*, 2 *P. mirabilis*, 1 *E. cloacae*, 1 *K. pneumoniae*, and 1 *Enterobacter aerogenes*) that grew on LB agar plates supplemented with 500 mg of arbekacin per liter were subjected to the typing of 16S rRNA methylase genes by a multiplex PCR. Primers used for the PCR amplification of bacterial 16S rRNA methylase genes were the following: RMTA-F 5′-CTA GCG TCC ATC CTT TCC TC-3′ and RMTA-R 5′-TTT GCT TCC ATG CCC TTG CC-3′, which amplify a 635-bp DNA fragment within *rmtA* gene (*3*); RMTB-F 5′-GCT TTC TGC GGG CGA TGT AA-3′ and RMTB-R 5′-ATG CAA TGC CGC GCT CGT AT-3′, which amplify a 173-bp DNA fragment within *rmtB* (*2*); RMTC-F 5′-CGA AGA AGT AAC AGC CAA AG-3′ and RMTC-R 5′-ATC CCA ACA TCT CTC CCA CT-3′, which amplify a 711-bp DNA fragment within *rmtC* (*4*); and ARMA-F 5′-ATT CTG CCT ATC CTA ATT GG-3′ and ARMA-R 5′-ACC TAT ACT TTA TCG TCG TC-3′, which amplify a 315-bp DNA fragment within *armA* (accession

Table 1. Gram-negative strains investigated during September and October, 2004

Bacterial species	Strains, n			
	Isolated	Resistant to all aminoglycosides tested	Harboring 16S rRNA methylase gene, n	Rate of 16S rRNA methylase-producing strains, %
Pseudomonas aeruginosa	18,037	384	14	0.08
Escherichia coli	14,701	39	3	0.02
Klebsiella spp.	12,293	11	1	0.008
Enterobacter spp.	6,398	26	2	0.03
Acinetobacter spp.	3,116	33	4	0.13
Serratia marcescens	3,009	14	0	0
Citrobacter spp.	2,422	1	0	0
Proteus spp.	2,389	8	2	0.08
Alcaligenes spp.	443	0	0	0
Other	24,818	8	0	0
Total	87,626	527	26	0.03

nos. AY220558 and AB117519). PCR results and clinical data from these 29 strains are summarized in the Table 2. Genes for 16S rRNA methylases were absent in 3 arbekacin high-level-resistant strains of *P. aeruginosa* by PCR analyses that used 4 sets of 16S rRNA methylase-specific primers. In these strains, simultaneous production of multiple aminoglycoside-modifying enzymes was suggested as reported previously (*12*). Twenty-six strains harboring any of the four 16S rRNA methylase genes were identified in 16 hospitals, with no apparent geographic convergence in the locations of the hospitals (Figure 1). In hospital L, 3 different bacterial species (*E. coli*, *E. aerogenes,* and *K. pneumoniae*) harbored the *armA* gene, which suggests probable conjugal transfer of *armA*-carrying plasmids among different bacterial species.

Pulsed-field gel electrophoresis (PFGE) was performed on 9 strains of *P. aeruginosa* and 3 strains of *A. baumannii* isolated from 4 separate hospitals where 16S

Table 2. Bacterial species and type of 16S rRNA methylase gene detected*

Strain no.	Bacterial species	PCR type of 16S rRNA methylase gene	Hospital	Clinical specimen
40	*Proteus mirabilis*	*rmtC*	A	Sputum
64	*Pseudomonas aeruginosa*	*rmtA*	B	Sputum
101	*P. aeruginosa*	*rmtA*	C	Otorrhea
103	*P. aeruginosa*	*rmtA*	C	Otorrhea
109	*P. aeruginosa*	*rmtA*	C	Otorrhea
113	*P. aeruginosa*	*rmtA*	D	Bile
127	*P. aeruginosa*	*rmtA*	D	Pharynx
157	*P. aeruginosa*	*rmtA*	D	Pharynx
158	*P. aeruginosa*	*rmtA*	D	Stool
231	*Acinetobacter baumannii*	*armA*	E	Wound
249	*P. aeruginosa*	*rmtA*	F	Pus
252	*P. aeruginosa*	*rmtA*	F	Pleural fluid
328	*P. mirabilis*	*rmtC*	G	Sputum
353	*P. aeruginosa*	*rmtA*	H	Sputum
386	*Escherichia coli*	*rmtB*	I	Urine
422	*P. aeruginosa*	UD	J	Urine
463	*P. aeruginosa*	*rmtA*	K	Urine
469	*E. coli*	*armA*	L	Skin
470	*Enterobacter aerogenes*	*armA*	L	Stool
471	*Klebsiella pneumoniae*	*armA*	L	Stool
479	*P. aeruginosa*	*rmtA*	M	Unknown
499	*E. coli*	*armA*	N	Urine
509	*Enterobacter cloacae*	*armA*	O	Urine
525	*P. aeruginosa*	UD	P	Urine
527	*P. aeruginosa*	UD	Q	Blood
593	*P. aeruginosa*	*rmtA*	R	Vaginal secretion
615	*A. baumannii*	*armA*	S	Sputum
617	*A. baumannii*	*armA*	S	Sputum
619	*A. baumannii*	*armA*	S	Pus

*Strains for which MIC of arbekacin was $\geq$512 mg/L are listed; UD, undetected.

Figure 1. Geographic distribution of hospitals where 16S rRNA methylase gene-positive strains were isolated. Of 16 hospitals, 4 were located in the Kanto area (Gunma and Tokyo), 6 in the Chubu area (Aichi, Gifu, and Shizuoka), 1 in the Koushin-etsu area (Nagano), 4 in the Kinki area (Osaka, Nara, and Hyogo), and 1 in the Kyushu area (Miyazaki). This distribution suggests a sparse but diffuse spread of 16S rRNA methylase-producing, gram-negative pathogenic microbes in Japan. Bacterial species and type of 16S rRNA methylase identified in each hospital are shown in Table 2.

rRNA methylase genes were isolated (Figure 2). Genomic DNA preparations from *P. aeruginosa* and *A. baumannii* were digested with *Spe*I and *Sma*I, respectively. Clonality was inferred based on the criteria of Tenover et al. (*13*) Two of 3 *rmtA*-positive *P. aeruginosa* strains isolated in hospital C were estimated to be the same clone. Among 4 *rmtA*-positive *P. aeruginosa* isolates recovered in hospital D, 2 different clonal lineages were observed. This finding suggests possible conjugal transfers of *rmtA*-carrying plasmids among genetically different strains of *P. aeruginosa*. Three *armA* gene–harboring *A. baumannii* identified in hospital S were obviously the same clone. These findings imply probable nosocomial transmission of 16S rRNA methylase gene–harboring strains in hospitals C, D, and S, as well as frequent conjugal transfers of plasmids carrying 16S rRNA methylase genes among gram-negative pathogenic bacterial species.

MIC determinations were performed according to the guideline of the CLSI (formerly National Committee on Clinical Laboratory Standards). All 16S rRNA methylase-positive strains were highly resistant (MICs >1,024 mg/L) of all 4,6-disubstituted deoxystreptamine group aminoglycosides (Table 3). In contrast, resistance to streptomycin and neomycin varied. Three16S rRNA methylase gene-negative *P. aeruginosa* strains were also highly resistant to arbekacin, but the MICs of some of the 4,6-disubstituted deoxystreptamine group aminoglycosides were relatively lower (256–512 mg/L) for these strains than those for 16S rRNA methylase gene–positive strains (>1,024 mg/L). Strains harboring 16S rRNA methylase genes tended to show resistance to oxyimino-cephalosporins such as cefotaxime and ceftazidime as well, but were susceptible to imipenem. As reported for the *armA*- or *rmtB*-bearing strains, the presence of β-lactamase genes was suggested in cefotaxime-resistant strains, and indeed the $bla_{CTX\text{-}M\text{-}14}$ gene was detected in several *rmtB*-positive strains tested in our study (data not shown). Some of these strains also demonstrated resistance to fluoroquinolones (Table 3).

Conclusions

The overall isolation frequency of 16S rRNA methylase-gene-positive gram-negative bacilli was very low (0.03%) in Japanese medical facilities in 2004, with the highest rates seen in *P. aeruginosa* and *Acinetobacter* spp. at 0.08% and 0.13%, respectively. Twenty-six bacterial isolates carrying 1 of the four 16S rRNA methylase genes were recovered from 16 (9.5%) of 169 hospitals that participated in this nationwide investigation. Of the 169 hospitals, 162 hospitals had ≥200 beds, accounting for 5.9% of all Japanese hospitals of similar scale. This implies that 16S rRNA methylase–producing strains might have been present in >250 Japanese hospitals during the investigation period, which in turn suggests sparse but diffuse spread of 16S rRNA methylase producers in Japan. Since several

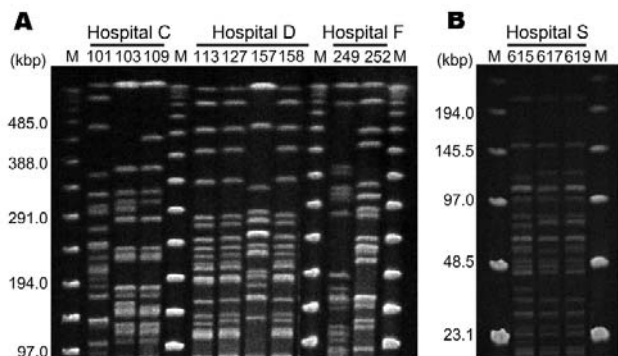

Figure 2. A) Pulsed-field gel electrophoresis (PFGE) fingerprinting patterns of *Spe*I-digested total DNA preparations from *Pseudomonas aeruginosa*. M, Lambda ladder PFGE molecular mass marker (Bio-Rad, Hercules, CA, USA). Strains 103 and 109 show similar patterns, which suggests probable nosocomial transmission of *rmtA*-positive strains in hospital C. Strains 113, 127, and 158 also demonstrate similar patterns, which implies possible nosocomial transmission in hospital D. However, 2 different PFGE patterns are observed in hospitals C, D, and F, which suggests transfer of plasmids carrying 16S rRNA-methylase genes among *P. aeruginosa* strains with different genetic backgrounds. B) *Sma*I-digested total DNA preparations from *Acinetobacter baumannii* isolated from hospital S. Three strains demonstrate the same PFGE pattern, which suggests probable nosocomial transmission of *arm*A-positive *A. baumannii* in hospital S. M, lambda ladder low-range PFGE molecular mass marker (New England Biolabs, Ipswich, MA, USA).

Table 3. MICs of antimicrobial agents for arbekacin-resistant strains*†‡

Strain no.	MIC (mg/L)										
	ABK	AMK	TOB	ISP	GEN	SM	NEO	CTX	CAZ	IPM	CIP
40	>1,024	>1,024	>1,024	>1,024	>1,024	8	>1,024	<0.06	0.125	0.125	64
64	>1,024	>1,024	>1,024	>1,024	>1,024	8	>1,024	<0.06	0.5	0.125	64
101	>1,024	>1,024	>1,024	>1,024	>1,024	32	>1,024	8	2	0.5	32
103	>1,024	>1,024	>1,024	>1,024	>1,024	32	16	64	2	0.5	<0.06
109	>1,024	>1,024	>1,024	>1,024	>1,024	8	16	64	16	0.5	<0.06
113	>1,024	>1,024	>1,024	>1,024	>1,024	128	512	16	2	16	0.125
127	>1,024	>1,024	>1,024	>1,024	>1,024	128	128	16	2	16	<0.06
157	>1,024	>1,024	>1,024	>1,024	>1,024	32	32	64	4	2	0.5
158	>1,024	>1,024	>1,024	>1,024	>1,024	128	512	32	8	16	0.125
231	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	128	4	16
249	>1,024	>1,024	>1,024	>1,024	>1,024	256	512	16	1	4	<0.06
252	>1,024	>1,024	>1,024	>1,024	>1,024	512	512	128	4	4	8
328	>1,024	>1,024	>1,024	>1,024	>1,024	8	512	>128	>128	2	32
353	>1,024	>1,024	>1,024	>1,024	>1,024	32	256	64	>128	4	32
386	>1,024	>1,024	>1,024	>1,024	>1,024	256	256	128	>128	0.5	>128
422	>1,024	>1,024	>1,024	256	>1,024	512	>1,024	>128	>128	8	128
463	>1,024	>1,024	>1,024	>1,024	>1,024	64	128	16	4	8	32
469	>1,024	>1,024	>1,024	>1,024	>1,024	64	32	>128	8	0.25	<0.06
470	>1,024	>1,024	>1,024	>1,024	>1,024	128	8	>128	>128	4	1
471	>1,024	>1,024	>1,024	>1,024	>1,024	64	8	128	4	0.25	<0.06
479	>1,024	>1,024	>1,024	>1,024	>1,024	256	1,024	64	4	0.25	0.25
499	>1,024	>1,024	>1,024	>1,024	>1,024	64	4	0.06	0.125	0.25	0.25
509	>1,024	>1,024	>1,024	>1,024	>1,024	64	1	>128	64	0.25	125
525	512	512	1,024	512	256	>1,024	>1,024	128	32	16	>128
527	1,024	512	1,024	>1,024	64	>1,024	>1,024	>128	>128	128	0.125
593	>1,024	>1,024	>1,024	>1,024	>1,024	128	64	>128	128	2	0.5
615	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	16	>128	>128	1	32
617	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	>128	1	32
619	>1,024	>1,024	>1,024	>1,024	>1,024	>1,024	32	>128	>128	1	32

*ABK, arbekacin; AMK, amikacin; TOB, tobramycin; ISP, isepamicin; GEN, gentamicin; SM, streptomycin; NEO, neomycin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin.
†MICs of kanamycin and sisomicin are not listed because values are >1,024 for all strain numbers.
‡See Table 2 for bacterial species and PCR type of 16S rRNA methylase gene of each strain number.

armA- or *rmtB*-positive strains have also been isolated in European and Asian countries, and given the potential for further dissemination, nationwide identification and ongoing surveillance of these isolates should be considered by all countries.

According to PFGE typing, nosocomial transmission of 16S rRNA methylase–producing *P. aeruginosa* and *A. baumannii* was suspected in 3 hospitals (hospitals C, D, and S). The banding patterns of *rmtA*-harboring *P. aeruginosa* isolated in hospitals C, D, and F were diverse, which excluded the possibility of an epidemic *P. aeruginosa* strain harboring the *rmtA* gene. Despite the observation of 2 different PFGE profiles among the 4 *P. aeruginosa* strains isolated in hospital D, they might share the same plasmids carrying the *rmtA* gene. For further characterization of genetic relations among *rmtA*-harboring *P. aeruginosa* strains, comparative analyses of plasmids and mobile elements that carry the *rmtA* gene (*14*) should also be pursued.

Nosocomial infections caused by multidrug-resistant, gram-negative bacteria have become a serious problem in clinical facilities. *P. aeruginosa* and *Acinetobacter* spp. have been especially efficient at developing multidrug resistance against broad-spectrum β-lactams, fluoroquinolones, and aminoglycosides (*3*,*6*,*7*,*9*). The identification of *armA* and *rmtB* genes in Europe and East Asia in both human (*1–11*) and livestock (*15;* EMBL/GenBank accession no. DQ345788) populations suggests that we must pay consistent attention to prevent further global proliferation. If 16S rRNA methylase–positive bacterial isolates disseminate widely and extensively, the high level of pan-aminoglycoside resistance will undoubtedly have an impact on illness, deaths, and costs of care in both clinical and livestock-breeding environments.

