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March 2009

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Liubov Popova (1889–1924)
The Traveler (1915)
Oil on canvas (142.2 cm × 105.4 cm)
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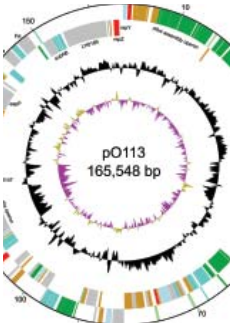

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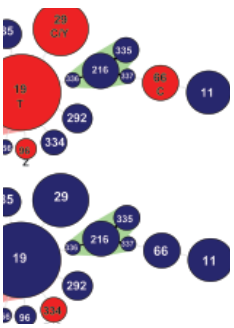

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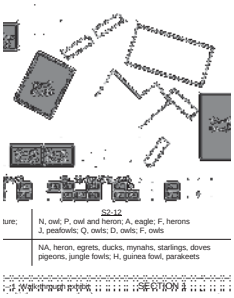

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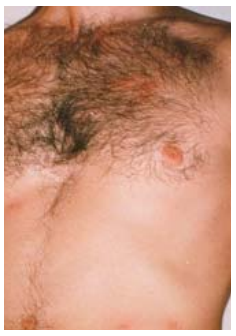

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Meeting the Challenge of Influenza Pandemic Preparedness in Developing Countries

David S. Fedson

Developing countries face unique difficulties preparing for an influenza pandemic. Our current top-down approach will not provide these countries with adequate supplies of vaccines and antiviral agents. Consequently, they will have to use a bottom-up approach based on inexpensive generic agents that either modify the host response to influenza virus or act as antiviral agents. Several of these agents have shown promise, and many are currently produced in developing countries. Investigators must primarily identify agents for managing infection in populations and not simply seek explanations for how they work. They must determine in which countries these agents are produced and define patterns of distribution and costs. Because prepandemic research cannot establish whether these agents will be effective in a pandemic, randomized controlled trials must begin immediately after a new pandemic virus has emerged. Without this research, industrialized and developing countries could face an unprecedented health crisis.

More than a decade ago, the first human cases of disease caused by avian influenza virus A (H5N1) appeared in Hong Kong Special Administrative Region, People's Republic of China. Six years ago, influenza virus A (H5N1) reemerged to cause highly lethal human disease in Southeast Asia. Health officials are concerned that these cases could be the harbinger of the next influenza pandemic. As a result, virtually all industrialized countries and many developing countries have mounted extensive pandemic preparedness efforts. However, as pointed out recently by Oshitani et al., industrialized countries face "unique and difficult issues, which make preparing for a pandemic more challenging" (*1*).

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Why a Top-Down Approach to Confronting the Next Pandemic Will Not Work

If a pandemic form of influenza virus A (H5N1) emerges within the next few years, all countries will have to depend almost entirely on egg-derived inactivated adjuvanted influenza vaccines. For developing countries, this approach will not succeed. Estimates show that within the first 6–9 months of a pandemic outbreak, vaccine companies will be only able to produce enough doses to vaccinate ≈700 million persons (*2*). This number is less than the combined populations of the 9 countries that produce almost all of the world's seasonal influenza vaccines. These countries will first use their vaccines to ensure that their own populations are protected. Non–vaccine-producing countries, both industrialized and developing, will have to wait.

In 2005, a representative of the World Health Organization (WHO) Global Programme on Influenza concluded that "most developing countries will have no access to a vaccine during the first wave of a pandemic and perhaps throughout its duration" (*2*). Since then, WHO has worked to build a stockpile of ≈150 million doses of vaccine against influenza virus A (H5N1) for developing countries (*3*), and 2 companies have pledged to provide WHO with 110 million doses. In 2007, a WHO scientific consultation on how to use this stockpile concluded, "If there is sufficient early warning that an outbreak of influenza (H5N1) is due to a virus that is capable of sustained human-to-human transmission, then theoretically there may be a relatively limited 'window of opportunity' to stop the spread of the virus before it spreads nationally or internationally. … However, a containment effort would be feasible only in settings where the number of localized cases are [sic] still limited, where adequate logistical support is available, and where the national government is supported by international assistance" (*3*). The vaccine

stockpile on which these efforts would depend does not yet exist.

Several industrialized countries are stockpiling vaccines against influenza virus A (H5N1) that might be used for prepandemic vaccination, but Oshitani et al. note that "both pandemic and prepandemic vaccines would not be available in developing countries unless an international mechanism exists to share such vaccine with them at low cost" (*1*). Even if limited supplies of vaccines could be produced for developing countries, no international mechanism is in place to pay for and distribute the vaccines, and WHO has yet to announce plans to set one up. Thus, when the next pandemic virus emerges, almost no vaccines will be available in developing countries to slow its spread (*1*,*2*).

Because global supplies of vaccines against pandemic viruses will be limited, government officials in a few industrialized countries have placed their hopes on stockpiles of antiviral agents, primarily oseltamivir, an expensive neuraminidase inhibitor. In 2005, WHO established its Southeast Asian Influenza Clinical Research Network to study neuraminidase inhibitor treatment of patients infected with viruses that possess pandemic potential (*4*). However, influenza virus A (H1N1) has developed resistance to oseltamivir, and similar antiviral resistance could develop in a future pandemic virus. Five million treatment courses (10 doses per patient) of oseltamivir have been donated to a WHO stockpile, but WHO has no plans to dramatically increase the size of this stockpile. On their own, the governments of a few countries that do not produce influenza vaccines or antiviral agents have purchased supplies of oseltamivir, but their stockpiles are sufficient to treat only 1% of their combined populations (D.S. Fedson, unpub. data). Not surprisingly, developing countries themselves "will not allocate scarce resources to stockpile significant quantities of oseltamivir for an unpredictable influenza pandemic" (*1*). Clearly, the limited supplies of antiviral agents available to developing countries where these infections now occur will scarcely have any effect on a pandemic after it starts to spread.

Influenza virologists report that recent isolates of highly pathogenic influenza viruses (H5N1 and H7N1) have acquired molecular characteristics suggesting they might become more easily transmissible among humans (*5*,*6*). In Indonesia, physicians have reported that everyone infected with the clade 2 influenza virus A (H5N1) who did not receive antiviral treatment has died (Table 1) (*7*). Given extremely limited global supplies of antiviral agents, this is a terrifying observation. If a pandemic virus were to emerge with a level of virulence approaching that of influenza virus A (H5N1) in Indonesia, it could lead to a global population collapse. Many influenza virologists doubt this will ever happen and believe instead that influenza virus (H7N7) or reemergent influenza virus (H2N2) could also cause the next pandemic. Chances are they might be right. Moreover, health officials in national governments and international agencies estimate that expected pandemic deaths will be no more than what can be extrapolated from the 1918–1920 pandemic (*8*). These officials seldom, if ever, use the phrases "population collapse" or "population die off," and their estimates may also be right. Nonetheless, in a seminal experiment reported in 1974, Webster and Campbell showed that genetic reassortment, the process that gave rise to pandemic viruses in 1957 and 1968, could give rise to a readily transmissible virus of extraordinary virulence (Figure) (*9*). This experiment and human experience with influenza virus A (H5N1) in Indonesia suggest it would be prudent for all countries to plan for something much worse than what occurred in 1918–1920.

The current approach to pandemic planning for all countries involves small groups of health officials, influenza scientists, and company executives, most of whom come from industrialized countries. For the foreseeable future, this top-down approach will be incapable of providing developing countries with timely supplies of affordable vaccines and antiviral agents. (Most industrialized countries that do not produce influenza vaccines will have similar difficulties, at least for the first pandemic wave.) The Indonesian Health Minister, for one, understands this. With little prospect that people in her country will be able to obtain vaccines against pandemic viruses, she precipitated a standoff with WHO by announcing in February 2007 that unless Indonesia is able to gain access to supplies of vaccines against pandemic viruses, her country will no longer share its influenza viruses A (H5N1) with WHO's

Table 1. Relationship between time of onset of antiviral treatment and case-fatality rate in persons with avian influenza A (H5N1) disease in Indonesia, 2003–2007*

Interval between onset of illness and treatment	No. cases	No. deaths	Case-fatality rate, %
≤24 h	2	0	0
0–4 d	11	5	45
0–6 d	37	24	65
>6 d	49	40	82
Any treatment	86	64	74
No treatment	33	33	100
All cases	119	97	82

*Adapted from (*7*).

laboratory-based surveillance system (*2*). Despite unorthodox arguments (*10*), her position has garnered wide support from the health ministers of many developing countries (*11*). Recently, Indonesia agreed to share influenza virus A (H5N1) sequences (not the viruses themselves) with the Global Initiative on Sharing Avian Influenza Data, but the country no longer promptly reports deaths from influenza virus A (H5N1), in defiance of new International Health Regulations. WHO has been unable to come up with a solution to this impasse.

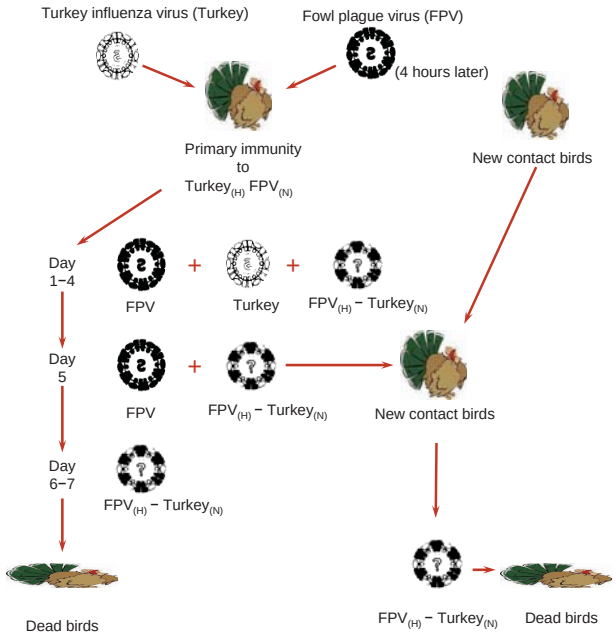

Figure. Genetic reassortment and genesis of a new pandemic influenza virus. This study was designed to determine whether the selection and transmission of a new reassortant influenza A virus could occur under experimental conditions in vivo that mimic what might occur in nature. Reassortment between 2 antigenically distinct influenza A viruses was studied in turkeys that had been previously immunized to induce low levels of antibodies to the hemagglutinin (H) of a nonlethal turkey influenza virus (Turkey), and to the neuraminidase (N) of a fowl plague virus (FPV), an avian virus that is highly pathogenic for chickens. Twenty-eight days after immunization, the immunized turkeys were sequentially infected, first with the Turkey virus and 4 h later with FPV. During the first few days, both parent viruses were isolated from the infected turkeys, but by day 4 a reassortant virus containing the FPV hemagglutinin and the Turkey neuraminidase ($FPV_{(H)}$–$Turkey_{(N)}$) was also isolated; within 2 days it became the dominant virus. All infected turkeys died, and only the $FPV_{(H)}$–$Turkey_{(N)}$ reassortant virus could be recovered. In a separate experiment, similarly immunized turkeys were again sequentially infected, but on day 5 a group of nonimmunized or selectively immunized turkeys ($Turkey_{(H)}$ $FPV_{(N)}$) were placed in the same room. All contact birds soon died of fulminant infection caused by the $FPV_{(H)}$–$Turkey_{(N)}$ reassortant virus. These experiments demonstrated that under conditions of selective primary immunity, a new virus could be generated through genetic reassortment in vivo and that this reassortant virus could be readily transmitted to contacts. The reassortant virus caused uniformly fatal disease in primary infected and contact birds. Thus, under the conditions of these experiments, genetic reassortment gave rise to a new influenza virus that led to a total population collapse. Adapted from Webster and Campbell (9).

In identifying the major issues and challenges of a pandemic threat facing developing countries, Oshitani et al. have called for better preparedness planning, improved systems for medical care and public health, expanded use of nonpharmaceutical interventions, and strengthened core capacities for seasonal influenza surveillance and vaccination (*1*). They recognize that this is a challenge few developing countries will be able to meet, but go on to say, "Preparing for a pandemic by simply strengthening preparedness within a single country is not possible. A pandemic is a global issue, and pandemic preparedness should be considered from a global perspective" (*1*). In practical terms, what exactly does this mean? The record thus far indicates that truly international efforts to prepare for pandemic vaccination and antiviral use have been meager. In almost all instances, these efforts have been vastly outweighed by efforts that reflect national concerns and interests.

A Bottom-Up Approach that Developing Countries Can Use to Confront the Next Pandemic

A top-down approach will not ensure that adequate and affordable supplies of vaccines against pandemic viruses and antiviral agents can be produced and distributed in time to protect populations in developing countries. Transferring technology for vaccine and antiviral agent production to a small number of developing countries will proceed slowly and will inevitably fail to meet the needs of neighboring countries not favored by these programs (*12*). Consequently, developing countries must consider an alternative bottom-up approach to pandemic control, an approach based on existing healthcare workers and institutions and that uses inexpensive and widely available generic agents that have intrinsic antiviral activities or that modify the host response (*13*,*14*).