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Spread of Extensively Drug-resistant Tuberculosis

To the Editor: An emergency has been declared in KwaZulu Natal, South Africa, where an outbreak of 53 cases of a highly lethal form of tuberculosis (TB) has occurred (*1,2*). This outbreak was caused by an extensively drug-resistant TB (XDR TB) strain.

XDR TB is defined as TB caused by *Mycobacterium tuberculosis* isolates resistant to isoniazid and rifampicin plus any fluoroquinolone and ≥1 of the 3 injectable second-line drugs (*3*). XDR TB may be considered an emerging disease but not a new disease. Nosocomial outbreaks of multidrug-resistant TB (MDR TB) occurred in Spain at the height of the HIV epidemic, when 49 TB cases were reported in an HIV ward in Madrid from 1991 through 1995 (*4,5*). Molecular epidemiology found that a particular strain caused 16 cases in another hospital in Madrid in 1993–1995 (*6*) and 31 cases in a hospital in Malaga in 1995–1998 (*7,8*). In total, 22 hospitals from 6 different regions of Spain were affected by this outbreak, which included at least 114 cases, caused by an *M. bovis* XDR strain (B strain) belonging to the *M. tuberculosis* complex. The patients included 1 from the Netherlands (*8*) and another from Canada (*9*).

The strain responsible for the 1991–1995 outbreak in Spain fits the XDR TB case definition; it was resistant to the 5 first-line drugs, as well as to ofloxacin, aminosalicylic acid, cycloserine, ethionamide, capreomycin, amikacin, and clarithromycin. Isolates were tested for drug susceptibility by the Canetti method on Lowenstein-Jensen medium supplemented with isoniazid, rifampicin, ethambutol, streptomycin, amikacin, and pyrazinamide (*6*). The isolates were also tested on 7H10 Middlebrook agar for susceptibility to aminosalicylic acid, ethionamide, capreomycin, clarithromycin, and ofloxacin (*6*). No effective medical treatment was available for these patients. In 2 of the hospitals affected, all patients died, with a short survival time (median of 44 and 49.5 days for the 2 hospitals) between diagnosis and death (*6,7*). A high rate of reinfection (45%) also was noted among HIV-positive patients treated with anti-TB drugs (*7*). As a result of this outbreak, Spanish hospitals now implement exhaustive control measures, such as maintaining respiratory isolation units under negative pressure; in addition, a national surveillance network for MDR TB was set up in Spain in 1998. From 1998 through 2003, we detected 22 new cases of infection with this strain (*10*), but no new cases have since been reported to the national MDR TB database.

Our experience indicates that the implementation of more stringent control measures and the use of new, more effective treatments for HIV infection can help to bring XDR TB outbreaks under control in developed countries. However, the outlook is bleak for developing countries like South Africa, in which coinfection with HIV and a highly transmissible and untreatable XDR TB strain could amplify the TB problem to levels unprecedented since the advent of antimicrobial drugs. These countries urgently require assistance with the establishment of control measures and the development of new drugs and effective vaccines against TB.

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Transfusion-associated Hepatitis E, France

To the Editor: Hepatitis E virus (HEV) is a leading cause of acute and fulminant hepatitis in developing countries (*1*). In industrialized countries, HEV seroprevalence rates of 0.4%–3% are common, and evidence is mounting that autochthonous transmission might account for a substantial proportion of infections (*1*–*3*). Whereas fecal-oral HEV transmission is preponderant in developing countries, other routes have been demonstrated in industrialized countries, among them consumption of pork or boar meat (*1*,*4*). Parenteral transmission was first suggested but not demonstrated (*5*,*6*). Because viruses cannot be fully inactivated in blood products, HEV recently emerged as a transfusion-transmitted pathogen, with 6 reported cases, including 3 in which comparative analysis of HEV sequences from blood donor and recipient was performed (*7*). We describe what is, to our knowledge, the first case in France and the first case worldwide involving a child as blood recipient.

A 7-year-old boy was examined in June 2006; he had erythematous-papulous skin and an increased level of alanine aminotransferase (ALT, 796 IU/L) of 1 week's duration. From October 2005 to May 2006, he had received several courses of combined chemotherapy for a rhabdoid tumor in his kidney. Therapy included carboplatin, cyclophosphamide, etoposide, adriamycin, and vincristine, and, because of the chemotherapy's hematotoxicity, the patient required 22 transfusions of concentrated erythrocytes or platelets. Peak levels of ALT and bilirubin were reached 4 weeks after onset of hepatitis (2,001 IU/L and 49 μmol/L, respectively), and ALT levels returned to normal range (8–45 IU/L) 6 weeks later. During a follow-up examination, the boy's prothrombin index remained >80%, and clinical signs mostly consisted of clinical jaundice. HEV diagnosis was established by detection in serum of HEV antibodies (EIAgen Kits, Adaltis Development Inc., Laval, Quebec, Canada) and HEV RNA with in-house assays. Other infectious or noninfectious causes of acute hepatitis were excluded. HEV immunoglobulin M (IgM) was weakly positive in June 2006 (optical density ratio = 1.6), then strongly positive the next month (ratio = 10.8); IgG remained negative. HEV RNA was detected from serum samples collected in June 2006 by an in-house real-time PCR that targeted the open reading frame 2 region of the HEV genome. Sequences of primers/probe are as follows: HevMrsRT fwd: 5′-AATTRATTTCGTCGGCY GG-3′; HevMrsRTRev: 5′- ACW GTCGGCTCGCCATTG-3′; HevMrsFam: 5′-FAM-ACTCYCGC CASGTYGTCTCA-TAMRA-3′.

Serum samples taken from 12 U of blood products that the child received during the 3-month period before onset of hepatitis were tested for HEV RNA; 1 sample was positive. Concentrated erythrocytes (310 mL) from this positive blood donation were transfused to the child in May 2006, 4 weeks after collection and 6 weeks before acute hepatitis developed. HEV IgG and IgM antibodies were not detected in the blood donation, which indicates that the blood donation occurred during the prodromic phase of HEV disease. HEV nucleotide sequences from the blood donor and recipient were identical. Phylogenetic analysis showed that they clustered together and were closely related to genotype 3f, which is prevalent in Europe (Figure [*8*]).

The blood donor was a 24-year-old man. He did not travel outside metropolitan France for 8 months before donating blood. Anti-HEV IgG seroconversion was observed on a serum sample collected 24 weeks after blood donation, whereas anti-HEV IgM and serum HEV RNA tested negative. ALT levels were within normal values at the time of blood donation and 24 weeks later. No clinical signs were reported at any time. No other recipient received blood products from this donor.

Our data show that HEV was transmitted from 1 blood donor to the recipient child. On the basis of retrospective HEV RNA detection, 6 other transfusion-transmitted HEV infections, all involving adult blood recipients, have occurred in HEV-hyperendemic and industrialized countries (*7*). Transfusion-transmitted HEV strains belonged to different genotypes/subtypes that corresponded to those found in the same geographic areas (Figure).

In France, neither HEV antibodies nor HEV RNA are systematically tested in blood donors, and blood donations currently are not tested for ALT. In the absence of systematic HEV RNA testing, HEV diagnosis in blood donations may be hampered by HEV viremia before clinical onset and anti-HEV seroconversion, the possible short persistence or absence of HEV antibodies, and the high frequency of subclinical infections

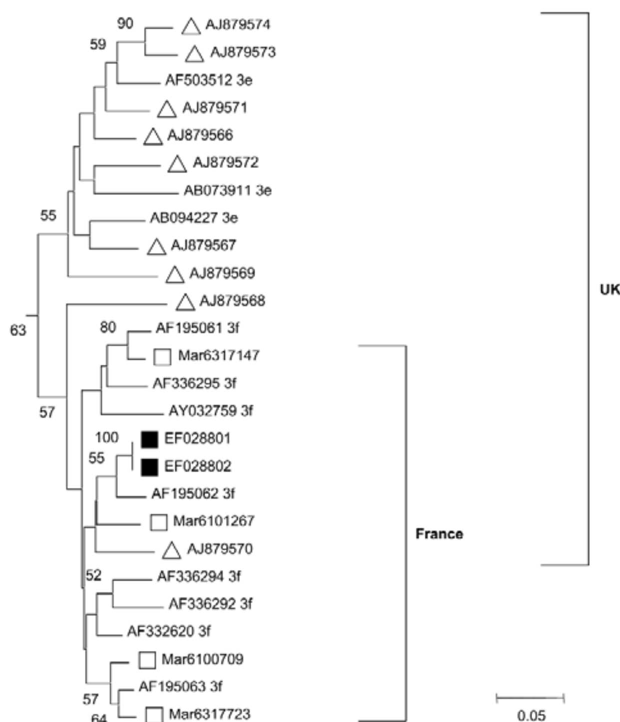

Figure. Outline of phylogenetic tree constructed by the neighbor-joining method based on partial nucleotide sequences of the open reading frame (ORF) 2 region of hepatitis E virus (HEV) genome obtained from the blood donor and blood recipient, with sequences of other local HEV strains previously identified and published HEV sequences (*8*). Bootstrap values are indicated when >50% as a percentage obtained from 100 resamplings of the data. Black squares indicate HEV sequences from the blood donor and recipient (GenBank accession nos. EF028801 and EF028802); white squares indicate HEV sequences from other individuals with hepatitis E living in Marseille and environs; white triangles indicate HEV sequences from the United Kingdom (*3*) that have an 80%–100% homology at the nucleotide level with those obtained by Boxall et al. (*7*). See online figure, available at http://www.cdc.gov/content/EID/13/4/648-G.htm, for complete phylogenetic tree and details of methods and sequences.

(*1*,*2*,*5*). On the basis of these data, improved knowledge of HEV epidemiology in the general population and in blood donors is needed in industrialized countries to guide HEV screening policy for blood products.

Another concern of transfusion-transmitted HEV infections is the potential severity of acute hepatitis E. Previously reported cases of contamination through transfusion and our case showed spontaneous recovery. However, although typically self-limited, hepatitis E is potentially life-threatening with mortality rates of 0.2%–4%, reaching 20% in pregnant women (*1*). Fatal outcome has also been described in children (*9*). In summary, HEV-contaminated blood donations are a challenge for diagnosis and virus safety of transfusion, including in industrialized countries.

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EMERGING INFECTIOUS DISEASES

Dengue and Relative Bradycardia

To the Editor: We have found that relative bradycardia is a notable clinical feature of dengue fever in Singapore. To our knowledge, this sign has not been previously associated with dengue. Awareness of this possible clinical finding could help in the early recognition of dengue and potentially reduce complications and death associated with dengue virus infection. Clinical features that can be used in the initial assessment of febrile patients are essential tools for clinicians, especially in limited resource settings.

Dengue fever is a potentially fatal illness; >2.5 billion persons are at risk and the disease is endemic in almost 100 countries (*1*). Singapore recorded >14,000 cases in 2005, its highest annual figure (*2*). No specific clinical features distinguish dengue from other febrile illnesses (*3*); thus, diagnosis relies heavily on results of laboratory investigations. Virus-specific immunoglobulin M (IgM) antibodies only become detectable after 5–7 days, and false-positive results can confound the diagnosis. PCR is a useful diagnostic tool; however, it is limited by the short duration of viremia and requirements for sophisticated laboratory support (*4*).

Relative bradycardia has been reported in many infectious diseases, including typhoid fever, Legionnaires' disease, psittacosis, typhus, leptospirosis, malaria, and babesiosis (*5*,*6*). During the 2005 Singapore outbreak, we observed relative bradycardia in several patients with dengue fever. We therefore performed a case-control study comparing febrile dengue patients to patients with other infectious diseases. The study was approved by our hospital's ethics committee.

The records of all patients admitted with a febrile illness to our general medical unit from June 1 to October 31, 2005, were reviewed. Patients with a clinical diagnosis of dengue fever and serologic confirmation (IgM or PCR positive) plus a temperature >38°C were included as case-patients. Age-matched controls were selected from the same general medical inpatient units and were admitted during the same period. All had fever, but they had a proven alternative diagnosis, including pneumonia (12 patients), upper respiratory tract infection (9 patients), urinary tract infection (6 patients), tuberculosis, liver abscess, viral fever other than dengue (3 patients each), meningitis, chickenpox, cellulitis, typhoid fever (2 patients each), and appendicitis, psoas abscess, typhus, infective endocarditis, pressure ulcers, and gastroenteritis (1 patient each). Exclusion criteria were the following: no laboratory confirmation, age <18 years or >60 years, preexisting substantial heart or lung disease or concurrent medication affecting heart rate, e.g., β-blockers, β-agonists, calcium channel blockers, or xanthine derivatives.

The peak temperature of all case-patients and controls was recorded within the first 24 hours of admission as well as heart rate and blood pressure at that point. Leukocyte count, hemoglobin concentration, hematocrit, and platelet counts were also noted. Data from 50 case-patients and 50 controls were tabulated and analyzed with Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

The mean age (± standard deviation) for dengue patients was 32.8 (±10.8) years and for controls was 36.5 (±10.2) years ($p = 0.08$). There were 39 male patients in the dengue group and 31 in the control group. Their mean peak temperatures were comparable: 38.6°C (±0.5) (dengue) and 38.8°C (±0.7) (controls) ($p = 0.09$). Mean heart rates were significantly lower in the dengue group: 87.6 (±12.5) beats/min (dengue) and 104.6 (±14) beats/min (controls) ($p<0.0001$).

Electrocardiographs (ECGs) were available for 10 of the dengue group, and all showed normal sinus rhythm. Three patients with bradycardia had an ECG. Results for 2 patients were normal; 1 showed mitral valve prolapse with mild regurgitation. Ten patients in the control group underwent an ECG, and none had any notable abnormality. Four controls had ECGs, results for 2 were normal; 1 had mild mitral regurgitation, and 1 had mild tricuspid regurgitation. The heart rates at peak temperatures for patients with dengue fever were compared with rates for controls at all temperatures. Our findings demonstrate a consistently lower heart rate at all peak temperatures recorded ($p<0.0001$) (Figure).

Dengue fever may adversely affect cardiac function. An echocardiographic study by Khongphatthanayothin et al. (*7*) showed depressed myocardial contractility and suboptimal heart rate response in some patients with dengue hemorrhagic fever. Acute reversible hypokinesia and reduction in left ventricular ejection fraction was also reported by Wali et al. (*8*). The underlying mechanisms were postulated to be immune in origin, although myocarditis may be a contributory factor. Fever production in response to exogenous pyrogens is believed to be mediated mostly by cytokine prostaglandin pathways, and neural input is important in the early phases of fever (*9*). Concentrations of cytokines, including tumor necrosis factor, interferon-γ, interleukin-8 (IL-8), IL-10, and IL-12, are substantially increased during dengue infection. Their levels likely correlate with specific clinical manifestations and illness severity (*10*). The relationship of cytokines to relative bradycardia is unknown. Further studies could consider the relative importance of immune and neural mechanisms and also any direct cardiac pathology in

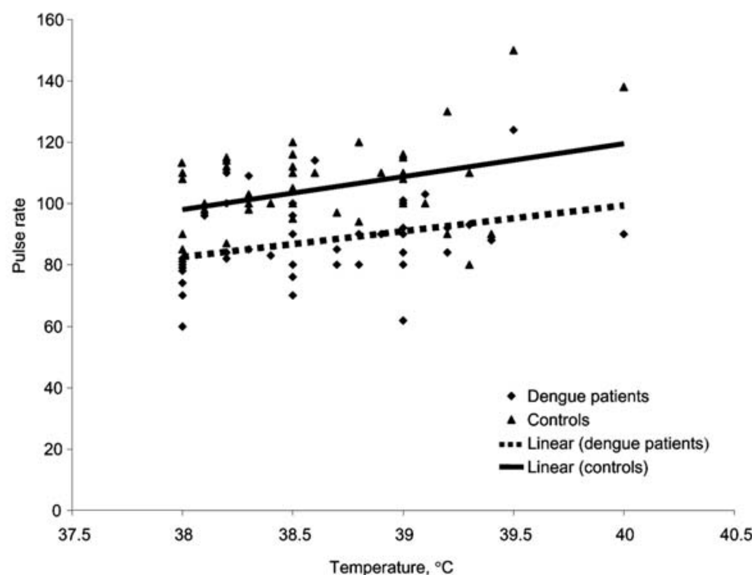

Figure. Temperature and heart rate relationship: scatter plot for patients with dengue fever and nondengue febrile illnesses.

the etiology of dengue-associated relative bradycardia.

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West Nile Virus, Venezuela

To the Editor: West Nile virus (WNV; genus *Flavivirus*; family *Flaviviridae*) has been perpetuating in North America since 1999 (*1*). However, its status as a self-perpetuating pathogen in South America remains uncertain. Infected horses and birds have been reported in various Caribbean Islands, Mexico, and northern Central America (*2*,*3*). In South America, isolated reports of infected dead-end hosts (horses) have come from northern Colombia and Argentina, but they lack evidence for infection in avian amplifying hosts (*4*,*5*). We report serologic evidence of establishment of WNV in South America.

Serum samples from birds and horses from 33 locations in Venezuela (Online Appendix Table, available from http://www.cdc.gov/EID/content/13/4/651-appT.htm) were screened for immunoglobulin G (IgG) antibodies against WNV antigen by ELISA (*6*) and confirmed by plaque reduction neutralization test (PRNT) as previously described (*7*). The flavivirus generating the IgG response was identified by using the following criteria: 90% inhibition of virus in serum diluted at least 1:40 and 4-fold greater neutralizing antibody titer compared with closely related flaviviruses. IgG antibody against flavivirus was detected by ELISA in 14 of 576 resident birds, including 5 *Turdus leucomelas*, 3 *Gallus gallus* (captive), 2 *Campylorhamphus trochilirostris*, and 1 each of *Elaenia flavogaster*, *Coereba flaveola*, *Thraupis palmarum*, and *Anisognathus flavinucha*.

WNV was confirmed as the etiologic agent of infection in 5 adult birds (3 *T. leucomelas* [pale-breasted thrush], 1 *C. flaveola* [bananaquit], and 1 *G. gallus* [domestic chicken] with the earliest collection date in February 2006); virus neutralization

titers ranged from 80 to 320. One serum sample cross-reacted with other flaviviruses tested, with equivalent titers to WNV, Saint Louis encephalitis virus (SLEV), and Ilheus virus (ILHV) and was thus considered infected with an undetermined flavivirus. Seven serum samples were negative (antibody titers <20), and 1 sample was not tested because of insufficient sample volume.

Antibody against flavivirus was detected by ELISA in 141 of 791 horses, and 34 (4.3%) were confirmed positive for WNV infection by PRNT; viral titers ≥640 occurred in half of these horses. The earliest collection date for a WNV-positive horse was February 2004 and the most recent was May 2006. Specific WNV-reactive equine serum samples were distributed in valley regions (prevalence 1.3%), savannah grasslands (2.4%), the western region of Zulia (0.4%) and the Central Lake Basin (0.3%). A total of 46 (5.8%) equine serum samples were positive for neutralizing antibody to SLEV, and 8 (1.0%) samples were positive for neutralizing antibodies to ILHV. Forty-nine samples neutralized at least 2 of the 3 viruses and were classified as undetermined flaviviruses. Serum samples from 2 horses were negative in neutralization assays; 2 others were not tested because of insufficient sample volume.

Detection of WNV-infected resident birds provides strong evidence of the establishment, rather than importation, of WNV in South America. We hypothesize that ornithophilic mosquitoes (such as some *Culex* spp.), which are present in the area in consistently high numbers, acquired the virus through hematophagous feeding on recently infected, migrating birds. Once introduced to local mosquitoes, virus is amplified among susceptible resident birds fed upon by ornithophilic mosquitoes. This pattern allows perpetuation and subsequent establishment of virus in a continuous transmission cycle, as opposed to infection of dead-end hosts, e.g., horses. This is the first report of WNV infection in South American birds and definitive establishment of the virus in South America.

We observed varying WNV seroprevalence rates in birds and horses across regions in Venezuela (Figure). These differences reflect the focal and stochastic nature of arbovirus transmission, which depends upon many ecologic factors. One possible explanation for the greater seroprevalence in the central and eastern llanos (savannahs) and valley regions, compared with the coastal western region of Zulia State ($p<0.0001$, by Pearson's χ^2 test) would be virus introduction by migrating birds by an eastern migration route.

Existence of several closely related flaviviruses in the American tropics (*8–10*) may convey cross-protection in animals (e.g., ILHV and SLEV) or humans (dengue viruses, yellow fever virus), thereby potentially diminishing disease caused by a newly introduced flavivirus such as WNV. Although ILHV infection has not been detected in Venezuela, this flavivirus is prevalent in Brazil, Peru, French Guyana, Trinidad, and Colombia. Our study demonstrated widespread distribution of ILHV in Venezuela. Other South American flaviviruses, such as Bussuquara, Cacipacore, and Iguape, and as yet undiscovered viruses may also circulate in Venezuela.

We encourage those involved in the public and animal health systems in Venezuela to consider zoonotic flaviviruses in the differential diagnoses of human and equine cases of encephalitis and to consider ecologic surveillance for zoonotic flaviviruses in mosquito and vertebrate host populations. We recommend monitoring blood and organ donations for flavivirus infections. Our study sheds light on flavivirus distribution in Venezuela. However, nothing else is known about the ecology of zoonotic flaviviruses in this country. Such knowledge will be essential for designing effective surveillance and control should these viruses be shown to cause human illnesses.

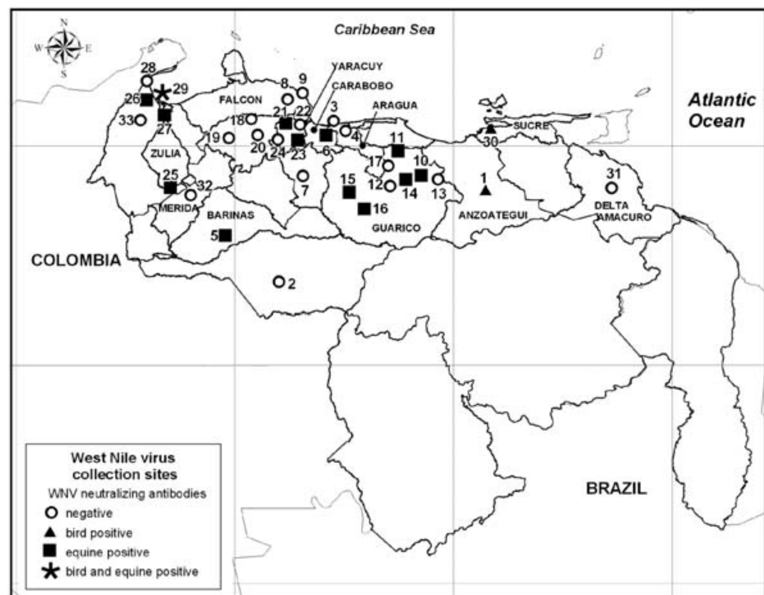

Figure. West Nile virus (WNV) collection sites in Venezuela, indicated by number (see online Appendix Table). Symbols represent results of tests for specific antibodies to WNV in serum samples of birds and horses (viral titers in a 90% plaque reduction neutralization test >40 and a 4-fold differential inhibition in a neutralization assay to WNV compared with other related flaviviruses). Source: Instituto Geográfico de Venezuela Simón Bolivar, Caracas.

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Novel Extended-spectrum β-Lactamase in *Shigella sonnei*

To the Editor: A 38-year-old French man with a history of chronic juvenile arthritis was referred to the Necker-Enfants Malades University hospital (Paris, France) with a dysenteric syndrome. The patient had returned the day before from a 1-month stay in Port-au-Prince, Haiti, where he spent most of his time in close contact with young children from an orphanage, most of whom had diarrhea. Clinical examination at admission showed fever (39°C), chills, diffuse abdominal pain, bloody diarrhea, and vomiting. The patient received ceftriaxone, which was stopped on day 4 because initial blood and stool cultures were negative for pathogens and clinical signs had completely resolved.

Ten days later, he reported the recurrence of diarrhea without fever. A novel stool culture grew *Shigella sonnei*. An extended-spectrum β-lactamase (ESBL) was detected by double-disk synergy test; the isolate was also resistant to aminoglycosides (except amikacin), tetracycline, and cotrimoxazole. The strain was susceptible to fluoroquinolones and fosfomycin. It also appeared susceptible to azithromycin (MIC 4 μg/mL), although azithromycin MIC for *Shigella* spp. should be interpreted with caution (*1*). The patient was successfully treated with azithromycin at a dose of 500 mg/day for 5 days. Azithromycin was preferred to fluoroquinolones to avoid the risk for tendinopathy because of the patient's history of chronic juvenile arthritis and because this antimicrobial agent was shown to be effective in the treatment of shigellosis caused by multidrug-resistant strains (*2*).

To identify the molecular basis of this ESBL, a series of PCR primers

were used for detection of TEM-, SHV-, or CTX-M–type ESBL *(3)*. Only the TEM PCR showed positive results. Sequencing of 2 independent PCR products showed a new allele (www.lahey.org/studies/temtable.asp). Analysis of the deduced amino acid sequence allowed characterization of TEM-137, derived from TEM-1 with 2 substitutions, Arg-16→Ser and Glu-240→Arg. This ESBL (and resistance to aminoglycosides and tetracyclines) was easily transferred to *Escherichia coli* J53-2 by conjugation.

MICs of β-lactams alone or in association with clavulanic acid, were determined by E-test, according to manufacturer's instructions (AB Biodisk, Solna, Sweden). High-level resistance to ceftazidime (MIC 32 μg/mL) and intermediate resistance to cefotaxime (MIC 8 μg/mL) were observed; the strain remained susceptible to cefepime and imipenem (MIC 0.5 and 0.25 μg/mL, respectively). Clavulanic acid did not restore susceptibility to ceftazidime (MIC 4 μg/mL) but did restore susceptibility to cefotaxime (MIC 0.5 μg/mL). With clavulanic acid, the MIC of cefepime was 0.06 μg/mL.

ESBL in *S. sonnei* is rare worldwide. In Argentina, a CTX-M-2 was found in an isolate of *S. sonnei* resistant to cefotaxime but not to ceftazidime (*4*). In South Korea, TEM-15, TEM-17, TEM-19, TEM-20, TEM-52, and CTX-M-14 were characterized in *S. sonnei* (*5*); TEM-52 and CTX-M-14 were also widely distributed, particularly in *Salmonella* spp. (*6,7*). In Turkey, an isolate of *S. sonnei* producing CTX-M-3 was reported (*8*). In Hong Kong, sequencing of 2 *S. sonnei* isolates showed the presence of CTX-M-14 and CTX-M-15 *(9)*. Finally, in Bangladesh, 2 isolates of *S. sonnei* with a class A ESBL were reported; they were not characterized at the molecular level, but the resistance phenotypes suggested a CTX-M type (*10*).

In our case, little information on antimicrobial drug resistance could be obtained from Haiti because no systematic investigation on resistance in *Enterobacteriaceae* is performed. Nevertheless, the emergence of TEM-137 (GenBank accession no. AM286274) harbored by this imported *S. sonnei* isolate clearly demonstrates that ESBL-associated shigellosis has emerged in Haiti and that potentially large and severe shigellosis outbreaks could occur, for which the use of azithromycin could be beneficial, as illustrated in our patient. Because treating shigellosis is becoming problematic, it is essential to focus on prevention measures such as simple rules of personal hygiene that might drastically decrease the risk of transmission.

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Vibrio parahaemolyticus O3:K6 Epidemic Diarrhea, Chile, 2005

To the Editor: Outbreaks of diarrhea and gastroenteritis caused by *Vibrio parahaemolyticus* have been recently reported in many countries and regions where this pathogen was previously unknown (*1,2*). In mid-January 2005 (Figure), the number of cases of acute diarrhea produced by *V. parahaemolyticus* dramatically increased in Puerto Montt (41°41′S), a major city in Region X of Chile. The epidemic subsequently peaked in February and then declined with isolated cases in March and April. A total of 3,725 cases of acute diarrhea were detected during the summer months of January–April, 2005 throughout Region X (39°15′S–44°4′S). This epidemic rapidly spread to other urban areas in Region X and to the rest of Chile because Region X is the source of ≈75% of the seafood consumed in Chile. By the end of March 2005, the total number of cases in Chile was 10,783, making this the largest documented occurrence of *V. parahaemolyticus* diarrhea in the world.

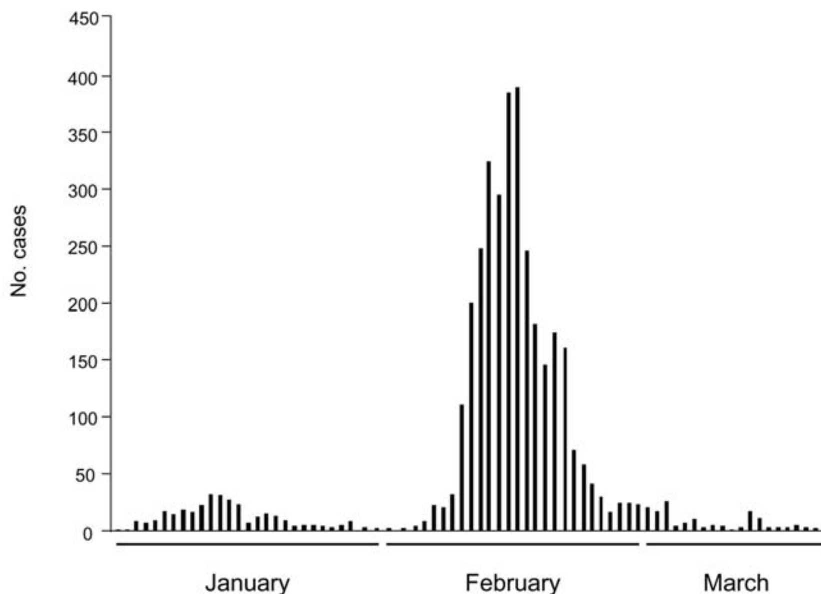

Figure. No. cases of acute diarrhea in Region X, Chile, January 4, 2005–March 21, 2005.