Many influenza scientists doubt this approach will work (*14*–*16*). Nonetheless, as reviewed elsewhere (*13*,*14*), several retrospective studies suggest that outpatient statins (drugs taken to lower cholesterol levels and prevent cardiovascular diseases) reduce 30-day pneumonia mortality rates by ≈50% (Table 2) (*17*–*22*). Most investigators agree that these observational studies must be interpreted with caution and that promising results should be followed by prospective clinical trials. One such trial is already under way, and a preliminary report has shown that in 67 pneumonia patients in intensive care units, treatment with statins reduced the hospital mortality rate by 51% ($p = 0.026$) (*23*). Pulmonary investigators also believe that peroxisome proliferator–activated receptor (PPAR) α and PPARγ agonists (fibrates and glitazones, respectively) could be used to treat acute lung injury (*14*). An important experimental

Table 2. Recent studies of patients with pneumonia treated with statins*

Investigator (reference)	Study design and population	Principal outcome	Adjusted odds ratio (95% CI) or % reduction (p value)
van der Garde et al. (*17*)	Case–control diabetes patients, 4,719/15,322	Pneumonia hospitalization	0.50 (0.28-0.89)
Schlienger et al. (*18*)	Case–control, 1,227/4,734	Pneumonia hospitalization	0.63 (0.46–0.88)
		30-day pneumonia mortality rate	0.47 (0.25–0.88)
Mortensen et al. (*19*)	Retrospective cohort, 1,566/7,086	30-day pneumonia mortality rate	0.54 (0.42–0.70)
Chalmers et al. (*20*)	Prospective cohort, 257/750	30-day pneumonia mortality rate	0.46 (0.25– 0.85)
Thomsen et al. (*21*)	Retrospective cohort, 1,372/28,528	30-day pneumonia mortality rate	0.69 (0.58–0.82)
Majumdar et al. (*22*)	Prospective cohort, 325/3,090	Hospital mortality rate and ICU admission (adjusted for administrative data)	0.88 (0.63–1.22)
		Hospital mortality rate and ICU admission (adjusted for age, propensity score, clinical data, and functional status)	1.10 (0.76–1.60)
Choi et al. (*23*)	Randomized controlled trial, ICU treatment; 33 with atorvastatin and 34 controls	ICU mortality rate	45.4 (0.08)
		Hospital mortality rate	51.2 (0.026)

*Except for the inpatient randomized controlled trial of Choi et al, (*23*), recent treatment in the observational studies was defined as a statin prescription within a period of 30 days (*18*) to 90 days before hospitalization for pneumonia. CI, confidence interval; ICU, intensive care unit.

study has shown that the fibrate gemfibrozil, a PPARα agonist used to prevent heart disease, reduced mortality rates in mice infected with influenza virus (H2N2) by 54% (*24*). Statins and PPAR agonists have antiinflammatory and immunomodulatory activities, and there is considerable molecular cross-talk between these agents (*14*). Moreover, combination treatment is safe, and in patients with cardiovascular diseases, clinical benefits are additive. Used either alone or together, this treatment might similarly benefit patients during an influenza pandemic.

Other generic agents, some with direct activity against influenza virus, should also be considered (*14*). Chloroquine, long used as an antimalarial drug, increases endosomal pH and acts as an antiviral agent by impairing virus release into the cytosol. Resveratrol, a polyphenol found in red wine, reduces influenza mortality rates in experimentally infected mice (*25*). Catechins (found in green tea) (*26*) and curcumin (turmeric; found in curry powder) (*27*) have numerous cell-signaling effects, suggesting that they too might be beneficial. A combination of agents that act on both the host response and the virus might be required.

It is becoming increasingly difficult for investigators to ignore arguments for treating the host response to influenza. Recently, investigators showed that giving a neuraminidase inhibitor to mice infected with influenza virus A (H5N1) was not nearly as effective as treating the mice with an antiviral agent and 2 immunomodulatory agents, mesalazine, a PPARγ agonist, and celecoxib, a cyclooxygenase (COX)–2 inhibitor (*28*,*29*). In this model, targeting the host response to infection was essential for improving survival rates and times. More important, 2 studies in mice showed that intratracheal administration of either a fragment of the PB1-F2 protein of the 1918 influenza virus (*30*) or an inactivated influenza virus A (H5N1) (*31*) caused severe acute lung injury similar to that seen in fatal human cases of influenza (either from the 1918–1920 pandemic or from the current H5N1 subtype). In these experimental models, there was no virus replication. Thus, antiviral agents would have had no effect. Although we still lack direct evidence that one or more antiinflammatory and immunomodulatory agents alone would effectively treat human influenza virus A (H5N1) infections, these results and those from the study of influenza virus (H2N2)–infected mice treated with gemfibrozil (*24*) suggest these agents might be effective.

What makes these agents so important is that many of them are currently being produced as generic drugs in developing countries (*13*,*14*). These drugs are inexpensive, could be produced in abundance, and could even be stockpiled and made available for use on the first day of a pandemic. No matter what is accomplished in the years ahead, adequate supplies of vaccines and specific antiviral agents will never be available to persons in developing countries on the first pandemic day.

A Research Agenda to Establish a Generic Approach to Pandemic Treatment and Prophylaxis

What types of research on generic agents do we need before the pandemic virus appears? First, experimental studies of several candidate treatment regimens must be undertaken in mice infected with influenza virus A (H5N1) or 1918-like viruses (Table 3). The agents used in these studies might have antiinflammatory and immunomodulatory or antiviral properties (some might have both), but all must be generic agents that are currently produced in developing countries. Admittedly, these experimental studies in mice will have limitations (*32*), but they should identify avenues for further research. Once a few treatment regimens have been shown to be effective in mice, they should be tested in

ferrets. Later, 2 or 3 of the most promising regimens should be tested in nonhuman primates.

After demonstrating the effectiveness of 1 or more treatment regimens in animals, influenza virologists should then use in vitro systems to define the molecular mechanisms responsible for their protective activity. However, some of these agents will have broader effects on the host response. For example, although administering a COX-2 inhibitor along with a PPARγ agonist improved survival rates and times in mice infected with influenza virus A (H5N1) (*21*), another study showed that selective COX-2 inhibition was detrimental to the resolution of acute lung injury (*33*). Most influenza scientists focus their research on the virus or on cell-signaling events associated with viral pathogenesis (*34*). Yet the pathophysiologic effects of severe infections involve the entire host, something well known to researchers who study sepsis (*35*–*37*). Their studies have shown that statins and PPAR agonists stabilize myocardial and microvascular function, preserve integrity of pulmonary endothelial cell tight junctions and prevent pulmonary edema, and promote resolution of acute inflammation (*13*,*14*). Thus, other investigators with laboratory and clinical expertise in critical care, cardiopulmonary diseases, and endocrinology and metabolism must be recruited to explore in animals the molecular mechanisms underlying these broad treatment effects on the host. However, in undertaking this research, investigators must not forget that their primary goal is to find effective ways to manage a pandemic in populations and not simply to explain in more precise terms the harmful effects of pandemic virus infection in individuals.

While these studies are under way, an analysis should be undertaken for each candidate agent to determine which companies produce them, where each is manufactured, annual levels of production (and surge capacity), patterns of distribution to other developing countries, and costs for public markets (Table 3). Special attention must be given to companies that follow Good Manufacturing Practices to minimize the risk that some of these agents might be counterfeit. When animal studies have defined 1 or more promising regimens, an international process must be set up to develop logistics for financing, producing, and distributing each agent.

Table 3. Research agenda to establish whether generic agents could be used for treatment and prophylaxis of a pandemic caused by a subtype H5N1–like influenza virus

1. Test candidate treatment regimens in mice, ferrets, and nonhuman primates to identify specific generic agents that might be effective in managing a pandemic
2. Study promising generic treatments in cell culture and animals to define the molecular mechanisms that explain their beneficial effects against influenza virus A (H5N1) and 1918-like influenza viruses
3. Conduct a global analysis to identify developing countries where these generic agents are produced and determine quantities produced, surge capacities, patterns of distribution, and costs to public programs
4. Establish an international process to coordinate or manage the stockpiling of generic agents and/or their distribution once a pandemic virus has emerged
5. Plan to conduct randomized controlled trials of promising generic treatments immediately after the emergence of a new pandemic virus

Where feasible, clinical trials of promising treatment regimens might be undertaken in patients with severe seasonal influenza. In a few instances, clinicians might choose to treat patients infected with influenza virus A (H5N1) on a compassionate basis (*12*). However, none of these limited studies will guarantee that promising treatments in the prepandemic period will be effective against a true pandemic virus. Thus, careful plans must be made during the prepandemic period that will enable investigators to conduct randomized controlled trials of promising generic regimens during the early weeks of a new pandemic. If the case-fatality rate is similar to that of influenza virus A (H5N1) (≈60%), trials will not need to be large (Table 4). Within a few days, investigators should be able to recruit sufficient numbers of patients to satisfy statistical requirements.

Planning for clinical trials during the prepandemic period must start with identifying clinical investigators who will conduct these trials and institutions that will sponsor their work. Supplies of the agents to be tested must be set aside, study protocols written, and ethical approval obtained. A mechanism for rapid regulatory approval must be developed to enable trials to be conducted wherever the pandemic virus first emerges. A financing mechanism must be established that enables immediate access to funds necessary to support the trials. Finally, an internet-based communication strategy must be devised that ensures prompt dissemination of study results to physicians and health officials worldwide.

None of this research on generic agents will be possible without international coordination. Thus far, the top-down approach that has characterized vaccine and antiviral research and development has lacked an international system for coordination and management to ensure rapid progress (*2*). Likewise, nothing has been done to ensure worldwide production and distribution of the vaccines and antiviral agents being developed. A similar approach must not be allowed to govern the research agenda for generic agents.

Experience with the severe acute respiratory syndrome (SARS) in 2003 shows us how we could do much better. When SARS first came to international attention, WHO quickly established 3 virtual networks of experienced virologists, clinicians, and epidemiologists (*38*). By sharing experiences and findings on secure websites and in daily teleconferences, investigators soon identified and sequenced the SARS coronavirus, defined the clinical fea-

Table 4. Sample size requirements for a randomized controlled trial of treatment to reduce deaths in a pandemic caused by a subtype H5N1–like influenza virus*

Case-fatality rate, %		Reduction in no. deaths, %	Total sample size (power)		
Untreated	Treated		80%	90%	95%
50	37.5	25	530	690	850
50	25	50	140	170	210
50	12.5	75	60	80	90

*1:1 randomization of persons to the 2 treatment groups, α = 0.05 (2-sided), χ^2 test (continuity corrected). The example shown assumes a case-fatality rate of 50%, which is similar to what has been seen for patients infected with influenza virus A (H5N1). If a new pandemic virus is associated with a lower case-fatality rate, sample sizes required to show similar reductions in case-fatality rates would have to be larger.

tures of the disease, and established practical measures for clinical management and epidemiologic control. Surprisingly, WHO has not set up a similar system to coordinate research and development of vaccines against pandemic viruses and antiviral agents, despite the far greater threat to global health inherent in an influenza (H5N1) pandemic (*2*). Given escalating pressure from developing countries, WHO can ill afford to adopt the same slow approach to establishing the scientific basis for using inexpensive and widely available generic agents for pandemic control.

Conclusions

Oshitani et al. correctly emphasize that preparing for the next pandemic requires a global perspective, but this does not necessarily mean that the measures used to confront the pandemic in developing countries must be supplied through an internationally organized top-down process. An international process will surely be required for distributing vaccines and antiviral agents, but experience indicates that the process will be slow and cumbersome and supplies of these agents will remain scarce (*2*). Nonetheless, developing countries will need abundant supplies of effective agents, and abundance will be guaranteed only if these agents are generic, inexpensive, and produced in developing countries themselves.

It is too soon to know whether generic agents could be used to confront the next influenza pandemic, yet developing countries lack realistic alternatives. For this reason, their leaders must convince scientists and international organizations, including WHO, of the urgent need for research to determine whether these inexpensive agents could mitigate the effects of a pandemic. Otherwise, developing and industrialized countries alike could be faced with an unprecedented global health crisis.

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Dr Fedson is a former professor of medicine at the University of Virginia and former Director of Medical Affairs in Europe for Aventis Pasteur MSD (now Sanofi Pasteur MSD). Since retiring in 2002, he has continued to write and lecture about influenza vaccination, pandemic preparedness, and the potential for using generic agents for pandemic treatment and prophylaxis.

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etymologia

Shigella

[shĭ-gel′ə]

Genus of gram-negative bacteria in the family *Enterobacteriaceae*, named for Japanese bacteriologist Koyoshi Shiga (1871–1957). In 1897, Japan experienced a severe dysentery epidemic; >91,000 cases were reported, and the case-fatality rate was >20%. Dr Shiga isolated the etiologic agent from patient stool samples: a bacillus, later called *Shigella dysenteriae*. He went on to describe the toxins that the organism produces; one that causes serious complications during infections is now known as Shiga toxin.