Analysis of a questionnaire prepared by the health authority of Region X and completed by 341 patients during January 2005 indicated that all patients had clinical signs compatible with acute diarrhea caused by *V. parahaemolyticus* (*3,4*). Stool samples of 60 patients with acute diarrhea were analyzed by standard procedures (*5,6*). Serotyping confirmed that all *V. parahaemolyticus* isolates were O3:K6 (*5,6*), did not produce urease, and showed the Kanagawa phenomenon (virulence-associated hemolysis) (*5–8*). PCR analysis indicated that the genome of these isolates contained *tdh*, *tlh*, and *toxRS*/new open reading frame 8 DNA sequences and lacked *trh* sequences (*1,8*), which are consistent with molecular characteristics of the pandemic clone O3:K6 (*1,5–8*). Pulsed-field gel electrophoresis confirmed that *V. parahaemolyticus* strains isolated from patients throughout the epidemic corresponded to pandemic clone O3:K6, as did the strains isolated in Chile from 2 smaller outbreaks in 1998 and 2004 (*1,8*). Strains of this clone also constituted the only pathogenic strain of *V. parahaemolyticus* detected in mussels and the only pathogenic strain that has persisted in shellfish throughout this period (*1,8*). The most common vectors in this outbreak were clams and mussels, not oysters, which reflect the pattern of consumption of shellfish in Chile during the summer (*1,8,9*).

This epidemic in 2005 points to the potential of *V. parahaemolyticus* O3:K6 to affect many susceptible persons if preventive measures are not taken and enforced quickly (*6–9*). Temperature and salinity have been reported as factors that influence concentrations of *V. parahaemolyticus* in the oceans (*2,5,7,8*). During summer 2005, seawater temperatures ≈19°C were recorded in several places along the coast of Region X where shellfish are collected (*8*). These temperatures were almost 3°C above 16°C, which is the average seawater temperature for January and February measured at the official weather station in Region X (http://www.shoa.cl/cendoc-jsp/index. jsp). Many of these areas with high seawater temperatures also have a wide tidal range, and shellfish in these locations are exposed to solar radiation in intertidal dry beds at ebb and low tides and can reach temperatures of 30°C. Elevated seawater temperatures and intertidal exposure to solar radiation can increase the concentration of *V. parahaemolyticus* in shellfish (and in the ocean), thereby increasing the risk for human infection after consumption.

Spread of *V. parahaemolyticus* toward the boreal and austral latitudes, as demonstrated by the course of this epidemic and the recent Alaskan outbreak, might be the result of climatic changes; a warming trend in seawater was noted in both events (*2,8*). Expansion of the *V. parahaemolyticus* O3:K6 pandemic clone may have also been facilitated by expansion of international trade

because bacteria could have been transported to Chile by ballast water from the Northern Hemisphere (*1,4,6*). As in previous outbreaks, shellfish responsible for this epidemic were harvested near international shipping lanes (*1,3,4,6*). The appearance of *V. parahaemolyticus* O3:K6 in Chile has thus converted the expansion of this strain into a real pandemic because this vibrio is now present in 5 continents. The persistence of *V. parahaemolyticus* in Region X might also have been encouraged by an expansion of finfish and shellfish aquaculture in that area. As in other parts of the world, expansion of these food industries could provide physical and nutritional substrates for vibrios to persist and propagate when growth is triggered by increases in temperature of seawater (*1,2,8*).

Emergence of *V. parahaemolyticus* in Region X has also coincided with expansion of harmful algal blooms in the same area. These blooms are triggered by increases in seawater temperature and degradation of the coastal environment (*9,10*). A connection has been established between algal blooms and the presence of *V. cholerae* and cholera epidemics in the Gulf of Bengal and off the coast of Peru at the start of the Latin America epidemic (*10*). Further research is necessary to ascertain whether persistence of *V. parahaemolyticus* and epidemics are related to algal blooms in this region of Chile.

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Toxoplasma gondii Prevalence, United States

To the Editor: We correct the prevalence of *Toxoplasma gondii* immunoglobulin (Ig) G antibodies published in Emerging Infectious Diseases in 2003 (*1*). An incorrect cutoff value in the computer program used to calculate seropositivity of anti–*T. gondii* IgG antibody resulted in some incorrect prevalence rates. We discovered this error when analyzing more recent National Health and Nutrition Examination Survey (NHANES) data.

The cutoff value for anti–*T. gondii* IgG seropositivity used in the prior publication (*1*) was >6 IU, which is the correct value for NHANES III 1988–1994 data (*2*) but not for NHANES 1999–2000 data. Because of a change by the *T. gondii* test kit manufacturer, the cutoff value for NHANES 1999–2000 seropositivity data was increased to ≥10 IU. This cutoff change from >6 to ≥10 IU does not cause a large difference in the *T. gondii* seroprevalence reported. In addition, it does not change the overall findings of the article or the overall relationship between NHANES III (1988–1994) and NHANES 1999–2000. However, it does produce a borderline change for 2 demographic subgroups (non-Hispanic white per-

Table. Comparison of *Toxoplasma gondii* IgG antibody seroprevalence, NHANES 1999–2000 and NHANES III (1988–1994)*†

	NHANES 1999–2000			NHANES III (1988–1994)		
	N‡	Prevalence	95% CI	N‡	Prevalence	95% CI
Total	4,234	14.3	12.3–16.2	11,132	16.0	14.5–17.5
Sex						
Male	2,013	15.2	12.4–18.0	5,144	16.7	14.8–18.6
Female	2,221	13.4	11.2–15.5	5,988	15.3	13.5–17.0
Race/ethnicity						
Non-Hispanic white	1,293	10.8	8.1–13.6	3,304	14.3	12.5–16.2
Non-Hispanic black	1,027	16.8	13.4–20.3	3,674	18.0	16.1–19.8
Mexican American	1,553	14.2	10.1–18.4	3,661	18.3	16.7–20.0
Age group, y						
12–19	2,105	7.3	4.7–10.0	2,749	8.5	6.4–10.5
20–29	735	11.9	9.5–14.4	3,100	15.2	12.1–18.3
30–39	726	17.0	12.9–21.2	2,960	16.1	14.6–17.6
40–49	668	18.7	15.0–22.3	2,323	22.2	19.4–25.0
Country of birth						
United States	3,211	10.5	8.3–12.8	8,606	14.1	12.7–15.5
Not United States	995	32.0	24.0–39.9	2,493	27.9	24.1–31.7

*IgG, immunoglobulin G; NHANES, National Health and Nutrition Examination Survey; CI, confidence interval.
†Sex, race/ethnicity, country of birth,and total values are age-adjusted to the 2000 census–estimated population using the 4 age categories shown.
‡Totals for the race/ethnicity and country-of-birth categories do not add up to the total number because an "other" race/ethnicity category was included in the totals but not shown in these categories or because persons did not provide a response to country-of-birth questions.

sons and persons born in the United States), for whom the difference from NHANES III to NHANES 1999–2000 data reached statistical significance at $p<0.05$ in the *t* test, but the 95% confidence intervals (CIs) for the prevalence estimates for these groups still overlapped between NHANES III and NHANES 1999–2000 (i.e., the *t* test is a less conservative measure of association than CI).

After this correction, the overall age-adjusted *T. gondii* antibody prevalence according to NHANES 1999–2000 data changed from 15.8% (95% CI 13.5%–18.1%) to 14.3% (95% CI 12.3%–16.2%). The Table shows the overall and stratified seroprevalence rates for NHANES 1999–2000 (corrected) compared with NHANES III (no corrections needed).

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Human Infection with *Schineria larvae*

To the Editor: Myiasis remains prevalent worldwide (*1,2*) and is infestation by larvae from fly species of live or dead tissues from vertebrate hosts (*1,3,4*). In humans, myiasis most frequently causes infection of exposed ulcers or traumatic wounds (*1*). In industrialized countries, most cases occur in tourists returning from tropical and subtropical areas (*5,6*), but autochthonous cases still exist. Several bacterial species have been associated with fly larvae, including species of the family *Enterobacteriaceae* and, more recently, *Schineria larvae* (*7,8*). *S. larvae*, a gram-negative bacterium, has been grown from larvae of *Wohlfahrtia magnifica*, a fly species responsible for myiasis (*7,8*). Its 16S rRNA gene has been amplified from a bacterial community of species involved in aerobic thermophilic bioprocesses (*9*). We report a case of *S. larvae* bacteremia in a man with wound myiasis.

On June 12, 2006, a 76-year-old man who had type 2 diabetes mellitus was examined at the emergency

department of Drôme North Hospitals, Romans, France, for inflammation of chronic cutaneous ulcers of both legs and intermittent fever. The patient lived alone in a rural, crowded area and had received no medical care. He reported owning sheep and denied any recent travel outside France. At the time of admission, his body temperature was 37.8°C, he was malodorous, and he had swelling and painful wounds on both legs. Maggots were found in the leg wounds, scrotum ulcers, and at the anal margin. A radiographic examination of both legs did not show any osteolytic lesion. Laboratory data were as follows: C-reactive protein 71 mg/L (reference value, <5 mg/L), leukocyte count 18.2×10^9/L (81% granulocytes), platelet and erythrocyte counts within normal limits, glucose 200 mg/dL, hemoglobin A1c level 13.8%. Serum protein electrophoresis showed hypoalbuminemia (20 g/L) and hypergammaglobulinemia (16.9 g/L) but no monoclonal gammopathy. Two blood samples and exudate from the leg wounds were collected for microbial cultures.

The patient was given a combination of amoxicillin-clavulanate and ofloxacin, and his cutaneous wounds were cleaned. After 24 h of incubation, leg wound cultures grew methicillin-susceptible *Staphylococcus aureus*, and the 2 blood cultures yielded the same *S. aureus* strain and an oxidative gram-negative bacterium (Romans strain). The Romans strain was found to be highly susceptible to β-lactams, aminoglycosides, chloramphenicol, cotrimoxazole, fluoroquinolones, and colistin. The antimicrobial drug therapy was changed to oxacillin and ofloxacin. The patient's condition improved rapidly, and his leg wounds healed progressively during hospitalization. He was discharged 27 days after initiation of antimicrobial therapy, which he continued for 7 more days. The patient was reexamined 1 month later and was considered cured.

The Romans strain was sent to the bacteriology laboratory at Grenoble University Hospital for identification. Using the API 20NE and Vitek II ID-GNB systems (bioMérieux, Marcy L'Etoile, France), we obtained, respectively, a "good" identification of *Psychrobacter phenylpyruvicus* and a "very good" identification of *Oligella ureolytica*. The nearly complete 16S rRNA gene of the Romans strain was amplified and sequenced; primers were Fd1 and rp2 (*10*) (GenBank accession no. EF120377). A BLAST search that used the network service of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) showed 99.6% identity between the determined gene sequence of 16S rRNA and that of *S. larvae* type strain L1/68^T (accession no. AJ252143). The 16S rRNA gene sequences from several species belonging to the Gamma Proteobacteria order were aligned by using the ClustalW package (www.ncbi.nlm.nih.gov/). A consensus phylogenetic tree was constructed from Jukes-Cantor evolutionary distances based on the neighbor-joining method using *Bacillus subtilis* as the root. The Romans strain clustered with previously characterized *S. larvae* strains (Figure).

Our report demonstrates that *S. larvae* can induce bacteremia in humans. Because *S. larvae* has been associated with only fly larvae, we can speculate that bacteremia originated from maggots infesting the patient's wounds. We cannot affirm that *W. magnifica* was the fly species involved because maggots were not saved for identification. Phenotypic identification of *S. larvae* is tedious (*7*). Because it is an asaccharolytic species, erroneous identification may occur. We can speculate that difficulties in phenotypic identification of this species may explain why it has not been previously reported as a potential human or animal pathogen.

In conclusion, myiasis remains an unresolved problem in animals and humans worldwide. Physicians and microbiologists should be aware of the possibility of *S. larvae* bacteremia and should specifically search for *S. larvae* infection in myiasis patients. Also, animal myiasis is still responsible for severe economic losses to the livestock industry worldwide. The occurrence of *S. larvae* bacteremia in animals with myiasis may explain the evolution from disease to death, especially in chronically infected animals.

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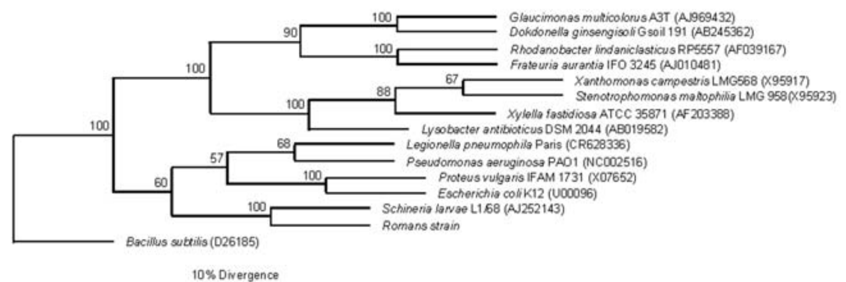

Figure. Phylogenetic position of the Romans strain within the Gamma Proteobacteria, determined by using Jukes-Cantor evolutionary distance calculation and neighbor-joining tree method. Bootstrap values (based on 500 steps) are indicated. GenBank accession no. of 16S rRNA gene of each bacterial species is indicated in parentheses.

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Isolation of *Schineria* sp. from a Man

To the Editor: *Schineria larvae* has been isolated from maggots of the fly *Wohlfahrtia magnifica* (*1*), which cause myiasis in animals and people in Eurasia and northern Africa. In industrialized nontropical countries, a range of species in the order Diptera cause facultative myiasis in patients with neglected wounds (*2*). Since the recent description of *S. larvae*, *Schineria* sp. isolates and clones have been detected in diverse environmental and animal sources, but in all cases a relation with flies could be established. We describe a case of bacteremia due to *Schineria* sp. in a human patient with myiasis.

In July 2005, a 39-year-old homeless man with medical history of polyneuropathy related to alcohol abuse was examined at Montpellier Hospital, Montpellier, France, and found to be in poor general health and to have an abnormal electrocardiogram, mild fever (38°C), metabolic disorders, increased C-reactive protein (254 mg/L) and fibrinogen (18.23 µmol/L), and a normal leukocyte count (7.8×10^9/L). Removal of his shoes and socks, which he had worn continuously for 2 months, showed advanced maceration of his feet (trench foot) with wounds invaded by maggots. The following organisms were found in wound samples: *Proteus mirabilis*, *Providentia stuartii*, group G *Streptococcus*, *Streptococcus* sp., and *Enterococcus* sp. Aerobic blood culture, after 2 days of incubation, was positive for a gram-negative rod, strain ADV1107. 05. Subculture on MacConkey medium showed positive reactions for oxidase, catalase, and gamma-glutamyltransferase. Positive malate reaction with API 20NE system (bioMérieux, Marcy l'Etoile, France) identified the strain as *Oligella urethralis,* whereas VITEK2 (bioMérieux) with ID-GN card failed to identify the strain. Disk diffusion assay showed the strain to be susceptible to β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, erythromycin, rifampin, and colistin but resistant to nalidixic acid and fosfomycin. Local therapy of debridement, bandaging, and sulfadiazin argentic, along with systemic antimicrobial therapy (ofloxacin 400 mg/day plus cefotaxime 6 g/day) for 2 weeks, led to clinical improvement and sterilization of the blood cultures. The local therapy was continued, and ofloxacin (400 mg/day) was prescribed for 15 days while the patient was in a rehabilitation center.