Sources: Trofa AF, Ueno-Olsen H, Oiwa R, Yoshikawa M. Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. Clin Infect Dis. 1999;29:1303–6; Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007.

Shiga Toxin–producing *Escherichia coli* Strains Negative for Locus of Enterocyte Effacement

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Most Shiga toxin–producing *Escherichia coli* (STEC) infections that are associated with severe sequelae such as hemolytic uremic syndrome (HUS) are caused by attaching and effacing pathogens that carry the locus of enterocyte effacement (LEE). However, a proportion of STEC isolates that do not carry LEE have been associated with HUS. To clarify the emergence of LEE-negative STEC, we compared the genetic composition of the virulence plasmids pO113 and pO157 from LEE-negative and LEE-positive STEC, respectively. The complete nucleotide sequence of pO113 showed that several plasmid genes were shared by STEC O157:H7. In addition, allelic profiling of the *ehxA* gene demonstrated that pO113 belongs to a different evolutionary lineage than pO157 and that the virulence plasmids of LEE-negative STEC strains were highly related. In contrast, multilocus sequence typing of 17 LEE-negative STEC isolates showed several clonal groups, suggesting that pathogenic LEE-negative STEC has emerged several times throughout its evolution.

Shiga toxin–producing *Escherichia coli* (STEC) strains are foodborne enteric pathogens associated with hemorrhagic colitis and the development of the life-threatening condition hemolytic uremic syndrome (HUS). Young children in industrialized countries are particularly at risk (*1*). The production of Shiga toxin (Stx) increases mortality rates for those persons who have STEC infections compared with those who have other varieties of *E. coli* infection (*1*). Therefore, the production of ≥1 Stx variants is central to pathogenesis; however, bacterial adherence and subsequent colonization of the intestinal epithelium also contribute to STEC virulence.

Many disease-related STEC serogroups, including the most prevalent O157:H7 clone, possess a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (*2*). STEC containing LEE are characterized by their ability to attach to the host intestinal mucosa and destroy the surrounding microvillus brush border, which causes substantial cytoskeletal rearrangements within the enterocyte (*3*). On the basis of this phenotype, LEE-positive STEC are classified as attaching and effacing (A/E) pathogens with the closely related human pathogen, enteropathogenic *E. coli* (*4*,*5*). Although many studies have demonstrated that LEE is essential for host colonization and virulence of A/E pathogens (*6*–*9*), others have demonstrated that some STEC isolates without LEE, such as STEC O113:H21, are associated with sporadic and outbreak cases of severe disease indistinguishable from that caused by STEC O157:H7 (*10*–*13*). In the absence of LEE, mechanisms are emerging by which these atypical or LEE-negative STEC interact with the host intestinal mucosa and induce disease. Recently, a potent new toxin, SubAB, was discovered in LEE-negative strains of STEC; this toxin induces cell death through cleavage of the endoplasmic reticulum chaperone, BiP/GRP78 (*14*). SubAB is more

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prevalent in LEE-negative than in LEE-positive strains of STEC and likely contributes to the progression to severe disease (*15*,*16*). In addition, LEE-negative STEC isolates from several serogroups, particularly STEC O113:H21, can invade tissue culture cells, a mechanism partially dependent on flagellin (*17*–*19*).

Large plasmids encoding EHEC hemolysin (Ehx) are found in almost all disease-associated STEC strains. Restriction fragment length polymorphism (RFLP) analysis of the *ehxA* gene from pO113 of STEC O113:H21 has suggested that pO113 evolved separately to the Ehx plasmids of LEE-positive STEC (*20*). To clarify the contribution of large plasmids to the virulence and evolution of STEC, we determined the complete nucleotide sequence of pO113 from EH41, a clinical (HUS) isolate of STEC O113:H21 (*21*). We compared the nucleotide sequences of pO113 and pO157 to examine the relationship between the 2 plasmids for their origin, gene content, and putative role in disease. In addition, we performed allelic profiling of *ehxA* and *repA* (a plasmid replication initiation gene of pO113) from LEE-negative and LEE-positive STEC isolates to model plasmid evolution compared with the evolution of the *E. coli* background, which was determined by multilocus sequence typing (MLST). Finally, to increase understanding of the evolutionary origins of STEC, we determined genetic features of LEE-negative STEC that may be used to improve diagnosis and detection.

Materials and Methods

Bacterial Strains and Culture Conditions

E. coli isolates used in this study are listed in Table 1; those shown in boldface were further examined for detailed analysis of *ehxA* and *repA* sequence and MLST. All *E. coli* strains were cultured aerobically in Luria broth or on Luria broth agar at 37°C. When required, chloramphenicol was added at a concentration of 12.5 μg/mL.

Sequencing and Annotation of pO113

The complete nucleotide sequence of pO113 from EH41 was determined from a series of overlapping 30–40 kb fragments cloned into the Copy Control pCC1FOS cosmid vector (Epicentre, Madison, WI, USA) and propagated in *E. coli* EPI300 (Epicentre). The final sequence was assembled using Sequencher version 4.7 (Gene Codes Corp. Ann Arbor, MI, USA). The sequence annotation was performed by using WASABI (*25*), a Web-based annotation system for prokaryotic organisms. WASABI was used to generate an automatic annotation of the sequence, which was followed by manual curation by the authors. The automatic annotation used GeneMarkS (http://exon.gatech.edu/genemark) to identify putative coding regions and BLAST (www.ncbi.nlm.nih.gov/blast) and reversed position-specific (RPS)–BLAST to assign function on the basis of sequence similarity. The final annotated sequence was deposited into GenBank under accession no. AY258503.

Presence of pO113 Genes in STEC Strains

DNA for PCR and sequencing was extracted from 1 mL of overnight culture by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). PCR was used to examine the prevalence of 9 genes found on pO113 in a cohort of LEE-negative and LEE-positive STEC strains. The plasmid locations of *pilQ*, *epeA*, *trbC*, *repA*, *ehxA*, *espP*, *iha*, *subAB*, and *repZ* are listed in Table 2; oligonucleotide

Table 1. *Escherichia coli* isolates used in this study*

Isolate	Serogroup	Origin†	LEE‡	Ref.
EH41	O113:H21	HUS	–	(*17*,*21*,*22*)
EH53	O113:H21	HUS	–	(*11*,*23*)
EH71	O113:H21	HUS	–	(*17*)
97025659	O113:H21	TTP	–	
95016910	O113:H21	Food	–	
95063160	O113:H21	Cow	–	
95063151	O113:H21	Human	–	
96037512	O113:H21	Food	–	
97001061	O113:H21	Food	–	
99008358	O113:H21	Dysentery	–	
EH42	O116:H21	HUS	–	(*17*)
EH43	O130:H11	HUS	–	(*11*)
EH48	O5:H–	HUS (UTI)	–	
9724772	O5:H–	Diarrhea	–	
EH69	O1:H7	HUS	–	(*11*)
EH52	NT:H7	HUS	–	(*11*,*23*)
9816261	O76:H7	HUS	–	
9611588	O128:H2	Diarrhea	–	
EH5	O91:H–	Diarrhea	–	(*17*)
EH32	O91:H–		–	
9730196	O87:H16	Asymptomatic	–	
9619262-1	OR:H–	Diarrhea	–	
96/4591	O123:H–	Cow	–	
85-170	O157:H7	HUS	+	
EDL933	O157:H7	HUS	+	(*24*)
84-284	O157:H7		+	
EH9	O157:H7		+	
9515477	O157:H7	HC	+	
9515480	O157:H7	HUS	+	
9515474	O157:H7	HUS	+	
9924822	O157:H7	HUS	+	
95005698	O157:H–	HUS	+	
95051613	O157:H–	HC	+	
EH70	O157:H–	HC	+	
E45035	O111:H–	HUS	+	
ED142	O111:H–	HUS	+	
EH38	O111:H–	HUS	+	
EH44	O26	HUS	+	
EH6	O26:H11		+	
EH34	O26:H11		+	
EH1	O26:H21	Diarrhea	+	
EH68	O147:H–	Diarrhea	+	
EH22	O145:H25		+	

*Isolates shown in **boldface** were used for allelic profiling and multilocus sequence typing phylogenetic analysis. LEE, locus of enterocyte effacement; Ref., reference; HUS, hemolytic uremic syndrome; TTP, thrombocytopenic purpura; UTI, urinary tract infection; HC, hemorrhagic colitis.
†Clinical information and source are provided where known.
‡Symbols indicate presence (+) or absence (–) of the *eae* gene.

Table 2. Gene names, plasmid location, and oligonucleotide sequences used to examine the prevalence of pO113-encoded genes among STEC strains*

Gene	Plasmid location, bp	Oligonucleotide sequences	Amplicon size, bp	PCR annealing temperature, °C
pilQ	9593–12105	F: 5'-TTGCAGACCCGCAGTTG-3' R: 3'-CAGGGCTTCGGCGATGT-5'	870	52
epeA	48716–52795	F:5'-CAGGTGGTACTGTCGGC-3' R: 3'-GCCCATGCCGCTCTGAA-5'	667	46
trbC	57357–59660	F: 5'-GCCACCACCGGTGGCGG-3' R: 3'-CAATCAGAATGCGGTCG-5'	230	50
repA	106112–107089	F: 5'-AAAGTCTTGTATAGCTC-3' R: 3'-GTTATCCATATCCAGGC-5'	871	44
ehxA	114136–117132	F: 5'-CCCAGGAGAAGAAGTCA-3' R: 3'-CTTCACCTGAGGCATCTT-5'	1,108	48
espP	134205–140707	F: 5'-AAACAGCAGGCACTTGAACG-3' R: 3'-GGAGTCGTCAGTCAGTAGAT-5'	2,000	52
iha	146066–149549	F: 5'-TCCAGTCAGTACCACGA-3' R: 3'-CTGTCGGAAAGTTTCAC-5'	981	48
subAB	150678–152419	F: 5'-GTGTACAGGACTCATGG-3' R: 3'-ATCACCAGTCCACTCAG-5'	783	48
repZ	163918–164949	F: 5'-ATACAGGAGTAAAACCG-3' R: 3'-CATATAACGCAGTACAC-5'	1,792	46

*STEC, Shiga toxin–producing *Escherichia coli*; F, forward primer; R, reverse primer.

sequences and annealing temperatures were used to amplify fragments of these genes with Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany).

Phylogenetic Analysis and MLST

A neighbor-joining tree was created from the nucleotide sequence of the 1,108-bp amplicon of *ehxA* and the 871-bp amplicon of *repA* for 30 *E. coli* isolates. The tree was inferred by using the neighbor-joining method as implemented in ClustalW (*26*,*27*). Significant nodes were identified by bootstrapping and Monte Carlo randomization; nodes present in >70% of the 1,000 bootstrap trees were identified as significant. MLST was performed on the basis of the nucleotide sequence of 580- to 672-bp amplicons of 7 conserved housekeeping genes: *aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*. A detailed protocol of the MLST procedure, including allelic type and sequence type (ST) assignment methods, can be found at the EcMLST Web site (www.shigatox.net/mlst). Sequences were concatenated for phylogenetic analyses. A neighbor-joining tree was constructed by using the Kimura 2-parameter model of nucleotide substitution with MEGA3 software (*28*), and the inferred phylogenies were each tested with 1,000 bootstrap replications.

Results

Sequence of the Large Plasmid pO113

The entire sequence of pO113 comprised 165,548 bases with an overall GC content of 49.64%; the sequence was predicted to encode 155 genes and 38 pseudogenes (Figure 1). Plasmid pO113 was considerably larger than the 92-kb pO157 because of the presence of a 63.9-kb transfer region (*21*,*29*). This region showed similarity to the *tra/trb* regions of the self-transmissible IncI plasmids R64 and ColIb-P9 (*30*); the number and order of genes within this region were relatively uninterrupted (Figure 2). Most genes acquired by pO113 encoding putative virulence determinants appear to have accumulated outside the transfer region (Figure 2), and many were associated with predicted insertion elements and DNA recombinases (Figure 1). Several pO113-

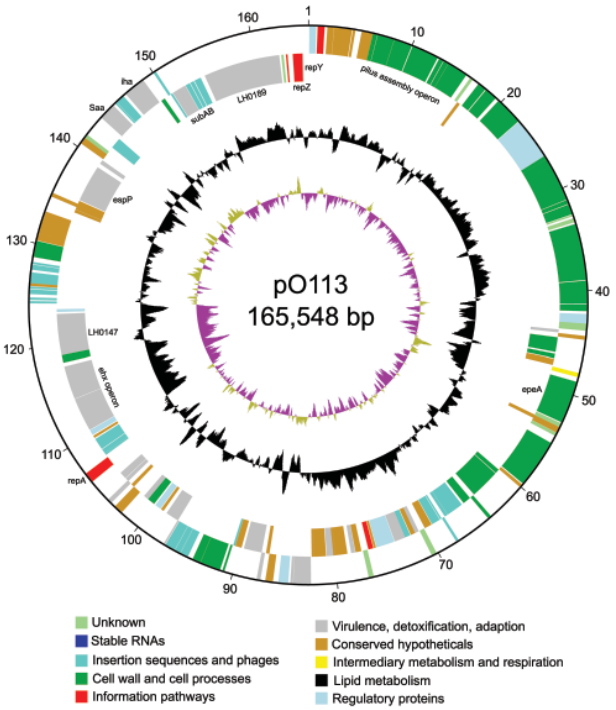

Figure 1. Circular map of virulence plasmid pO113 generated by using circular_diagram.pl (Sanger Institute, Cambridge, UK) and Inkscape software (www.inkscape.org). The locations of proteins encoded on the leading and lagging strand are shown on the outer 2 rings. The colors indicate the assigned GenoList functional category (Institut Pasteur, Paris, France). The black ring indicates GC content, with high GC content outermost. The innermost ring shows GC skew.

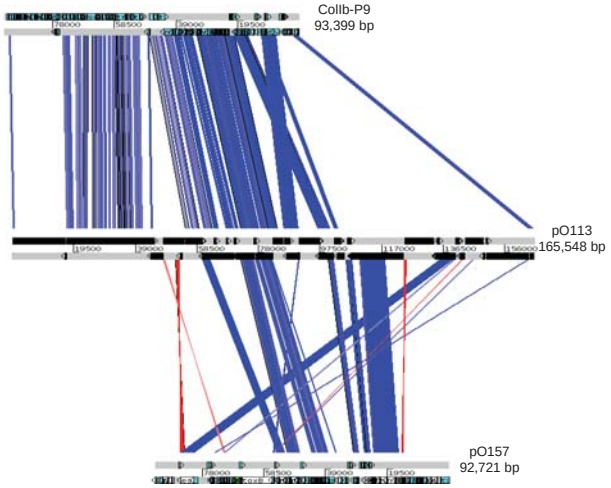

Figure 2. Graphic overview of sequences related to virulence plasmid pO113 in the plasmids ColIb-P9 and pO157. The overview was generated by ACT (www.sanger.ac.uk); related sequences are indicated as boxes between the horizontal bars representing each of the plasmid sequences. Similarity between sequences was established by using TBLASTX with the pO113 sequence as the subject and either ColIb-P9 or pO157 as the query sequence. Blue indicates that open reading frames occur in the same order; red indicates a DNA inversion.

encoded genes were shared by STEC O157:H7, including *ehxCABD* (110,523–117,649 bp), *espP* (135,505–139,407 bp), the putative adhesin *iha* (146,764–148,851 bp), and 2 replication genes, *repA* (106,112–107,089 bp) and *repZ* (163,918–164,949 bp) (*20*). However, pO113 lacked the pO157-encoded type II secretion system and a homologue of the adherence-promoting protein ToxB (*31*).

Plasmid pO113 encoded a number of unique virulence-associated determinants, including the autotransporter protein EpeA (48,716–52,795 bp), the autoagglutinating adhesin Saa (143,552–145,156 bp), and the subtilase-like serine protease toxin SubAB (151,027–152,070) (*15*). In addition, the complete nucleotide sequence of pO113 showed several novel putative factors that have not been described previously and that may contribute to host-pathogen interactions. These factors included the putative product of *LH0147* (118,905–123,200 bp), which may represent a novel member of the trimeric autotransporter family of exported adhesins (*32*). *LH0147* has a putative signal peptide cleavage site between amino acids 15 and 16 and a predicted location in the outer membrane. Similar to Saa, the predicted product of *LH0147* carries a YadA-like, Hia-like, C-terminal region denoted by the conserved domain pfam03895, which may be important for oligomerization and targeting of the protein to the outer membrane (*32*,*33*). In addition, *LH0189* (154,379–162,484 bp) encodes a putative hemolysin/hemagglutinin-like protein that shows significant similarity to several members of the ShlA/HecA/FhaA family of large outer membrane adhesins, including HecA from *Erwinia chrysanthemi* (49% similarity over 1,389/2,801 amino acids) (*34*). However, unlike other members of the HecA family, the product of *LH0189* did not have a recognizable signal peptide sequence for export, although the presence of ≥2 predicted transmembrane domains suggested that the protein localizes to the bacterial inner membrane. A partial 36,841-bp sequence for pO113 from STEC O113:H21 strain 98KN2 is available from GenBank under accession no. AF399919.3. A comparison of the overlapping regions of both plasmids showed that the nucleotide sequence was highly conserved and that the location, order, and predicted amino acid sequences of putative open reading frames were also highly conserved, exhibiting 99% identity. Although the putative HecA-like adhesin encoded by *LH0189* was apparently absent from STEC O113:H21 strain 98NK2, subsequent PCR analysis by using the primers 5′-TGA TAT TCT GTT GAG TG-3′ and 5′-ATC CGC CAC CTG ACT GC-3′ showed that this gene was also present in STEC O113:H21 98KN2 (data not shown). *LH0189* may be located in another region of the plasmid or on an island elsewhere in the genome of STEC O113:H21 98NK2.

Prevalence of pO113 Genes among LEE-negative and LEE-positive STEC

To investigate the prevalence of the pO113 encoded genes *pilQ, epeA, trbC, repA, ehxA, espP, iha, subAB,* and *repZ* among a range of LEE-negative and LEE-positive STEC isolates, we screened our collection of STEC isolates by using PCR. PCR conditions and oligonucleotide sequences are listed in Table 2. Of the 23 LEE-negative strains examined, only 10 (44%) were positive for all 9 genes (Table 3). The replication initiation gene, *repA*, was found in all LEE-negative strains tested, *espP* was present in 17 strains (74%), and *subAB* and *repZ* were each present in 18 strains (78%). Of the 20 LEE-positive STEC isolates examined, *ehxA* was present in 18 strains (90%) and *repA* in 19 strains (95%) (Table 4). In contrast, all LEE-positive STEC strains were negative for *epeA,* and only EH1, serogroup O26:H21, was positive for *subAB*.

Phylogenetic Analysis of *ehxA* and *repA*

To clarify the genetic relationship and origin of the large plasmids of LEE-negative STEC, we initially performed allelic profiling of the *ehxA* gene. The 1,108-bp amplicon of *ehxA* was sequenced for 17 LEE-negative and 13 LEE-positive STEC strains (Table 1). Overall, the nucleotide sequences of *ehxA* were closely related, exhibiting 96.8% nucleotide sequence identity across all 30 isolates. The nucleotide sequences were analyzed by using ClustalW (www.ebi.ac.uk/Tools/clustalw2) to produce a neighbor-joining tree demonstrating sequence relationships (Figure

Table 3. Prevalence of selected pO113 ORFs among LEE-negative strains of STEC*

Strain	Gene								
	pilQ	*epeA*	*trbC*	*repA*	*ehxA*	*espP*	*iha*	*subAB*	*repZ*
EH41	+	+	+	+	+	+	+	+	+
EH53	+	+	+	+	+	+	+	+	+
EH71	+	+	+	+	+	+	+	+	+
97025659	+	–	+	+	+	+	+	+	+
95016910	+	+	+	+	+	+	+	+	+
95063160	+	+	+	+	+	+	+	+	+
95063151	+	+	+	+	+	+	+	+	+
96037512	+	–	+	+	+	+	+	+	+
97001061	+	+	+	+	+	+	+	–	+
99008358	+	+	+	+	+	+	+	+	+
EH42	+	+	+	+	+	+	+	+	+
EH43	+	+	+	+	+	+	+	+	+
EH48	+	–	+	+	+	–	+	+	–
9724772	+	–	+	+	+	–	+	+	–
EH69	+	+	+	+	+	+	+	–	+
EH52	+	+	+	+	+	+	+	+	+
9816261	+	+	+	+	+	+	–	–	+
9611588	–	–	–	+	+	–	+	+	–
EH5	–	–	–	+	+	+	+	–	–
EH32	–	–	+	+	+	–	+	+	+
9730196	–	–	+	+	–	+	+	–	–
9619262-1	+	–	+	+	+	–	+	+	+
96/4591	+	–	+	+	–	–	+	+	+
% Positive	83	57	91	100	91	74	96	78	78

*ORF, open reading frame; LEE, locus of enterocyte effacement; STEC, Shiga toxin–producing *Escherichia coli*.

3, panel A). The *ehxA* sequences from LEE-negative and LEE-positive isolates segregated into 2 distinct clades, supporting a previous study that suggested pO113 belonged to distinct evolutionary lineage from pO157 (*20*). Irrespective of serotype, here we could show that the LEE-negative Ehx-encoding plasmids were genetically related, which suggests a common evolutionary origin (Figure 3, panel A). However, our study highlighted 3 exceptions to this delineation, including the LEE-negative STEC strain 9816261 (O76:H7), which possesses an *ehxA* sequence most closely linked to O111:H– strains of LEE-positive STEC, and the LEE-positive strains E45035 (O111:H–) and EH6 (O26:H11), which segregated with LEE-negative isolates.

To determine if the phylogenetic relationship between the LEE-positive and LEE-negative plasmids demonstrated by *ehxA* was evident in other pO113-encoded factors, we constructed a neighbor-joining tree for a second pO113 gene, the plasmid replication initiation gene *repA*. The 871-bp amplicon of *repA* was sequenced for 16 LEE-negative and 13 LEE-positive STEC strains. Similar to *ehxA*, *repA* was chosen because it was found in most LEE-negative and LEE-positive STEC strains. Overall, the nucleotide sequences of *repA* were closely related, exhibiting 94.5% nucleotide sequence identity. Phylogenetic analysis of the *repA* nucleotide sequence did not show the same clustering of strains as *ehxA* and showed less distinct delineation on the basis of LEE-positivity (Figure 3, panel B). These findings may indicate that *repA* was acquired by the large STEC plasmids independently of *ehxA* and suggests that *ehxA* is a more discriminating phylogenetic marker for allelic profiling.

Genetic Diversity and Ancestral Relationship between LEE-negative and LEE-positive STEC

Because the large plasmids of LEE-negative STEC appeared to be highly related, we examined the *E. coli* background of these strains by using MLST to understand if the strains also exhibited a clonal relationship. PCR amplification and sequencing of the 7 MLST loci in 17 LEE-negative and 13 LEE-positive STEC strains allowed ST classification of each strain (www.shigatox.net/mlst). The STs of the 30 STEC strains examined were intercalated with previously characterized pathogenic *E. coli* isolates, which showed that the current strains grouped into 7 known clonal groups (CGs) (Figure 4). Of the LEE-positive isolates, 6 belonged to CG 11 or the EHEC-1 group; 5 belonged to CG 14 or EHEC-2. One isolate EH22 (O145:H22), belonged to CG 42, and 1 isolate, EH1 (O26:H11), belonged to CG 36 and contained one of the LEE-negative isolates, EH43 (O130:H11). Among the other LEE-negative isolates examined, 7 belonged to CG 30 or the STEC-2 group, including all the O113:H21 isolates. One isolate, EH52 (NT:H7), belonged to CG 31, and 1 isolate, 9816261 (O76:H7), belonged to CG 47. Isolate 9816261 was most closely related to uropathogenic *E. coli*. Isolate 9611588 (O128:H2) was classified as a member of a new CG, CG 63, together with an enteropathogenic *E. coli* isolate (ST379). The remaining 6 LEE-negative STEC isolates examined here could not be assigned into CGs. These were 2 O5:H– isolates assigned ST811 (isolates 9724772 and EH48), 2 O91:H– isolates assigned ST814 (isolates EH32 and EH5), 1 O1:H7 isolate assigned ST818 (isolate EH69), and 1 OR:H– isolate as-

Table 4. Prevalence of selected pO113 ORFs among LEE-positive strains of STEC*

Strain	Gene								
	pilQ	*epeA*	*trbC*	*repA*	*ehxA*	*espP*	*iha*	*subAB*	*repZ*
85-170	–	–	–	+	+	+	+	–	–
EDL933	–	–	–	+	+	+	+	–	–
84-284	–	–	–	+	+	+	–	–	–
EH9	–	–	–	+	+	+	+	–	–
9515477	–	–	–	+	+	+	–	–	+
9515480	–	–	–	+	+	+	+	–	–
9515474	–	–	–	+	+	+	+	–	–
9924822	+	–	+	+	+	+	+	–	+
95005698	–	–	–	+	+	+	–	–	–
95051613	–	–	–	+	+	+	+	–	–
EH70	–	–	–	+	+	+	+	–	–
E45035	+	–	+	+	+	–	+	–	–
ED142	+	–	+	+	+	–	+	–	–
EH38	+	–	–	+	+	+	+	–	–
EH44	–	–	–	+	+	+	+	–	+
EH6	+	–	–	+	+	+	+	–	–
EH34	–	–	–	+	–	–	–	–	–
EH1	+	–	+	–	+	+	+	+	+
EH68	–	–	–	+	–	–	+	–	–
EH22	+	–	–	+	+	–	–	–	–
% Positive	35	0	20	95	90	75	75	0.05	20

*ORF, open reading frame; LEE, locus of enterocyte effacement; STEC, Shiga toxin–producing *Escherichia coli.*

signed ST810 (isolate 9619262). Overall, the MLST data showed that LEE-negative STEC do not exhibit clonality, and the large Ehx plasmid was acquired by horizontal gene transfer.