In October 2005, the patient was readmitted with the same symptoms. *P. mirabilis*, group A and group G streptococci, *Morganella* sp., *Bacteroides fragilis*, and *Candida albicans* were cultured from maggot-invaded wounds. Aerobic blood culture, after 1 day of incubation, was positive for strain ADV4155.05, which displayed the same phenotype as strain ADV1107.05 except for tetracycline resistance. Clinical improvement was observed after 2 weeks of the same local and systemic treatments as initially prescribed. The patient was transferred to an addiction care center and received oral antimicrobial therapy (ciprofloxacin 500 mg/day plus amoxicillin/clavulanic acid 3 g/day) for 20 days.

The 16S rDNA amplification and sequencing were performed with universal primers 27f and 1492r as described (*3*). The 1,414-bp sequences of the 2 isolates were identical and showed similarity level of 99.6% with the sequence of *Schineria* sp. 010793816 isolated from human urine (M. Vaneechoutte, pers. comm.) but only 98.3% with *S. larvae* L1/68^T 16S rDNA. This finding differed from the biochemical identification and underlined the usefulness of sequencing to precisely identify gram-negative bacilli that assimilate only a few

sugars. Phylogenetic analysis linked the 2 strains to the genus *Schineria* in the class Gamma Proteobacteria (Figure). However, whether the isolates are species *S. larvae* remains in doubt. Enterobacterial repetitive intergenic consensus–PCR and repetitive extragenic palindromic–PCR fingerprints (*6*) showed that the 2 strains were unrelated, thereby demonstrating that the second episode of bacteremia was a reinfection with a new strain and not a relapse.

The 16S rDNA of our isolates is most related to an uncultured bacterium found in swine waste (*7*), but its presence in such an environment could be correlated with fly larvae proliferation. Because of the lifestyle of *Schineria* sp., thinking that the strains in our patient originated from his wounds' maggots is reasonable. Unfortunately, the maggots were thrown away and could be neither analyzed nor identified. *Schineria* sp. could not be cultivated from the patient's wounds, perhaps because of its close association to larvae or to the abundant associated flora. Despite the presence of virulent bacteria in the wounds, *Schineria* sp. was the sole bacterium recovered from blood during the 2 independent episodes of bacteremia, which suggests its invasive potential. Invasiveness may be enhanced by the maggots' acting as a vector as they move through the necrotic tissues toward the bloodstream. Invasiveness also may be a specific characteristic of the bacterium; phylogenetic methods placed the genus *Schineria* in a subgroup that included human pathogens *Cardiobacterium*, *Francisella*, *Coxiella*, and *Legionella*. Indeed, all the phylogenetic methods tested excluded *Schineria* spp. of the family *Xanthomonadaceae* (Figure), which conflicts with current classification (*8*).

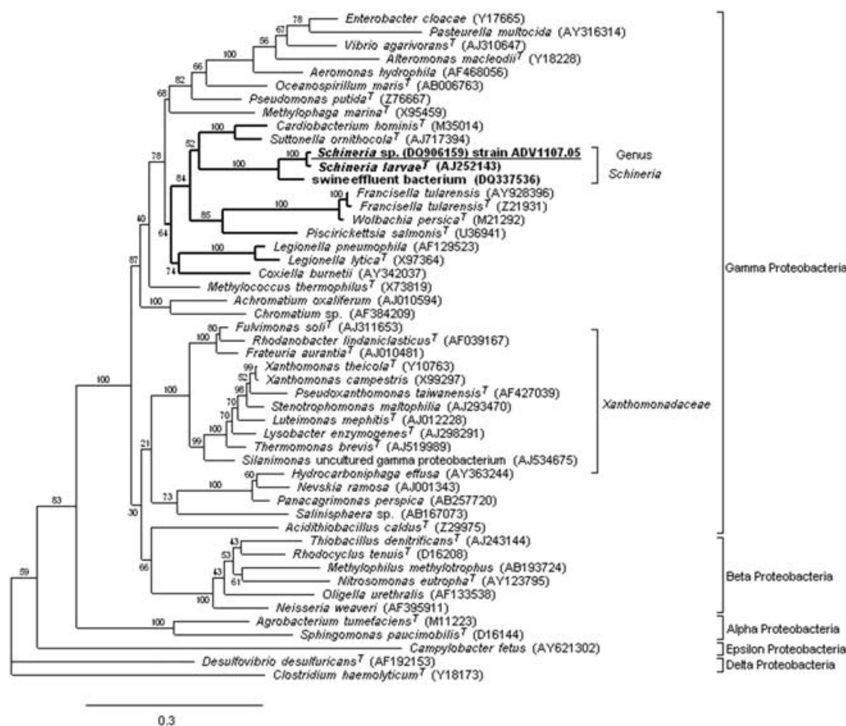

Figure. Maximum likelihood (ML) 16S rRNA gene phylogenetic tree showing the placement of the genus *Schineria* (**boldface**) and the isolate ADV1107.05 (underlined) in the phylum Proteobacteria. To reconstruct this tree, we used the strain ADV1107.05 sequence (DQ906159, 1441 bp) and 49 sequences selected from the GenBank database: 38 among the 15 orders of the Gamma Proteobacteria, 6 for the Beta Proteobacteria, 2 for the Alpha Proteobacteria, 1 for Delta Proteobacteria, 1 for Epsilon Proteobacteria and *Clostridium haemolyticum* (used as the outgroup organism). Accession nos. are in brackets. Alignment was performed with ClustalW 1.83 (*4*). ML phylogenetic analysis was performed by using PHYML v2.4.4 (*5*) with the general time-reversible plus gamma distribution plus invariable site (GTR + Γ + I) model found to be most appropriate according to Akaike information criteria. Bootstrap values given at the nodes are estimated with 100 replicates. The scale bar indicates 0.3 substitutions per nucleotide position. Strain ADV4155.05 sequence (DQ906158, 1414 bp) is not reported because it was identical to ADV1107.05. Trees were also obtained by distance methods (JC69, F84, and GTR models, and neighbor-joining), by parsimony, and by Bayesian inference. In all instances the genus *Schineria* branched out of the *Xanthomonadaceae* cluster.

No report has described bacteremia following myiasis with facultative parasites, but investigations of bacteria in reported myiasis cases have been conducted on cutaneous lesions and never on blood (*9*). Because of this association between maggots and risk for bacteremia, blood cultures should be performed for patients with myiasis and poor hygiene. Moreover, germ-free maggots bred for biosurgery use (*10*) should be checked, by molecular methods, for the absence of *Schineria* sp.

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Salmonella Kingabwa Infections and Lizard Contact, United States, 2005

To the Editor: Nontyphoidal *Salmonella* infections cause an estimated 1.4 million illnesses and 400 deaths in the United States annually (*1*). Among the >2,500 *Salmonella* serotypes, *Salmonella enterica* serotype Kingabwa rarely causes human illness. This serotype was first reported in a patient in the Belgian Congo in 1953 (*2*). From 1995 through 2004, only 30 human illnesses caused by *S.* Kingabwa were reported to the National *Salmonella* Surveillance System (*3*). No common source for *S.* Kingabwa human illnesses has been previously identified. We recently investigated an outbreak of *S.* Kingabwa infections associated with 2 lizard species: the water dragon and the bearded dragon.

Eighteen isolates of *S.* Kingabwa (antigenic formula: I 43:y:1,5) were received by PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, from 2001 through 2005. When digested with restriction enzyme *Xba*I and subtyped by pulsed-field gel electrophoreisis (PFGE), 13 isolates produced a single, indistinguishable pattern (KINX01.0001). Of these, 1 (8%) was isolated in 2001, 4 (31%) were isolated in 2002, 2 (15%) were isolated in 2004, and 6 (46%) were isolated in 2005. We defined a case as illness during 2005 caused by *S.* Kingabwa that matched pattern KINX01.0001 by PFGE. Of the 9 *S.* Kingabwa isolates received by PulseNet in 2005, 6 matched KINX01.0001. Antimicrobial drug susceptibility of 3 isolates was determined by the National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria at the Centers for Disease Control and Prevention (CDC), and the isolates were susceptible to each of 15 antimicrobial agents tested.

The 6 patients in the 2005 outbreak did not know each other and resided in 5 states: Maine (2 patients), Arizona, California, Idaho, and Ohio. Illness onset dates were in June, July, August, October (2 patients), and November 2005. Of the 6 patients, 4 (67%) were ≤1 year old (range <1–53 years), 4 were male, 2 were hospitalized, and none died.

Interviews with patients or their parents or guardians conducted during routine public health surveillance collected information on specific food items, water sources, restaurant venues, travel history, and animal contact. No common food or environmental source was identified. However, 4 (67%) of the 6 patients had known exposure to lizards: 3 water dragons (*Physignathus cocincinus*, Figure) and 1 bearded dragon (*Pogona* sp.). Of these 4 patients, 3 had >1 lizard in their own household as pets; the other patient was exposed to a lizard when visiting a family member. The 2 patients who did not recall lizard exposure might represent patients with background cases unrelated to lizards. Single cultures of the 2 lizards available for testing in February 2006 did not yield *S.* Kingabwa, which could mean that they did not carry this rare *Salmonella* serotype. However, this does not exclude lizards as the source of these illnesses because lizards intermittently shed salmonellae (*4*).

The lizards had been purchased from local pet shops and a traveling reptile show. Shipments of reptiles were mixed together at points of sale, and numerous distributors and importers were used, so determining the origin of individual reptiles was not feasible. However, water dragons and bearded dragons are imported into the United States from Asia and Australia and are rarely bred domestically.

Figure. Water dragon (*Physignathus cocincinus*). Three of the patients with *Salmonella* Kingabwa infections were exposed to this reptile species. Photo credit: Robert Lawton, rklawton@LawtonPhotos.com.

Two thirds of the patients in this outbreak had documented exposure to 1 of 2 lizard species, and half of the patients had pet lizards in their homes. In 2001, the estimated number of households with lizards was 545,000, which represents ≈0.5% of all American households (*5*). Using a standard binomial model, the probability of finding at least 3 of 6 persons chosen at random to be lizard owners is 0.000002. To our knowledge, this is the first investigation to identify a strong association between the rare serotype *S.* Kingabwa and lizards and the first instance of which we are aware that a serotype has been associated with a particular species of lizard dispersed in homes across the United States.

The association between reptile exposure and human salmonellosis is well-established (*6*–*8*). CDC has published recommendations for reducing the risk for infection from reptiles (www.cdc.gov/healthypets/animals/reptiles.htm); these include thoroughly washing hands with soap and water after handling reptiles or their cages and keeping reptiles out of food preparation areas. The young age of most patients in this outbreak supports the recommendation that reptiles should not be allowed in households with children <5 years of age.

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Influenza Virus Type A Serosurvey in Cats

To the Editor: Recent reports of cats positive for H5N1 type A influenza virus (*1*) raised the hypothesis that cats might have an epidemiologic role in this disease. Experimental findings seem to support this hypothesis. Experimentally infected cats might act as aberrant hosts (as do humans and other mammals), with symptoms and lesions developing and the virus subsequently spreading to other cats (*2*,*3*). The experimental conditions under which this occurs, however, can rarely be observed for domestic or wild cats. No spontaneous cases of transmission from cat to cat or cat to mammal have been reported, and scientifically validated reports about spontaneous disease in cats are rare

(*4–6*). Reports about cats with circulating influenza virus antibodies are even more rare and occur in unusual epidemiologic situations (*7*). The true susceptibility of cats to type A influenza viruses in field conditions thus remains to be elucidated.

Based on the assumption that partially susceptible animals should mount an antibody response, we investigated the possible presence of antibodies against the nucleocapsid protein A (NPA), a common antigen of type A influenza viruses, expressed by both avian and human strains (*8*), in feline serum samples stored at the University of Milan and collected from 1999 to 2005. Only samples for which complete information regarding the cat (owned vs. free-roaming) and its health status were included in the study. Cats were grouped as healthy or sick on the basis of clinical signs; a complete clinicopathologic screening that included routine hematologic tests, clinical biochemical tests, and serum protein electrophoresis; serologic tests for feline immunodeficiency virus and feline leukemia virus infection, which are known to induce immunosuppression; and information regarding the follow-up, including postmortem examination for dead animals. Specifically, 196 serum samples satisfied the inclusion criteria in terms of anamnestic information about the sampled cat and, according to the above-mentioned diagnostic approach, cats were grouped as reported in the Table. Owned cats were mainly living in the urban area of Milan. By contrast, approximately half of the free-roaming cats included came from rescue shelters from a rural area northwest of Milan. Sixty samples (58.8%) from owned cats and 51 samples (54.2%) from free-roaming cats were collected from September to February, when seasonal human influenza peaks.

Serologic tests for antibodies to type A influenza virus were performed with a competitive ELISA to detect NPA antibodies (*9*). Negative control serum from specific-pathogen-free chickens and positive control serum specimens from different species (avian, swine, and equine) were included in each plate to provide a full range of controls. Serum samples were considered positive when the absorbance value was reduced to at least 75% compared with 100% for negative control wells.

All cats were negative for type A influenza virus antibodies. The ELISA we used has been validated in several species, including humans (*9*). Antibodies against NPA are not a major response to influenza infection but likely would have been detected if infections of cat were widespread. Thus, although no positive feline serum samples were used as positive controls, the negative results are not likely false negatives. Indeed, the negative results of many cats included in the study (the free-roaming ones, especially those affected by severe illness, for which a natural cat/flu virus interaction is unrealistic) might be due to low exposure to the virus because avian influenza outbreaks never occurred in the sampling area included in this study (*10*). By contrast, many owned cats (those sampled during the winter) likely were exposed to human type A influenza viruses, since approximately half of the viruses responsible for human seasonal influenza isolated in Europe, especially in Italy, are type A (*8*,*10*). The close contact between pets and their owners probably exposed cats to these viruses; nevertheless, none of the pet cats seroconverted, even when they had severe systemic diseases or viral induced immunosuppression. Although the number of cats in this study might be statistically insufficient to show low seroprevalences, our results further support the hypothesis that, in field conditions, cats are most probably not susceptible to type A influenza viruses, especially to the human ones (e.g., H3N2, the most diffused among humans, which also did not induced symptoms or lesions in experimental conditions [*2*]) circulating in the "pre–cat flu era." In future studies, these results can be used to

Table. Survey of feline serum samples, collected from 1999 to 2005, for influenza A virus, Italy*

Clinical status	No.	Diagnosis	No.	Virus status	No.
Pet cats					
Nonsymptomatic	25			FIV	2
				FeLV	1
Symptomatic	77	FIP	36		
		Locally extensive inflammation	18	FIV	4
		Hematologic neoplasia	8	FIV	1
		Nonhematologic tumors	8		
		Systemic inflammatory or degenerative diseases	7	FIV	2
				FIV + FeLV	1
Free-roaming cats					
Nonsymptomatic	54			FIV	5
Symptomatic	40	Locally extensive inflammation	27	FIV	7
		Systemic inflammatory or degenerative diseases	7		
		FIP	6		

*FIV, feline immunodeficiency virus; FIP, feline infectious peritonitis; FeLV, feline leukemia virus.

compare the results of seroepidemiologic investigations among cats living in sites contaminated by avian viruses.