Discussion

We constructed a complete nucleotide sequence for a large plasmid from LEE-negative STEC. Previous studies have shown that many open reading frames on the plasmid are shared by other STEC, predominantly LEE-negative strains (*15*,*21*,*29*,*33*). Our PCR screen of 17 LEE-negative STEC isolates demonstrated a high degree of conservation of pO113-encoded genes, although not all genes examined were present in all strains. In addition, we found that although *repA*, *ehxA*, *espP*, and *iha* were common to LEE-positive and LEE-negative STEC, other pO113-encoded

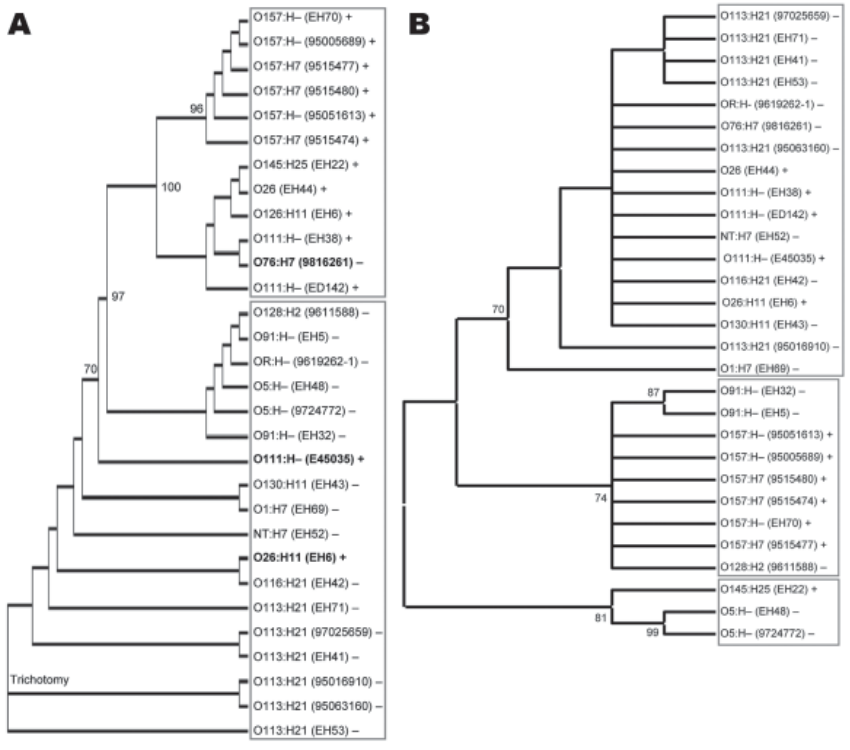

Figure 3. Neighbor-joining tree of *ehxA* (A) and *repA* (B) as implemented in ClustalW (www.ebi.ac.uk/Tools/clustalw2). This rectangular cladogram demonstrates the distinct clades (shown by boxes) for *ehxA* that delineate locus of enterocyte effacement (LEE)–negative and LEE-positive Shiga toxin–producing *Escherichia coli* strains. Exceptions to this pattern are shown in **boldface**, strain names are shown in parentheses, and + or – indicates the presence or absence of LEE. Significant nodes were identified by bootstrapping (Monte Carlo randomization); nodes were present in >70% of the 1,000 bootstrap trees highlighted and identified as significant.

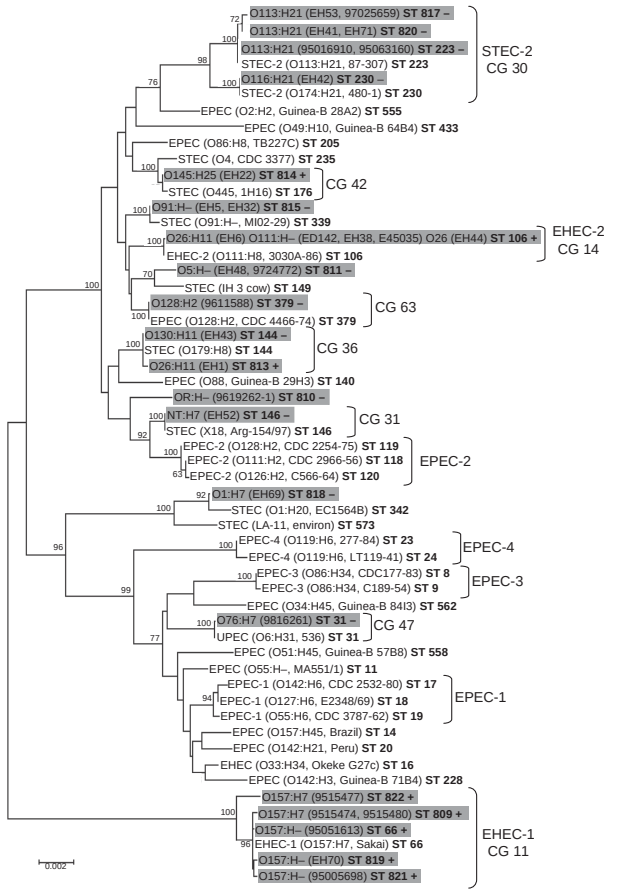

Figure 4. Phylogenetic relationships of 17 locus of enterocyte effacement (LEE)–negative and 13 LEE-positive Shiga toxin–producing *Escherichia coli (*STEC) strains (highlighted in gray) compared with a cohort of reference *E. coli* strains. Phylogeny was demonstrated by a neighbor-joining algorithm from 7 housekeeping gene sequences. Each isolate has been assigned a sequence type (ST) (in **boldface**), and assigned clonal groups (CGs) are displayed. The scale bar demonstrates the branch length that corresponds to 2 nucleotide substitutions per 1,000 nucleotide sites. Significant nodes were identified by bootstrapping (Monte Carlo randomization); nodes were present in >70% of the 1,000 bootstrap trees highlighted and identified as significant.

genes, *pilQ, epeA, trbC, subAB,* and *repZ*, were more commonly associated with LEE-negative STEC.

Generally, pO113 exhibited a mosaic structure similar to pO157, encoding a large number of transposases and insertion elements clustered outside a plasmid transfer region. Although the remnant transfer region places pO157 in the F-family of conjugative plasmids, the functional transfer region of pO113 places it within the IncI group of self-transmissible plasmids, which includes R64 and ColIb-P9. This finding indicates that pO113 and pO157 have a different evolutionary history. Allelic profiling of the *ehxA* gene in diverse STEC isolates confirmed that the large plasmids belong to different evolutionary lineages, independent of serotype.

In addition to the previously characterized virulence-associated determinants EpeA, Saa, and SubAB (*16*,*21*,*33*), a comparison of the coding capacity of pO113 with pO157 showed that pO113 encoded an array of predicted adhesins and toxins that may contribute to host colonization and disease. Some of the pO113-specific genes have homologs present on the chromosome of STEC O157:H7, which suggests that they may have a common role in STEC pathogenesis and may represent an adaptation to human infection. The unique virulence genes encoded by pO113 (especially those encoding putative adhesins and toxins) may compensate for the lack of A/E lesion formation by STEC O113:H21 and may account for the high virulence of this clone. Although the exact contribution of many of these novel and unique pO113-encoded factors to the pathogenesis of STEC infections in humans remains to be determined, the presence of several genetic determinants shared between pO113 and pO157 suggests that pO113 may contribute to STEC pathogenesis in a manner analogous to pO157. In addition, given the different evolutionary origins of pO157 and pO113, the presence of similar genes in both plasmids suggests that several types of gene transfer have occurred, including transposition, phage insertion, and recombination. Indeed, in pO113 the *ehx* operon, *repA*, *espP,* and *iha* are all closely associated with remnant transposases.

MLST is commonly used to examine the relationship between *E. coli* lineages. Previous MLST studies have used examples of parallel evolution in *E. coli* clones to show that the high virulence of particular clones is not an ancestral state derived from primordial *E. coli* but rather a condition developed from parallel acquisition of bacteriophage-encoded and plasmid-borne virulence determinants (*35*). One such example of parallel evolution is seen in CGs EHEC 1 and EHEC 2. These CGs are characterized by the distinct insertion sites of the LEE pathogenicity island and different intimin subtypes (*35*). On the basis of the *ehxA* sequence, 2 LEE-positive EHEC-2 clones (isolates E45035 and EH6) did not branch according to their LEE profile, which showed a closer relationship to the LEE-negative *ehxA* sequences. This finding suggests that some LEE-positive STEC strains have acquired LEE-negative plasmids. In addition, LEE-negative O76:H7 (isolate 9816261) was more closely related to LEE-positive *ehxA* sequences. MLST demonstrated that the isolate 9816261 represented an unusual STEC ST belonging to CG 47, showing the closest evolutionary link to UPEC.

Phylogenetic analysis of a second pO113 and pO157 gene, *repA*, did not lead to the same clustering of strains as *ehxA*. The 3 clades shown in the *repA* sequence showed less distinct delineation on the basis of the presence or absence of LEE. This disagreement may result because *repA*

is not a virulence factor and, therefore, is not subject to the same selective pressure as virulence determinants, such as *ehxA*. Moreover, in pO113 and pO157, *repA* is located next to remnant transposases, and the *repA* sequence may reflect the mosaic structure of the large plasmids rather than their ancestral genetic origin. In addition, we cannot be certain that all the *repA* sequences examined here were located on Ehx-encoding plasmids. Indeed, 2 LEE-positive STEC strains in our study, O26:H11 (EH34) and O147:H– (EH68), were positive for *repA* but not *ehxA,* indicating that *repA* may be present without *ehxA*. In this context, *ehxA* would appear to be a more useful and reliable marker for allelic profiling of the large plasmids.

A previous study that examined the evolution of 56 LEE-negative O174 STEC isolates demonstrated that they fell into 4 separate evolutionary clusters (*36*). Similarly, our study of LEE-negative STECs of various serogroups demonstrated divergent evolution; several clonal groups were represented and 6 other STs remain unassigned. STEC O113:H21 (strain EH41), from which we established the sequence of pO113, is a member of CG 30 or the STEC2 group and is closely related to other O113:H21 isolates by MLST and *ehxA* profiling, suggesting that STEC O113:H21 strains are clonal. The PCR screen that was conducted to examine the presence of pO113 genes in different STEC-2 isolates supports this idea with all but 1 of the O113:H21 strains possessing all 9 of the pO113 genes examined. The study of serogroup O174 isolates also demonstrated that the virulence gene content of these evolutionarily divergent strains is similar, confirming the idea that multiple independent lineages of STEC have acquired and maintained the same virulence gene repertoire (*36*). Similarly, we have observed this phenomenon with genes of pO113. For example, STEC strains EH43 and EH52, of CG 36 and 31, respectively, possess all 9 of the pO113 genes examined in this study, indicating their independent acquisition of pO113 or a closely related large plasmid.

We determined the genetic makeup of pO113 and highlighted the similarities and differences of pO157. We also demonstrated that LEE-negative STECs are not a clonal group of human pathogens; instead, they encompass a population of evolutionarily distinct STECs that share virulence features but appear to have acquired these features independently and in parallel, rather than from a common ancestor. Therefore, pathogenic STEC may arise when a given set of virulence genes come together in an *E. coli* host. What drives the selection of particular genes to create a STEC pathogen is unknown. However, because the existence of a primarily bovine animal reservoir of infection is a major difference between STEC and other pathotypes of *E. coli*, some genes, such as *ehxA* and *espP*, may be acquired by STEC to facilitate survival and persistence in the bovine gut (*37*,*38*). Therefore, although some determinants may not be considered essential virulence factors for human infection, they may confer an advantage to STEC survival and transmission in a different environment, such as an animal reservoir of infection.

As more plasmid and genome sequences become available, assessing the degree of genetic conservation across LEE-negative serotypes of STEC will be possible. Therefore, persistent public health surveillance and analysis of all STEC associated with human infection is essential to clarify the combination of virulence genes that lead to a STEC pathogen capable of causing serious disease, such as hemorrhagic colitis and HUS. It is critical for public health and clinical laboratories involved in pathogen diagnosis and surveillance to recognize LEE-negative STEC as a cause of human infection.

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Sources of Hepatitis E Virus Genotype 3 in the Netherlands

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Non–travel-related hepatitis E virus (HEV) genotype 3 infections in persons in the Netherlands may have a zoonotic, foodborne, or water-borne origin. Possible reservoirs for HEV transmission by water, food, and animals were studied. HEV genotype 3/open reading frame 2 sequences were detected in 53% of pig farms, 4% of wild boar feces, and 17% of surface water samples. HEV sequences grouped within 4 genotype 3 clusters, of which 1 is so far unique to the Netherlands. The 2 largest clusters contained 35% and 43% of the animal and environmental sequences and 75% and 6%, respectively, of human HEV sequences obtained from a study on Dutch hepatitis E patients. This finding suggests that infection risk may be also dependent on transmission routes other than the ones currently studied. Besides the route of exposure, virus characteristics may be an important determinant for HEV disease in humans.