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Pneumonic Plague, Northern India, 2002

To the Editor: A small outbreak of primary pneumonic plague took place in the Shimla District of Himachal Pradesh State in northern India during February 2002. Sixteen cases of plague were reported with a case-fatality rate of 25% (4/16). The infection was confirmed to the molecular level with PCR and gene sequencing (*1*). A previous outbreak in this region during 1983 was suggestive of pneumonic plague (22 cases, 17 deaths) but was not confirmed. In India, the last laboratory-confirmed case of plague was reported in 1966 from Karnataka State (*2*).

The index patient for the 2002 outbreak lived in a hamlet in the Himalayas. He went hunting on January 28, 2002, in a nearby forest at a height of ≈500–600 m from his house. There, he killed a sick wild cat and skinned it. He returned home on February 2 and sought treatment for fever, chills, and headache. On February 4, breathlessness, chest pain, and hemoptysis developed; radiologic findings were suggestive of lobar pneumonia, and treatment with augmentin was begun. He died the next day. Subsequently, 13 of his relatives exhibited a similar illness, although 2 additional patients acquired infection in the hospital. The incubation period for those patients was 1–4 days, which is consistent with that of pneumonic plague.

A team of microbiologists, epidemiologists, and entomologists visited the village after 7 more cases were reported until February 12, 2002, followed by a team from the National Institute of Communicable Diseases (NICD), New Delhi. The following case-patient definition was used: a person who sought treatment for fever of rapid onset, chills, chest pain, breathlessness, headache, prostration, and hemoptysis. A total of 16 cases were reported from 3 hospitals in the area: a local civil hospital, the state medical college, and a regional tertiary care hospital. Clinical material collected from the case-patients and their contacts was initially processed in the laboratories of these hospitals. Wayson staining provided immediate presumptive diagnosis, and confirmatory tests were performed at NICD. Diagnosis of plague was confirmed for 10 (63%) of 16 patients (*1*).

NICD conducted the following laboratory tests on 2 suspected culture isolates, 2 sputum specimens, 1 lung autopsy material specimen, and 1 lung lavage sample (Table): 1) direct fluorescent antibody test for *Yersinia pestis*; 2) culture and bacteriophage lysis test; and 3) PCR and gene sequencing to detect *Y. pestis*–specific genes (*pla* and *F1*). All these tests confirmed that isolates were *Y. pestis* and met all the World Health Organization's recommended criteria (*2*).

Antibodies against F1 antigen of *Y. pestis* were detected by passive hemagglutination testing of paired serum samples. Although 5 patients showed a >4-fold rise, 1 patient showed a >4-fold fall in antibody titer. In contrast, samples from 6 patients were negative for *Y. pestis*, and no change was found in the titers from 1 patient. No serum sample was collected from the index patient; for the 2 other patients who died, 1 of the single serum samples became contaminated, and the other was positive for *Y. pestis* (*3*). Paired serum samples from the case-patients were collected on a single day 4 weeks apart during the visit of the NICD team, regardless of the duration of symptoms.

Antimicrobial drug sensitivity testing was carried out by the Kirby-Bauer disk diffusion method. All isolates were sensitive to doxycycline, tetracycline, chloramphenicol, streptomycin, ciprofloxacin, gentamicin, and amikacin but were resistant to penicillin.

Table. Epidemiologic characteristics and laboratory findings of patients with suspected cases of pneumonic plague, India, 2002 (*1*)*

Patient no.	Relation to index patient	Age, y/sex	Onset of symptoms	Outcome	Wayson staining	Blood c/s	Sputum c/s	Molecular results	Serologic results
1	Index patient	35/M	Feb 2	Died Feb 5	–	–	–	–	–
2	Wife	29/F	Feb 6	Died Feb 14	–	–	–	Confirmed	Single sample positive
3	Brother	26/M	Feb 7	Discharged Mar 8	–	*Yersinia pestis*	–	Confirmed	Negative
4	Sister	31/F	Feb 9	Died Feb 18	–	*Y. pestis*	–	Confirmed	–
5	Sister	27/F	Feb 12	Discharged Feb 25	–	–	–	–	Negative
6	Brother-in-law	35/M	Feb 12	Discharged Mar 8	–	–	–	–	Negative
7	Brother-in-law	35/M	Feb 10	Discharged Feb 21	–	–	–	–	Negative
8	Sister-in-law	38/F	Feb 9	Discharged Feb 25	–	–	–	–	>4-fold rise
9	Companion on hunting trip	36/M	Feb 10	Discharged Feb 28	–	–	–	–	Same titer in paired serum specimens
10	Sister-in-law	37/F	Feb 12	Discharged Mar 11	–	–	–	–	>4-fold rise
11	Relative of sister-in-law	40/F	Feb 12	Died Feb 14	Positive	*Y. pestis*	*Y. pestis*	Confirmed	Negative
12	Aunt	57/F	Feb 10	Discharged Mar 4	Positive	Negative	*Y. pestis*	Negative	>4-fold rise
13	Neighbor	46/F	Feb 11	Discharged Feb 27	–	–	–	–	>4-fold rise
14	Son of neighbor	22/M	Feb 8	Discharged Feb 27	–	–	–	–	>4-fold fall
15	Patient hospitalized with epilepsy	47/F	Feb 11	Discharged Feb 18	–	–	–	–	Negative
16	Husband/attendant of patient 15	60/M	Feb 11	Discharged Mar 11	Positive	*Y. pestis*	*Y. pestis*	–	>4-fold rise

*c/s, culture/sensitivity; –, sample not submitted; paired serum samples were tested 4 weeks apart.

No fleas or other ectoparasites were found on the 6 cats, 8 dogs, 6 cows, 4 calves or 2 trapped rodents in the village. One serum sample, with pooled blood from 3 dogs was negative for antibodies against F1 antigen. Before these infections occurred, a heavy snowfall in the region had reduced the activity of rodents and was unfavorable for the survival and multiplication of rat fleas. The snow also helped restrict the spread of the infection because of reduced movement of the local population (*1*).

Primary pneumonic plague is acquired by inhaling infective droplets from persons or animals and rarely by accidental aerosol exposure. *Y. pestis* is a category A agent of bioterrorism (*4*). It is not truly airborne; person-to-person transmission requires face-to-face exposure within 2 m of a coughing patient (*2*). During 1977–1998, in the western United States, 23 cases of cat-associated human plague were reported. Bites, scratches, or other contact with infectious material while handling infected cats resulted in 17 cases of bubonic plague, 1 case of primary septicemic plague, and 5 cases of primary pneumonic plague (*5*).

In our report, close and prolonged contact with the index patient while providing care (for example, wiping his face during hemoptysis, supporting him during a bout of coughing, taking him to the hospital in a vehicle) resulted in secondary cases. Because of the severe winter, poor ventilation in houses further helped the illness spread. All patients acquired infection before plague was suspected. Initially, patients were treated for community-acquired pneumonia, which delayed the proper treatment and led to deaths. A patient admitted for status epilepticus was infected by her attendant, who in turn, acquired infection from a terminally ill plague patient for whom he provided some care. The patient with epilepsy and her attendant shared a common room with the terminally ill wife of the index patient, which was small and poorly ventilated. Surprisingly, the relative of the index case-patient who had accompanied him to the forest survived the infection; whereas, the wife and sister of the index patient died. No spread to healthcare workers was noted.

When plague was suspected immediate preventive measures were taken, for example, fumigation of the index patient's residence and any vehicles used for transporting the patients; active surveillance and education; standard work precautions; chemoprophylaxis for patient contacts and paramedics; and isolation and treatment of patients (*1*). The transmission rate for primary pneumonic plague is relatively low compared with that of many other communicable diseases; the average number of secondary cases per primary case is 1.3, according to a study done by Gani and Leach (*6*).

The key element in the control of small outbreaks of primary pneumonic plague could be the intensity of disease surveillance system (*6*). As a result, the state government has estab-

lished a Plague Surveillance Unit in District Shimla of Himachal Pradesh in 2002 (*1*).

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Francisella tularensis, Portugal

To the Editor: Tularemia is a zoonosis caused by *Francisella tularensis*. Recently, tularemia has emerged in new locations, populations, and settings (*1*). After an outbreak in Spain in 1997 (*2*), it was expected that the disease would spread toward Portugal, a country with an extended area that borders the affected areas.

To evaluate the situation, a surveillance project, including a seroepidemiologic study in human populations and detection of the nucleic acid of *F. tularensis* in biologic samples, was initiated. The district of Bragança, in northern Portugal, was selected as study area for its vicinity with tularemia-endemic areas of Spain and because *Dermacentor reticulatus* and *Ixodes ricinus* are well documented there (*3*).

Biologic samples were collected from 74 persons living in the study region whose activities represented an increased risk for contact with ticks and wild mammals. Serum samples were available from 48 and were analyzed with the microagglutination test (*4*). From the other 26 persons, blood samples were collected and frozen. Because of hemolization these samples were only subjected to PCR. DNA was extracted by using the QIAamp blood kit (QIAGEN GmbH, Hilden, Germany).

A total of 110 ticks were collected from vegetation by using the flagging method (n = 5) or from vertebrate hosts (n = 105) and were identified at the species level and processed individually (*5*). Of these ticks, 79 were *D. reticulatus,* 1 *I. ricinus,* 15 *D. marginatus,* 11 *Rhipicephalus sanguineus,* and 4 *Hyalomma marginatum*.

A fragment of the gene encoding the 17-kDa lipoprotein (Tul4) of *F. tularensis* was amplified, as described previously (*6*). Resulting products were subjected to electrophoresis on 0.8% low-melt agarose gels (Roche Diagnostics GmbH, Mannheim, Germany), and the bands were purified by using the QIAquick gel extraction kit (QIAGEN GmbH) and sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 377 DNA sequencer. The sequences were aligned with other sequences from databases by using ClustalX (*7*). Pairwise distance matrices were determined by the Kimura 2-parameter method, with MEGA3 software. Phylogenetic trees were constructed with the neighbor-joining algorithm, by using bootstrap analysis with 500 replications for evaluation of the matrices' topology. Also, 1 region with short sequence tandem repeats (SSTR9) of *F. tularensis* was amplified as described previously (*8*). Resulting products were subjected to electrophoresis on a 3% MS-4 agarose gel (Pronadisa, Madrid, Spain).

The 48 samples studied by serology were negative. From the 26 human samples available for PCR, 1 was positive in the amplification of Tul4, which represented a prevalence rate of 3.8% of the samples studied. This result was confirmed by repeating both the DNA extraction and the PCR 3×. The amplification of SSTR9 in this case was negative. The difference between the results of the PCR methods targeting Tul4 and SSTR9 in the human sample is not surprising, since Tul4 PCR has higher sensitivity than that of SSTR9, which is a method not optimal for direct use in clinical samples (*8*,*9*). This positive result was for a 43-year-old man, a hunter who had frequent contact with lagomorphs. At the time of the collection, he was asymptomatic, but a history of a recent febrile illness was reported. He also stated that he had no recent occupational or recreational exposure in Spain. For the ticks, 1

female *D. reticulatus*, collected from a sheep, was positive in the amplification of Tul4 and SSTR9, with a prevalence rate of 1.3% for *D. reticulatus* and 0.9% considering the total of ticks studied.

Sequence analyses of the 2 positive samples obtained (PoHuF1 and PoTiF1 for human and tick, respectively) showed a homology of 100% with *F. tularensis*. A phylogenetic analysis based on the same sequence also grouped the samples from tick and human with *F. tularensis* subsp. *holarctica* live vaccine strain (Figure).

This study enabled the first report of *F. tularensis* DNA detection in humans and ticks from Portugal. When studying asymptomatic persons, the likelihood of obtaining a PCR-positive result in a sample from a bacteremic patient would be expected to be much lower than finding a seropositive result. However, we did obtain a PCR-positive result from a blood sample of a person, who, as mentioned before, had a previous tularemia-compatible febrile illness. Indeed, in a previous study of 203 blood donors performed in the same area during 2001–2002, a seroprevalence rate of 8.9% in asymptomatic persons was found (*10*).

The low prevalence rates we detected contribute to the assumption that this disease should have a low incidence in Portugal, as it does in Spain. The results of this study represent the first direct evidence of *F. tularensis* in Portugal. Further studies to confirm the occurrence of human cases are needed.

GenBank accession numbers for the sequences generated in this study are DQ459299 for PoHuF1, DQ459300 for PoTiF1, and DQ665890 for FT1.

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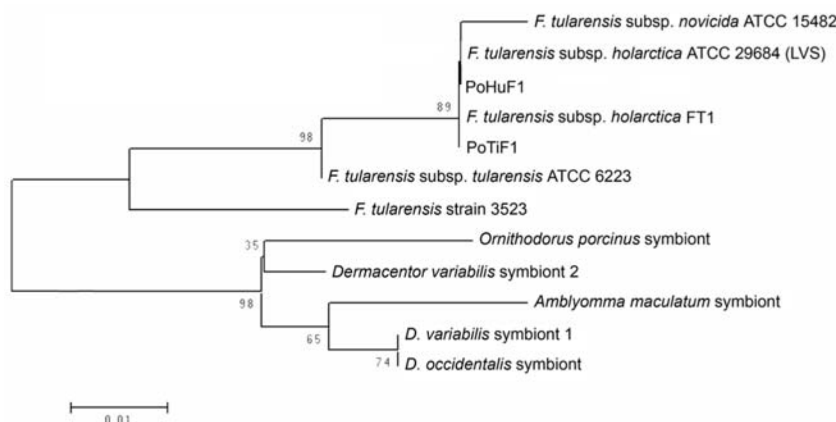

Figure. Neighbor-joining phylogenetic tree based on partial sequences of the gene coding the 17-kDa lipoprotein of *Francisella tularensis*.

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VIM-2–producing *Pseudomonas putida*, Buenos Aires

To the Editor: *Pseudomonas putida* infections (0.03% of isolates from the culture collection of inpatients, SIR Program 2003–2004, www.aam.org.ar) are mainly reported in immunocompromised patients, such as newborns, neutropenic patients, and cancer patients. They are usually susceptible to extended-spectrum cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems. However, isolates have been identified that produce acquired metallo-β-lactamases (MBLs) and are resistant to most β-lactams, including carbapenems.

Two multidrug-resistant *P. putida* isolates were obtained from clinical samples at the Sanatorio Mater Dei in Buenos Aires. One isolate was obtained in March 2005 from a urine specimen of a 76-year-old woman with a urinary tract infection who was using a urethral catheter. The second isolate was obtained in May 2005 from a tracheal aspirate of a 67-year-old man with nosocomial pneumonia.

Bacteria were identified by using conventional biochemical tests and the API 20NE System (API, bioMérieux, Lyon, France). Susceptibility tests were performed according to standard procedures. Both isolates were resistant to imipenem and meropenem (MICs >32 µg/mL) but were susceptible to amikacin and colistin. Susceptibility data are shown in the Table.