Hepatitis E virus (HEV) is an RNA virus that causes liver inflammation in humans, predominantly in developing countries. In the 1990s, serologic studies among blood donors in industrialized countries showed that anti-HEV seropositivity also occurred among a small percentage (1.1%–1.4%) of persons without a travel history to a hepatitis E–endemic region (*1*,*2*). Later studies confirmed sporadic hepatitis E cases contracted in Europe, the United States, and other industrialized regions (*3*). HEV strains detected in mammals can be classified into 4 major genotypes that are represented by Burmese isolates (genotype 1), Mexican isolates (genotype 2), US isolates (genotype 3), and recent Chinese isolates (genotype 4) (*3*). In addition, virulent and avirulent HEV strains that infect birds have recently been identified (*4*,*5*). In industrialized countries, non–travel-related HEV infections are caused by genotype 3 (Europe, United States, Japan, New Zealand, Argentina) and genotype 4 (Japan, People's Republic of China).

A possible role for zoonotic transmission in the epidemiology of human HEV episodes has been suggested after viral RNA was detected in different animal species, and these viruses were found to be closely related to HEV strains found in humans. The first animal in which HEV genotype 3 was identified and characterized was pig in the United States (*6*). HEV strains of genotypes 3 and 4 have since been detected in pigs in many other countries, and these strains were found to be genetically closely related to HEV strains originating from humans in the same geographic region (*7*,*8*). Serologic studies have also indicated a broad host range of HEV. In many animal species such as cows, cats, dogs, rodents, and mongooses, immunoglobulin G to HEV was detected by using several serologic tests. However, HEV RNA was not detected in these animals and because of the lack of positive reference materials to evaluate these tests, the results must be interpreted with caution (*9*–*12*).

Assuming a zoonotic source for HEV infections, exposure to reservoirs of HEV might occur through contact with infected animals and animal products. Consumption of contaminated food or drinking water or contact with contaminated surface waters may also expose humans to HEV. In Japan, identical fragments of HEV were obtained from strains isolated from deer, wild boar meat, and patients with hepatitis E who had consumed this meat (*13*–*15*).

In the Netherlands, the first report of non–travel-related HEV infections was published in 2003 (*16*). Since then, ≈10 cases/year of non–travel-related HEV infections have been diagnosed (*17*,*18*). In 2004–2006, a descriptive case study was performed to generate hypotheses about possible risk factors and transmission routes for non–travel-related

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HEV infections in the Netherlands (*19*). However, no conclusive evidence for a specific transmission route was observed. Therefore, we studied possible reservoirs for HEV transmission by water, food, and animals in the Netherlands. To assess possible differential risks of exposure, we compared these environmental sequences with sequences obtained from Dutch HEV patients whose conditions had been diagnosed in the same period.

Materials and Methods

Fecal Samples

Pooled fecal samples were collected from 97 pig farms located throughout the Netherlands (20–60 fresh stool specimens per farm) throughout 2005 (*20*). Individual fecal samples from 50 pigs (5 pigs from each of 10 farms) were collected at a slaughterhouse in the southern part of the Netherlands in November 2006. Fecal samples were collected from 150 muskrats (*Ondatra zibethicus*) that were caught in 2 regions in the Netherlands, in the northeast (Groningen) and in the southeast (Limburg) (*21*). Individual fecal samples were collected from 26 wild boars in National Park De Hoge Veluwe in the center region of the Netherlands (Gelderland) in 2005. Two fecal samples obtained from 2 pigs on 1 farm in 2002 were included in this study. Fecal samples were stored as 50% suspensions in 15 g/L of Trypton Soya broth (CM 129; Oxoid, Cambridge, UK) and 10% glycerol at –70°C until testing.

Water Samples

From September 2004 through July 2005, twelve large-volume water samples (230 L–260 L) were collected from the Meuse River monthly by using a conventional filter adsorption–elution method and concentrated by ultrafiltration by using a cellulose-acetate filter (nominal molecular weight limit of 10,000) under high pressure (3 bar) (*22*). Resulting concentrates were stored at –70°C.

Extraction of RNA

RNA was extracted from 100 μL of 10% pooled fecal suspensions from pigs or 0.1% fecal suspensions from muskrats according to the method Boom et al. (*23*). The QIAamp viral RNA mini kit (QIAGEN, Venlo, the Netherlands) was used to extract RNA from 140 μL of 10% fecal suspensions from individually sampled slaughterhouse pigs and 1% fecal suspensions from wild boars as described by the manufacturer. RNA was isolated from 12.5 μL of ultrafiltered water concentrate as described (*22*).

Reverse Transcription–PCR for HEV

Reverse transcription–PCR (RT-PCR) was performed on 10-fold serially diluted RNA samples with primers HEVORF2con-s1 and HEVORF2con-a1, which were specific for the conserved open reading frame (ORF) 2 region, as described (*24*,*25*). An internal control RNA was included in the RT step to monitor for inhibition of the RT-PCR (*20*). A nested RT-PCR was used to detect ORF1 sequences coding for nonstructural proteins by using primers HEVConsORF1-s1 and HEVConsORF1-a1 (*24*) for the first round of amplification and primers ConsORF1-s2 and ConsORF1-a2 (*26*) for the second round as described (*25*). Negative controls were used, and measures were taken to prevent contamination by complete separation of activities for first- and second-round PCRs in time and space. Animal and environmental samples were analyzed in the Laboratory for Zoonoses and Environmental Microbiology of the National Institute for Public Health and the Environment (RIVM) in Bilthoven. Human sequences were obtained from the Laboratory for Infectious Diseases and Perinatal Screening of RIVM. Use of 2 physically separated laboratories excluded possible cross-contamination of human, animal, and environmental samples. Numbers of viral genomes in samples by RT-PCR (PCR-detectable units [PDUs]) were estimated as most probable numbers as described (*27*).

Sequencing and Phylogenetic Analysis

HEV RT-PCR products positive by hybridization (ORF2) or of the correct size (ORF1) were subjected to electrophoresis on a 2% agarose gel, excised, purified by using a Qiaquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). HEV RT-PCR products for which no sequences were obtained by direct sequencing were cloned into a pCRII-TOPO vector (Invitrogen, Breda, the Netherlands); ≥5 clones per product were sequenced. Nucleotide sequences without primer sequences were aligned and clustered by maximum-parsimony analysis in BioNumerics version 4.6 (Applied Maths, Kortrijk, Belgium) and corresponded with analysis of a 242-nt fragment of ORF1 and a 148-nt fragment of ORF2. Human HEV sequences were obtained from human serum samples used for diagnosis of acute viral hepatitis (*18*,*19*).

Results

Survey of Possible Sources of HEV

Ten-fold serially diluted RNA samples extracted from animal samples and river water were analyzed for HEV ORF2 sequences by RT-PCR. The highest prevalence of HEV ORF2 RNA in 51 (53%) of 97 pooled fecal samples was detected in pig farms housing pigs 5–27 weeks of age (Table 1). A prevalence of HEV RNA of 14% was detected in feces of pigs ≈6 months of age and ready for slaughter, which corresponded with a prevalence of 30% on pig farms.

Table 1. Detection of hepatitis E virus RNA by RT-PCR for the ORF2 region of the genome in environmental samples, the Netherlands*

Source	Origin	Sample type	Sampling year	Matrix	No. samples	ORF2 (nt 6298–6494)†	
						No. (%) PCR positive	Sequence
Pig	Pig farm	Pooled	2005	Feces	97	51 (53)	36‡
		Individual	2002	Feces	2	2 (100)	2
	Slaughterhouse	Individual	2006	Feces	50	7 (14)	7
	Butcher shop/ supermarket§	Individual	2005	Liver	62	4 (6)	3
Wild boar	National Park	Individual	2005	Feces	26	1 (4)	1
Muskrat	Southeast	Individual	1998–1999	Feces	150	0	0
Water	Meuse River	Filtered	2004–2005	Concentrate	12	2 (17)	2

*RT-PCR, reverse transcription–PCR; ORF, open reading frame.
†Position in Burmese hepatitis E virus strain (GenBank accession no. M73218).
‡Obtained by direct sequencing.
§Obtained from Bouwknegt et al. (*28*).

HEV RNA was detected in 1 (4%) of 26 wild boar fecal samples. Muskrat fecal samples contained many inhibitory factors for RT-PCR. Therefore, samples were tested at several dilutions (10%–0.1% vol/vol feces). HEV RNA was not detected in any of the muskrat fecal samples, although the possibility that some of the samples had false-negative results cannot be excluded because in 24 fecal samples no internal control RNA was detected.

HEV RNA concentrations in positive fecal samples varied from 10^3 to 10^6 PDU/g. In 2 (17%) of 12 river water samples analyzed, HEV RNA was detected at concentrations of 2 PDU/L to 100 PDU/L.

Phylogenetic Analyses of HEV in Animal and Environmental Samples (2004–2006)

Thirty-six newly generated nucleotide sequences of 148 nt of HEV ORF2 were obtained from pooled fecal samples from pig farms that were previously shown to be positive for HEV ORF1 sequences (*20*). Nine nucleotide sequences of HEV ORF2 were obtained from individually sampled pigs either on a pig farm or during slaughter. The latter samples represented the infectious status of pigs at the time of consumption. One HEV ORF2 sequence was obtained from an HEV-positive wild boar fecal sample and 2 from HEV-positive water samples (Table 1). These sequences, together with 3 previously published HEV sequences detected in Dutch pig livers (*28*), were compared with available genotype 3 HEV strains from animals and humans (www.ncbi.nlm.nih.gov) by phylogenetic analysis. This comparison showed that all belonged to HEV genotype 3.

Sequences grouped within 4 previously proposed genotype 3 clusters (3a, 3c, 3e, and 3f) (*3*) (Figure 1), of which cluster 3c is unique to the Netherlands. Comparison of newly generated HEV ORF2 sequences obtained from pooled fecal pig samples with the previously published HEV ORF1 sequences from the same samples showed identical clustering in 4 HEV genotype 3 clusters (*20*). HEV genotype 3e sequences were not detected in the Netherlands until 2005. At 1 pig farm sampled in 2002, an unrelated HEV genotype 3 variant was detected. Most HEV sequences obtained from animals and water detected during 2004–2006 grouped within cluster 3f (43%): 18 HEV sequences isolated from pig farms, 1 from pig liver, and 1 from water, with identities of 87.2% to 97.3% (Table 2). The second largest cluster (3c) included 35% of environmental HEV sequences and contained 14 HEV sequences obtained from pig farms, 1 HEV sequence from pig liver, and 1 HEV sequence from wild boar. The identities ranged from 88.5% to 100%. Six sequences obtained from pig farms grouped within clusters 3a, with similarities ranging from 96.0% to 99.3%. Three HEV sequences obtained from pig liver, feces, and water grouped within cluster 3e. Analysis of the geographic distribution of all Dutch environmental HEV sequences by postal code showed that HEV sequences belonging to the 2 major clusters (3c and 3f) were distributed randomly. Too few HEV strains within clusters 3a and 3e were available to be informative for geographic distribution.

Phylogenetic Analyses of HEV ORF1 Sequences

Two sequences in cluster 3f obtained from different pig farms were 100% identical within the 148-nt fragment of ORF2. A total of 242 nt of ORF1 were sequenced and showed that the 2 sequences were 98.8% identical within this region of the HEV genome. This result indicated that the strains were similar but not identical.

Comparison of Environmental HEV Sequences with Human HEV Sequences

Dutch environmental and animal HEV sequences obtained during 2004–2006 were compared with HEV ORF2 sequences from a study on Dutch hepatitis E patients without a travel history who received a diagnosis during 2004–2006 (Figure 2) (*19*). Nucleotide identities between environmental isolates and between human and environmental isolates were similar, as shown in Table 2. These results suggest that variability in HEV strains circulating in Dutch pigs, wild boar, and surface water is similar to variability in strains circulating in humans.