Screening for MBLs was performed by using a double-disk diffusion method. Disks containing 1 µmol EDTA (metal chelator) were placed on Mueller-Hinton agar plates containing the 2 isolates. Disks containing carbapenem were placed 15 mm from disks containing EDTA. An increase in the inhibition zone of the disk containing drug near the disk containing EDTA was observed for both isolates, which suggested the presence of MBLs.

PCR amplification of *imp* and *vim* genes was conducted by using primers based on conserved regions of the *imp* and *vim* genes (*bla*IMP-F: 5′-GAAGGCGTTTATGTTCATACTT-3′, *bla*IMP-R: 5′-GTTTGCCTTACCATATTTGGA-3′, *bla*VIMG-F: 5′-GGTGTTTGGTCGCATATC-3′, and *bla* VIMG-R 5′-TGGGCCATTCAGCCAGATC-3′) and heat-extracted DNA as template. Reactions were performed in a T-gradient instrument (Biometra, Göttingen, Germany) with the following reaction conditions: 1 cycle at 95°C for 5 min, 52°C for 15 min, and 72°C for 6 min, followed by 30 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final reaction at 72°C for 20 min. Amplified fragments were sequenced on both strands by using an ABI Prism DNA 3700 (Applied Biosystems, Foster City, CA, USA), and nucleotide sequences were compared by using BLAST (National Center for Biotechnology Information, Bethesda, MD, USA, www.ncbi.nlm.nih.gov/Tools/). Nucleotide sequences were completely homologous to the *vim-2* coding gene.

Two repetitive-element–based PCR (rep-PCR) assays (ERIC-PCR and REP-PCR) with primers REP-1 (5′-IGCGCCGICATCAGGC-3′), REP-2 (5′-CGTCTTATCAGGCCTAC-3′), ERIC-1 (5′-CACTTAGGGGTCCTCAATGTA-3′), and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used to characterize isolates. PCR conditions were 94°C for 2 min, 30 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 4 min, and a final reaction at 72°C for 7 min. Banding patterns were visually analyzed after electrophoresis of samples. Variations in band intensity were not considered to indicate genetic differences. Banding patterns obtained by REP-PCR and ERIC-PCR assays were identical in both isolates (data not shown).

Among the MBLs acquired by *P. putida*, IMP-1 was reported by Senda et al. in Japan in 1996 (*1*) and later reported in Taiwan and Japan (*2*). IMP-12 was the first IMP MBL described in *P. putida* in Europe (*3*). VIM-1 in *P. putida* was first reported in Europe (*4*), and VIM-2 in *P. putida* was first reported in Taiwan, Republic of Korea, Japan, and France (*5,6*). Our isolates were resistant to aztreonam (MIC 64 µg/mL). However, carbapenem-susceptible *P. putida* had low

Table. Antimicrobial drug susceptibility profiles of 2 bla_{VIM-2}-carrying *Pseudomonas putida* isolates, Argentina

Drug	MIC (µg/mL)	
	Isolate 1	Isolate 2
Imipenem	32	64
Meropenem	64	64
Ertapenem	128	128
Piperacillin	64	64
Piperacillin-tazobactam	32	32
Ceftazidime	128	128
Cefepime	32	32
Aztreonam	64	64
Amikacin	4	4
Gentamicin	16	16
Ciprofloxacin	>64	>64
Gatifloxacin	>64	>64
Levofloxacin	>64	>64
Moxifloxacin	>64	>64
Doxycycline	64	64
Colistin	2	2

levels of susceptibility because the MIC_{50} was only 1 dilution below the current breakpoint (*7,8*). Aztreonam resistance could not be transferred by conjugation between IMP-1–producing (aztreonam-resistant) *P. putida* and *P. aeruginosa* (*2*) and is not associated with a transposon carrying blaVIM-2 (*6*). No evidence of extended-spectrum β-lactamases was detected in our isolates by classic synergy assays with clavulanate plus aztreonam, ceftazidime, or cefotaxime. VIM-6–producing *P. putida* isolates from Singapore (*9*) were more resistant to aztreonam (MIC >128 μg/mL), ceftazidime, and cefepime (MIC >256 μg/mL).

Detection of bla_{VIM-2} in *Pseudomonas* in South America was initially reported by the SENTRY Antimicrobial Surveillance Program (*10*) and included 1 *P. fluorescens* isolate in Chile and 3 *P. aeruginosa* isolates in Venezuela. To the best of our knowledge, our report is the first of VIM-2 in *P. putida* in Latin America. VIM-2–producing *P. putida*, which were originally restricted to East Asia and only very recently found in France, may represent an emerging pathogen or function as reservoirs for resistance because of their widespread presence in the hospital environment.

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Multidrug-resistant *Acinetobacter baumannii*, Russia

To the Editor: During the past decade, nosocomial infections due to multidrug-resistant *Acinetobacter baumannii* have been described with increasing frequency, mostly in intensive care units (ICUs), resulting in therapeutic difficulties (*1*). The main mechanism for resistance to extended-spectrum cephalosporins in *A. baumannii* is attributed to the overexpression of chromosome-encoded cephalosporinases or to plasmid-encoded Ambler class A, B, and D β-lactamases (*2*). *A. baumannii* that produce PER-1 extended-spectrum β-lactamase (ESBL) are rarely isolated outside Turkey and remain susceptible to carbapenems (*3*). Here we describe what we believe is the first ESBL-producing *A. baumannii* isolate resistant to carbapenems and the first characterization of a PER-1 *A. baumannii* isolate from Russia, further supporting the emergence and dissemination of PER-1 *A. baumannii* strains in eastern Europe and outside Turkey (*3,4*).

On April 17, 2005, a 79-year-old-man was hospitalized in the cardiology ward of a private hospital in Moscow, Russia, with cardiac arrhythmia and a pulmonary infarction subsequent to a pulmonary embolism. After 1 week, he was transferred to the ICU for multiple organ failure related to a nosocomial infection caused by an *A. baumannii* strain

susceptible to several antimicrobial drugs, including imipenem (with positive lung aspiration and blood cultures). He received imipenem and amikacin at high doses. On May 5, 2005, he was transferred to the internal medicine ward of the American Hospital of Paris, Neuilly-sur-Seine, France. On the day of admission, bacterial cultures taken from sputum showed a multidrug-resistant *A. baumannii* MOS-1 strain, susceptible only to colistin and rifampin. The patient received intravenous and aerosolized colistin 3 times a day plus rifampin at 1,200 mg/d so that he could return to Russia. Rapid identification of *A. baumannii* MOS-1, increased awareness as a result of a French national alert signaling the emergence of ESBL VEB-1–producing *A. baumannii* (*5*), and implementation of strict barrier precautions prevented dissemination of this strain. No other multidrug-resistant *A. baumannii* isolate with a similar resistance profile has been isolated in the hospital before, during, or after this period.

A. baumannii MOS-1 was susceptible to colistin and rifampin only, and no synergy image could be observed between clavulanic acid and cefepime or ceftazidime discs on a routine antibiogram performed by the disc diffusion method. Only the use of cloxacillin-containing Mueller-Hinton agar plates (200 μg/mL) to inhibit the activity of the naturally occurring cephalosporinase (AmpC) allowed detection of a synergy image, signature of the presence of an ESBL (*5*). MICs for imipenem, determined by the agar dilution method (*6*), were >64 μg/mL. Clavulanic acid addition (2 μg/mL) decreased ticarcillin MIC from >512 to 256 μg/mL and ceftazidime MIC from 512 to 128 μg/mL but did not affect MIC of imipenem.

Genes coding for ESBLs and for class B and D carbapenemases were sought by PCR as previously described (*4,5*). Primers used for detection of TEM and PER β-lactamases gave 894-bp and 825-bp PCR products, respectively (*4*). Sequence analysis showed identity with bla_{TEM-1} and bla_{PER-1} genes (*4*). Results of isoelectric focusing showed 3 isoelectric point values (5.3 for PER-1, 5.4 for TEM-1, and >8.5 for AmpC) in *A. baumannii* MOS-1 (*4*). A crude β-lactamase extract of that isolate had no significant imipenem hydrolysis activity, which suggests that the carbapenem resistance may have emerged through a nonenzymatic mechanism such as mutations in porins (*7*). Bla_{PER-1} gene in *A. baumannii* MOS-1 was located on a composite transposon, Tn*1213*, identical to that characterized by Poirel et al. (*8*). Attempts to demonstrate plasmids or transfer genes encoding TEM-1 or PER-1 failed (data not shown), which suggests that the genes were chromosomally encoded. *A. baumannii* MOS-1 was not clonally related to well-characterized PER-1 *A. baumannii* strains from Turkey, France, and Belgium (*4,9*) (Figure), further supporting genetic heterogeneity of PER-1 *A. baumannii* isolates, even though the immediate genetic environment of bla_{PER-1} gene was similar (*8*).

The emergence and spread of ESBL-producing *A. baumannii* strains are of concern because they will increase carbapenem use, thus raising the risk for emergence of carbapenem-resistant isolates. *A. baumannii* MOS-1 is, we believe, the first description of an ESBL-producing *A.*

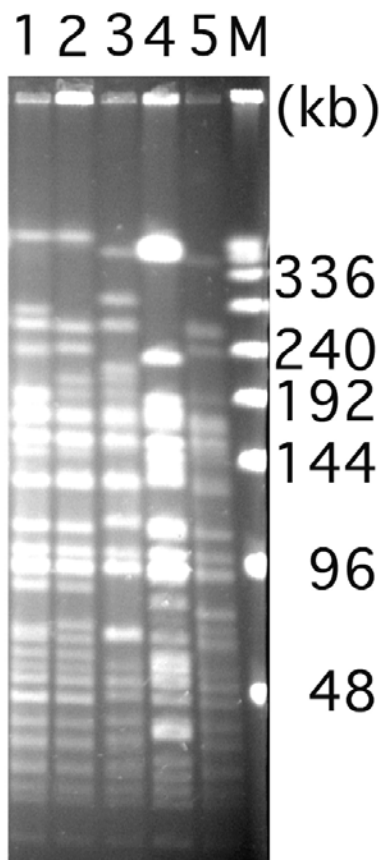

Figure. Pulsed-field gel electrophoresis of *Apa*I restricted analysis of *Acinetobacter baumannii* isolates. Lane 1, *A. baumannii* AMA-1 from France (*4*); lanes 2 and 3, *A. baumannii* IST-1 and *A. baumannii* IST-2 from Turkey (*4*); lane 4, *A. baumannii* isolate from Belgium (*4*); lane 5, *A. baumannii* MOS-1 from Russia (current study); lane M, bacteriophage lambda molecular mass ladder. Numbers on the right side of the figure represent the sizes in kb.

baumannii isolate also being resistant to carbapenems. This resistance was likely acquired in vivo under imipenem treatment, but the susceptible strain was not available for strain comparison. This is also the first description of a PER-1 *A. baumannii* isolate from Russia, a country from which little epidemiologic data on antimicrobial drug resistance are available, except for the emergence of ESBLs of CTX-M type in *Enterobacteriaceae* (*10*).

This study highlights the importance of international patient transfer in the spread of antimicrobial drug resistance, thus emphasizing the need for hospitals to isolate and screen for multidrug-resistant pathogens in all patients admitted to hospitals from foreign countries. This is particularly critical when the foreign country is known for a high prevalence of multidrug-resistant bacteria or when no antimicrobial drug resistance data are available.

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Human Infection with *Rickettsia* sp. related to *R. japonica*, Thailand

To the Editor: Although rickettsioses caused by scrub typhus and typhus group rickettsiae are well recognized in Thailand, few spotted-fever group rickettsiae (SFGR), including *Rickettsia honei* TT118 and *R. felis,* have been documented to be associated with human illnesses (*1*,*2*). We report a case of human infection with an SFGR species closely related to *R. japonica* in Thailand.

In January 2005, a 36-year-old man with prolonged fever, pneumonia, and septic shock was transferred from a private hospital to Phramongkutklao Army Hospital in Bangkok. Two weeks before the onset of fever, the patient had camped at Khao Yai National Park, ≈175 km northeast of Bangkok. The park is a popular location for tourists and the largest national park declared as a natural wildlife reserve area. The patient reported the presence of wild deer around the camping area but did not recall being bitten by an arthropod. Ten days before hospitalization, he developed flulike symptoms, fever, and sore throat. Six days later, he noted petechiae on his lower extremities, and his condition worsened. At the time of hospital admission, the patient had fever of 38.6°C, tachycardia, dyspnea, hypotension, nausea, vomiting, generalized maculopapular rash, and subconjunctival hemorrhage. Laboratory investigation showed thrombocytopenia (platelets 64,000/mm^3), leukocytosis (14,000/mm^3), and elevated levels of serum hepatic enzymes (aspartate aminotransferase 287 IU/L [reference 5–50 IU/L]; alanine aminotransferase 186 IU/L [reference 5–40 IU/L]). Chest radiograph showed interstitial pneumonitis. Serum antibody test results were negative for leptospira and

dengue virus; blood smear was negative for malaria.

Samples of the patient's whole blood were collected in EDTA on days 10, 18, 20, and 25 after illness onset, and each sample was sent at the time of collection to the Armed Forces Research Institute of Medical Sciences, Bangkok, to be investigated for rickettsial infection. Plasma was separated and tested for scrub typhus, typhus group, and SFGR-specific immunoglobulin M (IgM) and IgG by immunofluorescence assay by using *Orientia tsutsugamushi* Karp-Kato-Gilliam strains and *R. typhi* Wilmington and *R. honei* TT118 whole cell antigens. No antibodies to rickettsiae were detected in the initial sample. On day 18, only antibodies against *R. honei* TT118 antigen were detected at a low titer, 50 for IgM and 200 for IgG, while antibodies to scrub typhus and typhus group rickettsiae remained negative (titers <50). Antibody level was unchanged on days 20 and 25.

At the time of admission, the patient began receiving 2 g of intravenous ceftriaxone and 200 mg of oral doxycycline daily. Three days later, treatment with doxycycline was stopped because the initial serologic results for rickettsia were negative. However, doxycycline was resumed on day 21, after antibodies to Thai tick typhus agent were detected in a second specimen. Within 3 days, the patient was afebrile and asymptomatic. He was discharged from the hospital and continued oral doxycycline for an additional 7 days. At 2-week follow up, he had completely recovered.

To identify which SFGR was responsible for the patient's illness, we used molecular approaches. We extracted DNA from the patient's blood specimens by using QIAamp Mini blood kit (QIAGEN, Valencia, CA, USA) and subjected it to duplex nested–PCR assays targeting a 343-bp fragment of the rickettsial genus–specific 17-kDa antigen gene (*3*) and a 690 bp-portion of the *Orientia* 56-kDa antigen gene (*4*). An appropriate control panel included DNA from a reference sample of human blood, *Coxiella* sp., and *Leptospira interrogans*. Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) enzyme mixture was used in PCR. By resolution on agarose gel, a PCR fragment of the expected size for the 17-kDa antigen gene was observed from the day-10 sample but not from the control samples. *Alu*I restriction pattern of amplified 17-kDa fragment was similar to that of SFGR. Additional rickettsial gene fragments, 630 nt-*ompA* (nt 70–701) and 945 nt-*gltA* (RpCS.193F-5′-GTAGGGTATCTGCGGAAGCC-3′, RpCS.1143R-5′-GAGCGAGAGCTTCAAGTTCTATTGC-3′), were also amplified from the day-10 specimen. All amplicons were excised from agarose gels, purified by QIAEX II Gel Extraction Kit (QIAGEN), and then sequenced. BLAST analysis of 17-kDa antigen gene (GenBank accession no. DQ909071), *gltA* (DQ909073), and *ompA* (DQ909072) segments obtained from this patient showed 99% identity to corresponding genes of *R. japonica*. Phylogenetic analysis of these 3 genes indicates that the *Rickettsia* sp. from this patient is closely related and clustered within the same clade of *R. japonica* (Figure). Isolation of this rickettsial agent from the patient's blood by animal inoculation and by cell culture methods is ongoing.

Persons visiting Khao Yai National Park are at risk for rickettsioses, particularly SFGR. Vectors for SFGR have been found in this area (*5*). The clinical and molecular findings in this case add to the accumulating data on the emerging rickettsial agents and their geographic distribution in Thailand.

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Figure. Phylogenetic relationships between *Rickettsia* sp. and rickettsial genes amplified from the patient (PMK 94) inferred from comparison with the rickettsial 17-kDa antigen gene, *gltA*, and *ompA* sequences by the neighbor-joining method. Bootstrap values of 1,000 replicates are indicated.

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Global Mapping of Infectious Diseases: Methods, Examples, and Emerging Applications

Simon I. Hay, Alastair Graham, and David J. Rogers, editors

Academic Press, London, United Kingdom, 2007
ISBN-10: 012-031762-1, ISBN-13: 978-0120317622
Pages: 399 + 34 plate pages and DVD; Price: US $169.95

In 1849, John Snow pioneered the application of mapping to public health by producing a map depicting locations of cholera cases around the Broad Street pump in London (*1*). Thus, any book describing recent advances in mapping infectious disease is potentially of interest to practicing public health officials. The topics covered in the 11 chapters in this book range from the very technical, such as descriptions of satellite-obtained environmental data, to the geographic and climatic distribution of dengue and yellow fever, plotted in risk maps for those diseases. However, most public health officials will likely find this book overly specialized, particularly the first 4 chapters. These contain detailed descriptions of the technical aspects of measuring, modeling, and analyzing climatic and geospatial data. Public health officials are likely to appreciate the chapters describing the distribution and factors potentially affecting further spread of disease. These chapters present data on the distribution of malaria, dengue, yellow fever, soil-transmitted helminths, and tickborne diseases, and information on how global transport systems and climate changes could alter the distribution of diseases.

Some of the authors have fallen prey to the rather regrettable tendency to address "hot topics," such as bioterrorism and the spread of pandemics, even if such topics are somewhat outside the domain of the rest of the book. The result is that in 1 chapter there are 1 or 2 pages in which the authors briefly, and mostly uncritically, review some of the most well-known literature on these topics. Readers would have been better served had the authors of that chapter focused on vectorborne diseases, for which they are justly well known. Furthermore, even in chapters focusing on practical aspects of disease distribution, many sections contain detailed descriptions of methods that most public health officials are likely to want to skip over. Placed at the back of the book are the color plates of maps (the central feature of such a book). This placement is annoying because it makes it difficult to quickly find the figures being described in a given chapter. Overall, this book is more likely to appeal to the specialist, who will find it a useful addition to a technical library, while most public health officials will likely be better served in seeking a book containing more general descriptions of mapping infectious diseases.

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Handbook of Helminthiasis for Public Health

D.W.T. Crompton and Lorenzo Savioli

Taylor & Francis CRC Press, London, United Kingdom, 2006
ISBN: 0849333288
Pages: 362; Price: US $139.95

"I'll never forget the day I read a book." Daniel Pinkwater begins his book commentaries for National Public Radio with this Durante-Barnett tune. This came to mind when I realized I have never before read a textbook on parasitology from start to finish. I usually rely on a reference to refresh my memory about a detail of this or that life cycle or to reinforce a grant application with the number of those afflicted across the globe. Handbook of Helminthiasis for Public Health by D.W.T. Crompton and Lorenzo Savioli, however, bears reading straight through. What makes a complete reading so well worthwhile and also sets the book apart from most on either public health or parasitology is its successful marriage of these 2 points of view. This book covers the most common helminths by focusing on the parts of their biology that are most relevant to public health. Methods for rapid inexpensive surveys, international health initiatives, the economics of boreholes, and latrine design are discussed next to metacercarial development.

The focus on helminths also sets this book apart. In the first place, helminths are naturally engaging because of their ability to integrate their own complex biology with human biology and culture. Second, a significant re-evaluation is under way regarding the influence of parasitic worm infection on health. This refutes the perception in some circles that

most helminthic infections are less harmful than the common cold. The first chapter of this book presents the more complete recent analyses that give a broader view of the true consequences for health.

Handbook of Helminthiasis for Public Health is structured in 3 parts: Human Health and Helminth Infection, Helminthology, and Control Interventions. Part 1 establishes the context for understanding the effects of helminth infections, the economics of these infections, and the resources required to control these infections. These are important aspects of infection because poverty, sanitation, national politics, and economic influences all contribute to the spread of helminthiasis but are often the most neglected aspects of helminth epidemiology. The book also recognizes the importance of urban settings for these infections, which is appropriate for a year during which, many projections say, the global human population becomes predominantly urban. The first chapter returns to specifics by ending with a comprehensive list of helminths that have been found to infect humans, a list that extends for 10 pages.

Part 2, Helminthology, is organized in part by phylogeny and in part by common transmission characteristics; thus, cestodes and schistosomes are presented in separate chapters while the soil-transmitted and foodborne helminths are grouped together. This section is devoid of the usual life-cycle diagrams but does a good job of describing the biology most relevant to public health surveillance and control measures. Useful diagrams are provided of parasite morphology and a large number of tables and charts about age and geographic distribution, illness rates, response to control campaigns, and drug dosages.

In part 3, Control Interventions, problems such as drug resistance, health education, assessment of health awareness in a population, the structure of latrines, and the place of helminth control in the international political arena are addressed and made concrete by reference to many specific infections covered in the preceding section. The book also offers 4 appendices: a glossary, a list of journals about helminthology and control intervention, detection methods in helminthology for stool samples, and a model framework for control of foodborne trematodes. A list of the 63 tables is provided, and the table of contents is well organized.

No book is without its faults; there are a few frank errors. Death from *Schistosoma mansoni* and *S. japonicum* is primarily a result of portal hypertension and esophageal bleeding with preservation of hepatic function, rather than hepatic failure as indicated in the book. The immunology references are old, except where current vaccine development is discussed. The book also fails at times from overgeneralization. Cysticercosis is diagnosed in the United States and Canada; it is just rarely transmitted in these countries. More abbreviations (e.g., U5MR, FBT, MDG, FECRT, KAP) should be included in the glossary or index. An abundance of useful figures and tables are provided, but more maps would be useful in Part 2 of the book.

Handbook of Helminthiasis for Public Health is very readable. The core audience, according to the jacket cover, is readers who have a public health background and workers involved in control programs. However, the book should interest parasitologists and even basic researchers who wish to understand the full context of helminth biology.

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Utagawa Hiroshige (1797–1858). Plum Garden at Kameido (1857) (detail). From the series One Hundred Views of Edo. Color woodblock print (37.7 cm × 26.5 cm). Honolulu Academy of Arts, Hawaii, USA, Gift of James A. Michener, 1991 (24103)

The Darkest Place Is under the Light House

– Japanese proverb

Polyxeni Potter*

"Hiroshige's death cannot be too much deplored," read the note next to his name on a list of the famous who perished in the 1858 cholera epidemic (*1*). Utagawa Hiroshige himself, before his death at age 62, is said to have written in verse, "I leave my brush at Azuma and go on the journey to the Holy West to view the famous scenery there" (*2*). Metaphorical meaning and questionable authenticity aside, when interpreted in Western terms, the farewell becomes prophetic since the artist, long overlooked in his native Japan, was discovered and brought to light in the West.

The low cost of production made woodblock prints, Hiroshige's main medium, widely accessible—a print sold for the price of a bowl of noodles (*3*). The subjects, eloquent snapshots of the provinces, remained unknown to the cultured class in Japan, until his popularity rose in Europe and the United States. How the prints first found their way to Western artists, who appreciated and imitated them is legend. James McNeill Whistler (1834–1903), who was very influential in introducing Japanese art to England, might have seen his first Hiroshige at a rundown Chinese tea house near London Bridge or on the wrapper on a pound of tea (*2*).

Vincent van Gogh so admired Japanese prints that he copied some, among them Plum Garden at Kameido, on this issue's cover. "I envy the Japanese artists," he wrote, "for the incredible neat clarity which all their works have" (*4*). Hiroshige's influence was greatest on the impressionists (Claude Monet, Pierre August Renoir, Mary Cassatt) and postimpressionists (Paul Cézanne, Henri de Toulouse-Lautrec, Paul Gauguin). His landscapes, "simple as breathing," "easy as buttoning one's waist-coat," influenced Western painting away from representation toward light, color, and emotion (*4*).

Hiroshige's biography is pieced together from anecdotes as there are few authentic records of his life. He was born Andō Tokutarō in Edo, present-day Tokyo, a precocious child with an eye for the unusual and the detailed. Orphaned in his early teens, he managed to receive art instruction, first from amateur painter Okajima Rinsai, a friend and neighbor, and at age 15, from the art establishment. In the short course of a year, he was admitted to the Utagawa School as designer of color prints. According to Japanese custom, an accomplished apprentice was given a name that generally incorporated part of the master's name. Apprentice of Toyo**hiro**, Andō was named **Hiro**shige. The diploma, in Toyohiro's own writing, read Utagawa Hiroshige. The artist later also used the names Ichiryūsai and Ryusai.

Hiroshige's father, Gen'emon Andō, a hereditary retainer of the shōgun, was a fireman, and when he died, Hiroshige kept his modest post, eking out a living until he could relinquish the post to another member of the family and devote himself to art. Then he set off to see and draw the provinces. A wanderer and bon vivant, he lingered on the road, pausing to

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observe and sketch, mixing with country folk, dining at local eateries. His discriminating tastes and humorous accounts of people and places, recorded in his diaries, were largely lost in the 1896 earthquake, but his absorption with natural beauty, which he held "exquisite, beyond capability of describing with brush" (*1*), survived in his landscapes.

Legend has it that seeing the work of master printmaker Katsushika Hokusai (1760–1849) inspired Hiroshige to become a *ukiyo-e* artist, to create images of the "floating world." These images, drawn from the transient world of actors and others in Edo's theater district, expanded to encompass scenes of nature and eventually the life of the common people: "Living only for the moment, turning our full attention to the pleasures of the moon, the snow, the cherry blossoms, and the maple leaves; singing songs, drinking wine, diverting ourselves in just floating, floating…like a gourd floating along with the river current; this is the floating world" (*5*).

Hiroshige formed his own interpretation of the floating world, which he summed up in the inscription on The Hundred Views of Mount Fuji: "…the old man [Hokusai] had drawn grasses, trees, birds, animals, and other things in his usual talented brush….his work focused upon making things interesting….I simply reproduce sketches of what I had seen before my eyes" (*6*). But true to life as Hiroshige was, he captured the pathos, not the details, favoring white spaces, flattened forms, organic scenes in brilliant color. Like van Gogh, he tried to draw not "a *hand*, but the gesture, not a mathematically correct head, but the general expression….In short, *life*" (*7*).

Hiroshige was very prolific. He created thousands of images of his beloved Edo and surrounding provinces—bridges, roads, temples—under all manner of weather, day and night. He named them all personally, as he also had a talent for verse, which he dispersed generously and with wit. His prints were copied and reprinted freely.

Plum Garden at Kameido, in a series created just a year before his death, shows the master's unparalleled facility with Japanese topography. This close-up of a plum tree, thick trunk framing the famed gardens, young sprouts shooting out the edge, blossoms placed seductively against a sky flushed red, is an essay in perspective. The tree trunk, a main attraction, is what we are less likely to see, its spare immediacy too close to the lens. Hiroshige wants us to venture past it into the spring extravaganza beyond.

Perspective, a Western influence, graces many of Hiroshige's later works, which having excelled in capturing life as he saw it, now explored its depth. Master of illusion, he brought what he saw into focus, knowing full well that the scene was but a composition of life elements, not life itself.

Hiroshige's dilemma with perspective is not unlike the scientist's, who also draws selected objects closer for a better look. But magnification and clarity are no guarantee of true perspective in the laboratory any more than in art. Out in the open, under the proverbial lighthouse, lies always the risk of missing the obvious in close and plain view. And despite the science frame, zoonotic and vector-borne interactions and connections within the natural environment, like the strolling visitors in the garden at Kameido, can easily be overlooked.

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Upcoming Issue

Look in the May issue for the following topics:

Options and Associated Challenges in Responding to Malaria Epidemics in Africa

Respirator Usage by Public in Post-Hurricane New Orleans and Protection from Airborne Hazards

Bubonic Plague in Lushoto district, Tanzania, and Human Flea *Pulex irritans*

Geographic Trends in Influenza-related Deaths during Pandemic and Epidemic Seasons, 1969–2001, Italy

Apoptosis and Pathogenesis of Avian Influenza A (H5N1) Virus in Humans

Genome Analysis Linkage of Recent European and African Influenza A (H5N1) Viruses

Pet Rodents and Fatal Lymphocytic Choriomeningitis in Transplant Patients

International Spread of Multidrug-resistant *Salmonella* Schwarzengrund in Food Products

Rudolf Virchow and the Recognition of Alveolar Echinococcosis

Disseminated *Acanthamoeba lenticulata* Acanthamebiasis in Heart Transplant Recipient

Leptospirosis in Urbanized Wild Boars, Berlin, Germany

Mycobacterium liflandii Infection in European Laboratory Colony of *Silurana tropicalis*

Complete list of articles in the May issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 14–17, 2007
Society for Healthcare Epidemiology (SHEA) Annual Scientific Meeting
Baltimore, MD, USA
Contact: 703-684-1006
http://www.shea-online.org

April 30–May 2, 2007
10th Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org

May 31–June 1, 2007
2nd International Conference on Avian Influenza in Humans: Recent Developments and Perspectives
Institut Pasteur
Paris, France
http://www.isanh.com/avian-influenza

June 24–28, 2007
Association for Professionals in Infection Control and Epidemiology 34th Annual Conference and International Meeting
San Jose, CA, USA
Contact: 202-454-2638
http://www.apic.org

July 16–18, 2007
Public Health Congress
Mandarin Oriental
Washington, DC, USA
http://www.publichealthcongress.com

July 29–August 1, 2007
17th ISSTDR Meeting | 10th IUSTI World Congress
Seattle, WA, USA
http://www.isstdr.org

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@ cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

* ★ New infections resulting from changes or evolution of existing organisms.
* ★ Known infections spreading to new geographic areas or populations.
* ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
* ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

* ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
* ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
* ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

* ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
* ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
* ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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March 2007

Extensively Drug-resistant TB

Courtesy of Oglethorpe University Museum, Atlanta, Georgia, USA. Photographed by James Gathany

EMERGING
INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete 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.