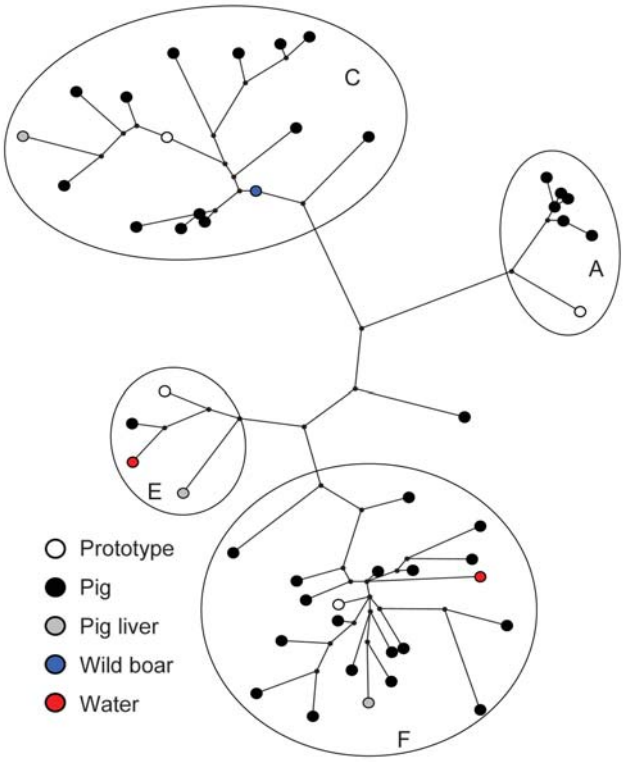

Figure 1. Maximum-parsimony tree of hepatitis E virus (HEV) sequences detected in pig, wild boar and water samples, based on a 148-nt sequence of open reading frame 2 (nt 6322–6469 of strain M73218). Sources of Dutch sequences and genotype 3 clusters are indicated. Sequences are compared with prototype sequences of different clusters of HEV genotype 3. Prototypes correspond with the following GenBank accession nos.: A) US1, AF060668; C) NLSW105, AF336298; E) UK-swine p354, AF503511; F) G1, AF110391. The following accession numbers have been used for phylogenetic analysis of isolates from surface water: EU526620, EU526626, from wild boar; EU526642, from pig liver; DQ916142–DQ916144, from pig feces; and DQ996399, EU526606–EU526619, EU526621–EU526625, EU526627–EU526641, EU526643–EU526647.

Human sequences grouped within the same 4 HEV genotype 3 clusters, with most of the sequences clustering within Dutch cluster 3c. A relatively high percentage of the sequences in cluster 3c is of human origin (43%). In this cluster, 1 human sequence was 100% identical with a porcine HEV sequence. For confirmation of homology, ORF1 RT-PCRs were performed, but only the pig sample yielded an ORF1 sequence. No direct geographic evidence could be established for an association between the patient and the pig farm; the patient who contracted the HEV infection in 2005 lived in the northern region of the Netherlands, but the pig farm was located in the eastern region. Comparison of postal codes of other Dutch patients without a recent travel history with postal codes of environmental sample locations did not show any geographic clustering.

In cluster 3f, only 1 (5%) of 21 sequences identified during 2004–2006 was of human origin. Comparison with HEV sequences derived from human serum samples showed 2 close homologies, 1 between sequences from a hepatitis E patient in 2005 and a pig (96.6%) and 1 between sequences from a patient in 2006 and a surface water sample in 2005 (97.3%).

Discussion

Evidence was obtained for the presence of HEV in food, water, and animals in the Netherlands. Although 4 genotypes of HEV are known, 3 of which have been detected in pigs, each HEV ORF2 fragment sequenced in our study was identified as genotype 3. The highest prevalence (53%) was found on pig farms housing pigs 5–27 weeks of age, which is consistent with studies reporting that most HEV infections in pigs occurred between 2 and 4 months of age (*6*,*29*). However, our study on pigs of 6 months of age, the approximate age at slaughter, showed that HEV RNA was present in 14% of the pig feces samples, which corresponds to 30% of the pig farms. HEV RNA was detected in 6% of commercial pig livers, which indicated that pigs may still be a source of HEV during slaughter. This finding suggests that swine veterinarians (*30*,*31*) and other professionals, such as sewage workers (*32*), slaughterhouse workers, and butchers, who have close contact with pigs or pig products, may be exposed to the virus by working with pigs. This suggestion is supported by the fact that we detected HEV RNA in wastewater resulting from rinsing pig intestines during slaughter (S.A. Rutjes, unpub. data).

The percentage of HEV RNA–positive commercial livers was approximately half that of HEV-positive feces at the time of slaughter, which may be caused by metabolic degradation of the virus in liver tissue or by frequent freezing of livers before they are sold in supermarkets or butcher shops, which may reduce the amount of viral RNA. Alternatively, differences in HEV prevalence detected in livers and that detected in feces may be caused by differences in assay sensitivity or different distribution patterns in the 2 sample matrices. In 2 recent studies, contaminating virus in pig livers was shown to be infectious, but the risk for infection by consumption of properly cooked pig livers was extremely small (*33*,*34*).

Rodents have been suggested to be a reservoir for HEV on the basis of high seroprevalences (≤73%) (*11*,*35*). We used RT-PCR to examine whether muskrats are a potential reservoir for HEV. Although no RNA was detected in muskrat fecal samples, muskrats cannot be excluded as a reservoir because of high concentrations of RT-PCR inhibitors in feces, which implies that false-negative results may have been obtained. Alternatively, HEV strains present in muskrats may not have been detected by the HEV RT-PCR used in the current study.

Table 2. Nucleotide identities between environmental and human HEV strains that circulated during 2004–2006, the Netherlands*

Cluster	Nucleotide identities between environmental HEV strains†			Nucleotide identities between human and environmental HEV strains†			Nucleotide identities between human HEV strains†		
	No. strains compared	Minimal, %	Maximal, %	No. strains compared	Minimal, %	Maximal, %	No. strains compared	Minimal, %	Maximal, %
3a	6	96.0	99.3	7	96.6	99.3	1	–	–
3c	16	88.5	100	28	87.2	100	12	88.2	100
3e	3	91.2	96.6	5	92.6	97.3	2	93.3	97.3
3f	20	87.2	97.3	21	88.5	93.9	1	–	–

*HEV, hepatitis E virus; –, no identities (1 human HEV sequence was present).
†Based on a 148-nt sequence of open reading frame 2 (nt 6322–6469 of strain M73218).

In addition to animal sources, HEV genotype 3 was detected in 17% of samples studied from the Meuse River. Several studies have shown that HEV originating from pigs and humans is consistently present in sewage water (*36*,*37*), which implies that surface waters may be contaminated by sewage overflows or discharge of insufficiently treated sewage water. The Meuse River runs from France through Belgium into the Netherlands and is used for recreational purposes and drinking water. Thus, water from this river is a potential source for exposure to HEV.

HEV concentrations ranging from 10^3 PDU/g feces to 10^6 PDU/g feces and from 0.002 PDU/mL to 0.1 PDU/mL were detected in surface water. Although these are results for genome copies and not infectious viruses, it may be concluded that in the sources examined in this study, pig feces contained the highest numbers of (infectious) virus. To confirm this hypothesis, samples should be tested for infectious virus by in vivo infection experiments (*28*) or cell culture (*38*,*39*).

In 1998–1999, HEV sequences detected in Dutch pigs grouped within clusters 3a, 3c, and 3f (*25*). In the current study, strains of an additional fourth cluster of HEV, genotype 3e, were detected in pig and surface water samples and in 2 patients whose illnesses had been diagnosed in 2005 and 2006. This result indicates that although this 3e variant was not previously detected in the Netherlands, it is now present in various sources and may have emerged a few years ago. Cluster 3c comprises 35% of animal and environmental HEV sequences and 75% of human HEV sequences and is unique to the Netherlands (*20*). This geographic clustering of genotype 3 strains has been reported in several countries (*3*). Sequences of subtypes 3e and 3f have also been detected in other European countries and Japan, whereas subtype 3a sequences have been detected mainly in the United States, Japan, and South Korea, which suggests that these subtypes may have been introduced in the Netherlands by travelers or commercial trade involving HEV-infected pigs. One genotype 3 variant detected on 1 pig farm was unrelated to variants in available databases or any human strain so far detected in the Netherlands.

The percentage of human sequences within the 2 largest clusters (3c and 3f) showed large differences (43% vs. 5%). This finding suggests that HEV 3c strains may be more pathogenic to humans, more stable in the environment, or are shed in higher numbers. Strains of feline calicivirus with different pathogenicities obtained from distinct outbreaks did not show conserved changes in virus genomes. Nevertheless, strains with higher pathogenicity infected tissue culture cells more efficiently and showed earlier cytopathic effects (*40*). To determine whether such differences in pathogenicity are also present between 3c and 3f HEV variants, a cell culture assay for HEV is needed. Despite increasing knowledge of replication and packaging of HEV in somatic cells, an efficient cell culture method is currently not available (*38*,*39*).

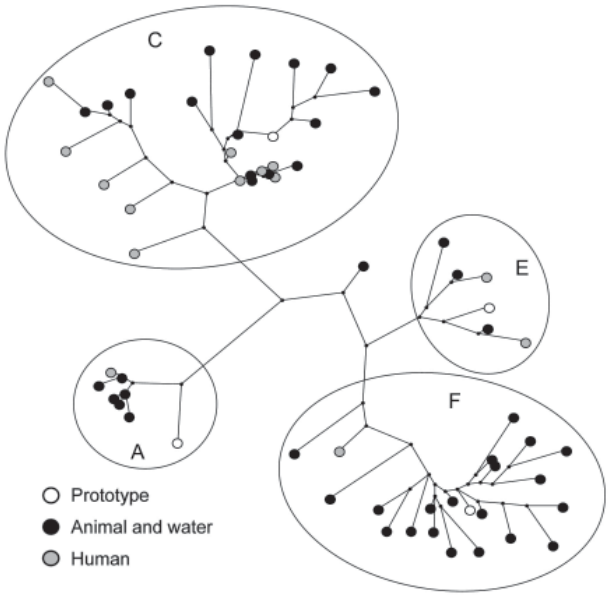

Figure 2. Maximum-parsimony tree of hepatitis E virus (HEV) sequences detected in environmental samples and patients during 2004–2006, based on a 148-nt sequence of open reading frame 2 (nt 6322–6469 of strain M73218). Origins of HEV sequences and genotype 3 clusters are indicated. Sequences are compared with prototype sequences of different clusters of HEV genotype 3. Prototypes correspond with the following GenBank accession nos.: A) US1, AF060668; C) NLSW105, AF336298; E) UK-swine p354: AF503511; F) G1, AF110391. The following accession numbers have been used for phylogenetic analysis of human isolates: AB385844–AB385848, AB385850–AB385852, DQ200279, DC200282–DQ200284, DQ200287, DQ200289, DQ200292, DQ200293. Accession numbers of animal and environmental samples are those in Figure 1.

Of 46 animal and environmental HEV sequences, 2 strains were 100% identical on a fragment of 148-nt of ORF2, encoding the viral capsid protein. Of 16 human isolates, 2 strains were 100% identical. One HEV sequence of 148 nt of ORF2 from a patient whose condition was diagnosed in 2005 was identical to a porcine HEV strain detected in the same year. The fact that of the 46 animal and environmental HEV sequences only 2 sequences were identical indicates that sequence variability in this short fragment is high, which is suggestive of a high mutation rate in this part of the genome. Conversely, variation within herds appeared to be low (S.A. Rutjes, unpub. data) (*15*), which argues against a high mutation rate. To better understand the role of similarities of ≈100% between mutual environmental HEV strains and human strains, mutation rates of HEV in individual pigs should be studied by longitudinal follow up.

In this study, several sources of HEV have been identified that are suggestive for risk factors such as contact with pigs or wild boars or their food products, as well as consumption of those products in undercooked conditions. To reduce exposure and infection by introduction of efficient intervention measures, transmission routes have to be identified. Furthermore, different distributions of human HEV sequences between the 2 largest clusters (3c and 3f) suggest that the route of exposure and the virus subtype will play a role in HEV infection and disease in humans.

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Integron-mediated Multidrug Resistance in a Global Collection of Nontyphoidal *Salmonella enterica* Isolates

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Salmonella enterica bacteria have become increasingly resistant to antimicrobial agents, partly as a result of genes carried on integrons. Clonal expansion and horizontal gene transfer may contribute to the spread of antimicrobial drug–resistance integrons in these organisms. We investigated this resistance and integron carriage among 90 isolates with the ACSSuT phenotype (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) in a global collection of *S. enterica* isolates. Four integrons, *dfrA12/orfF/aadA2*, *dfrA1/aadA1*, *dfrA7*, and *arr2/blaOXA30/cmlA5/aadA2*, were found in genetically unrelated isolates from 8 countries on 4 continents, which supports a role for horizontal gene transfer in the global dissemination of *S. enterica* multidrug resistance. Serovar Typhimurium isolates containing identical integrons with the gene cassettes *blaPSE1* and *aadA2* were found in 4 countries on 3 continents, which supports the role of clonal expansion. This study demonstrates that clonal expansion and horizontal gene transfer contribute to the global dissemination of antimicrobial drug resistance in *S. enterica*.

Salmonella enterica bacteria are a leading cause of foodborne disease worldwide (*1*,*2*). In the United States, as many as 1.4 million cases of *S. enterica*–associated disease occur annually (*3*,*4*). While usually self-limiting, salmonellosis may require antimicrobial drug treatment in infants, the elderly, or immunocompromised persons. However, antimicrobial drug resistance has become increasingly common in *S. enterica*, which can complicate therapy. The National Antimicrobial Resistance Monitoring System reported that in 2004, 15.0% of non-Typhi isolates were resistant to ≥2 classes of antimicrobial drugs, and 8.1% were resistant to ≥5 classes. The most common *S. enterica* multidrug-resistance pattern in 2004 was ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) (*5*).

Antimicrobial drug resistance can occur by point mutations in the bacterial genome or through horizontal transfer of genetic elements carrying resistance genes. Resistance may be disseminated through clonal expansion of drug-resistant strains or through horizontal transfer of genetic elements coding for resistance determinants. *S. enterica* populations change through the introduction of strains that expand and displace existing populations (*6*,*7*). Such population dynamics enable antimicrobial drug resistance in *S. enterica* to spread as a result of clonal expansion. The global dissemination of the multidrug-resistant (MDR) *S. enterica* serovar Typhimurim phage type DT104 clone is an example of the role of clonal expansion in the spread of antimicrobial drug resistance determinants across multiple countries and continents (*8*). Clonal expansion is also probably responsible for the dissemination of nalidixic acid resistance in *S. enterica* serovar Typhimurium isolates obtained in southern Asia and Africa (*9*).

Horizontal transfer of genetic material among *S. enterica* or from other bacterial species also plays an important role in the dissemination of drug resistance in this pathogen (*10*). Evidence indicates that horizontal gene transfer plays a major role in the dissemination of antimicrobial drug re-

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sistance in other bacterial species, such as *Escherichia coli* (*11*) and *Stenotrophomonas maltophilia* (*12*). The location of antimicrobial drug–resistant genes on mobile genetic elements, such as plasmids, transposons, and integrons, facilitates the mobilization of resistance from one organism to another (*13*).

Integrons are genetic structures capable of capturing and excising gene cassettes, which usually encode antimicrobial drug resistance determinants. Although integrons are not self-mobilizable, they are usually found in association with transposons and are often located on plasmids, facilitating their mobility (*13*). Integrons are thus ideally suited for the dissemination and recombination of antimicrobial drug–resistance genes. Integrons are common in *S. enterica* and make an important contribution to the extent of antimicrobial resistance in this species (*10,13,14*). Because of their plasmid and transposon association, integrons are assumed to be mobilized predominantly through horizontal gene transfer (*10*). However, the clonal nature of *S. enterica* suggests that clonal expansion may also play a role in dissemination of drug resistance. An example of clonal expansion of integron bearing *S. enterica* is the global distribution of the serovar Typhimurium DT104 clone, which harbors a genetic resistance island known as the *Salmonella* genomic island 1 (SGI1). This region contains a number of drug resistance elements including 2 integrons with the gene cassettes *blaPSE1* and *aadA2* and genes for tetracycline and chloramphenicol resistance, which are not integron associated (*15*).

Clonal expansion of integron-bearing *S. enterica* would account for the occurrence of a particular genetic lineage with a specific integron in a variety of regions. Horizontal gene transfer would account for the existence of identical integrons in isolates of different genetic lineages. To explore the roles of clonal expansion and horizontal gene transfer in the dissemination of antimicrobial drug resistance caused by class 1 integrons, we investigated the integron structure and genetic lineage of 90 MDR nontyphoidal *S. enterica* isolates from a global collection comprising >1,900 isolates from 13 countries and 6 continents. A goal of this study was to improve our understanding of the contributions of clonal expansion and horizontal gene transfer to the dissemination of integrons carrying antimicrobial drug–resistance genes in *S. enterica* to enable the development of improved strategies for the control of antimicrobial drug resistance in this organism as well as other emerging pathogens of public health importance.

Materials and Methods

Bacterial Isolates

A total of 1,920 *S. enterica* isolates were investigated; 1,743 isolates were collected by laboratories in Argentina, Australia, Belgium, Canada, Denmark, Germany, Italy, the Philippines, South Africa, Spain, Taiwan, Uganda and the United States during September 2001–August 2002 (Table 1). These isolates were collected as part of a separate study that attempted to identify a genetically and geographically diverse group of *S. enterica* isolates with reduced susceptibilities to fluoroquinolones. The isolates were not selected but rather were collected consecutively, without regard to their antimicrobial drug susceptibility. In addition, 179 isolates were collected by the Allegheny County Department of Health in Pennsylvania during 2002–2003 as part of routine surveillance. Serotyping was performed by the collecting laboratories, except for isolates from Taiwan, which were serotyped by the Pennsylvania Department of Health.

The ACSSuT resistance phenotype has become increasingly prevalent in *S. enterica*, and that phenotype has been commonly associated with class 1 integron carriage in this species. For these reasons, we selected a subset of isolates from the collection that exhibited the ACSSuT resistance phenotype for further investigation. Isolates selected for integron investigation were confirmed to be *S. enterica*

Table 1. Laboratories that provided *Salmonella enterica* isolates for this study

Country	Institution	Contact person
United States	Centers for Disease Control and Prevention, Foodborne and Diarrheal Diseases Laboratory Section, Atlanta	Timothy J. Barrett
Canada	Ontario Public Health Laboratory, Toronto	Frances Jamieson
Canada	Laboratory for Foodborne Zoonoses, Population and Public Health Branch, Guelph	Cornelius Poppe
Argentina	Centro de Estudios en Antimicrobianos, Buenos Aires	Jose Maria Casellas
Australia	Queensland Health Scientific Services, Archerfield	John Bates
Belgium	Antwerp University Hospital, Antwerp	Herman Goossens
Germany	Bundesgesundheitsministerium für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin	Andreas Schroeter
South Africa	South African Institute for Medical Research, Johannesburg	Karen Keddy
Spain	Institute of Health Carlos III, Enteric Bacteria Laboratory, Madrid	Miguel Usera
Italy	Istituto Superiore di Sanita, Rome	Alessandra Carattoli
Denmark	Hvidovre Hospital, Copenhagen	Dennis Hansen
Taiwan	National Cheng Kung University, Tainan City	Wen-Chien Ko

by PCR with primers specific for the *invA* region of the *inv* locus (*16*) (Table 2).

Antimicrobial Drug Resistance Testing

Antimicrobial drug resistance was determined by using the disc diffusion method on Mueller–Hinton agar (Becton, Dickinson and Co., Sparks, MD, USA), according to the manufacturer's directions. Susceptibility to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline was determined according to the manufacturer's breakpoints.

Integron Detection and Characterization

Genomic DNA from *S. enterica* isolates was prepared using the DNeasy Tissue Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's directions. Class

Table 2. Primers used for PCR amplification of *Salmonella enterica* integrons

Primer	Sequence (5′ → 3′)	Target	Reference
5'CS	GGCATCCAAGCAGCAAGC	5′ conserved segment	(*17*)
3'CS	AAGCAGACTTGACCTGAT	3′ conserved segment	(*17*)
int_F	CGATGCGTGGAGACCGAAACCTT	intI1	(*18*)
int_R	GTAACGCGCTTGCTGCTTGGATGC	intI1	(*18*)
invA_F	ACACAGCTCGTTTACGACCTGAAT	invA	(*16*)
invA_R	AGACGACTGGTACTGATCGATATT	invA	(*16*)
sul1_F	GCGCGGCGTGGGCTACC	sul1	This study
sul1_R	CCGCAAGGCTCGCTGGAC	sul1	This study
aadA1_R	CGATGACGCCAACTACCTCTGATA	aadA1internal primer	This study
arr2_F	ATTGTTGGCGTTGTTGAAGACTGG	arr2 internal primer	This study
cmlA5_F	GAATGGGAATGGGATGCCTGATAG	cmlA5 internal primer	This study
oxa10_R	TTTACAAAGCACGAAGACACCATT	blaOXA10 internal primer	This study
cmlA_F	GCAGGTCGCGAGGAAAGTAATG	cmlA 5′ forward primer	This study
cmlA_R	ACACCGCCCAAGCAGAAGTAGA	cmlA 3′ reverse primer	This study
blaOXA30_F	TCGCAAGAAATAACCCAAAAA	blaOXA30 internal primer	This study
aacA4_F	AAGCGGGGTTTGAGAGG	aacA4 forward primer	This study
aacA4_R	CGCGTACTCCTGGATCGGTTTCTT	aacA4 reverse primer	This study
dfr_1_F	TTTAGGCCAGTTTTTACCCAAGAC	dfrA1 internal primer	This study
ere_est_R	GCGCCAGCAGAATTATCCTTACAT	ereA2 internal primer	This study
aac(6')IIC_F	CCGCGGGATTGACCAGT	aac(6′)IIC internal primer	This study
dfrA12_F	GCTGCGCATTTTGGTTCC	dfrA12 internal primer	This study
aadA2_R	TGTCATTGCGCTGCCATTCTCC	aadA2 internal primer	This study
qacH_F	GCGTCGCCGTTCTAAATCTGCTAT	qacH internal primer	This study
aac_R	GGGCGCCGGGTGTCTGGAG	aacA4 internal primer	This study
IS_F	GTCACGCCCCGACCATCACCTTCC	IS1247 internal primer	This study
TNP_F	CCGCGCTGGCCGACCTGAAC	Transposase A internal primer	This study
ere_F	CCTAACCGGGCGATTCAA	Erythromycin esterase internal primer	This study
cmlA_R_internal	ATCACACGCCCCATAAAACGAG	cmlA internal primer	This study
arr_R2	GCGGGATCCAGAACCAGGCGACAT	arr-2 internal primer	This study
arr_accA_R	AGAGCGGCTTTGGTTCC	Internal primer arr-2–accA junction	This study
ere_F2	CGCTGATTTCGCTGTCCTGA	ereA internal primer	This study
dfrA17_F	AAAAAGGCTAACAAGTCGT	dfrA17 internal primer	This study
cml_R2	GCTGAATTGTGCTCGCTGTCGTA	cml internal primer	This study
aadA_con_F	CGACATCATYCCGTGGCGTTAT	aadA forward consensus primer	This study
aadA_con_R	CGGCAGCCACATCCTTC	aadA reverse consensus primer	This study
aacA4_F	ATGACCTTGCGATGCTCT	aacA4 internal primer	This study
aacA4_R	CTCGATGGAAGGGTTAGG	aacA4 internal primer	This study
blaOXA30_F	ACACAATACATATCAACTTCGC	blaOXA30-aadA internal primer	This study
aadA1_R_S	GGATAACGCCACGGAATGATGTC	aadA1 internal primer	This study
albany_PSE1a_F	CCTTTGGGGCCACCTACAG	blaPSE1 primer	This study
albany_PSE1b_F	ATCAAAATTATGGGGTTACTTACA	blaPSE1 primer	This study
albany_dfr1_F	ATGGTAGCTATATCGAAGAATGGA	dfr primer	This study
albany_dfr2_F	AAGTACTGGCTATTGCCTTAGGAG	dfr primer	This study
U7-L12	ACACCTTGAGCAGGGCAAAG	SGI1 left junction	(*15*)
LJ-R1	AGTTCTAAAGGTTCGTAGTCG	SGI1 left junction	(*15*)
104-RJ	TGACGAGCTGAAGCGAATTG	SGI1 right junction	(*15*)
104-D	ACCAGGGCAAAACTACACAG	SGI1 right junction	(*15*)

1 integron carriage was determined by PCR using primers specific to the *intI* region of the integrase gene. Isolates positive for integrase were further characterized by PCR using primers specific for the 5′ and 3′ conserved segments (CS) of the integron structure. PCR was performed in a 50-μL volume consisting of 1× PCR buffer II (Applied Biosystems, Foster City, CA, USA), 1.5 mmol/L $MgCl_2$, 0.1 mmol/L dNTPs, 0.33 μM forward and reverse primers, and 1.66 units Amplitaq Gold polymerase (Applied Biosystems). PCR conditions were an initial denaturation at 95°C for 5 min; 35 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 5 min plus 5 s each cycle (5 s longer in each subsequent cycle than in the previous cycle); and a final extension at 72°C for 7 min.

Long-range PCR was performed to detect integrons >2.0 kb by using the Gene Amp HiFidelity Kit (Applied Biosystems), according to the manufacturer's directions. Long-range PCR conditions were an initial denaturation at 94°C for 2 min; 10 cycles at 94°C for 15 s, 58°C for 30 s, and 68°C for 4 min, followed by 20 cycles at 94°C for 15 s, 58°C for 30 s, and 68°C for 4 min plus 5 s each cycle (5 s longer in each subsequent cycle than in the previous cycle); and a final extension at 72°C for 7 min. PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized using UV illumination on a Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA, USA). For isolates that amplified multiple integrons, PCR products were separated by gel electrophoresis and purified by using either the Qiaquick Gel Extraction Kit (QIAGEN) or the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Column Kit (Bio-Rad). Some PCR products were cloned before sequencing by using the Topo TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions.

Isolates in this study were investigated for the presence of SGI1 and variant SGI1s by PCR using published primers specific for the right (104-RJ, 104-D) and left (U7-L12, LJ-R1) junctions of the chromosomal insertion site (*19*). Isolates were considered positive for the left or right junctions of the SGI1 if they generated PCR product of the appropriate size with primers specific for that junction. By this method, isolates with the SGI1 would be positive for the left junction but not for the right junction because of the presence of a retronphage between the 104-RJ and 104-D primer sites. Isolates with variant SGI1s would be positive for the left and right junctions.

Gene Cassette Identification

Class 1 integron gene cassettes were identified by PCR and sequence analysis by using the 5′ and 3′ CS primers. Sequencing was performed with the BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Capillary sequence analysis was performed on a 3730 DNA sequence analyzer (Applied Biosystems). Sequences were analyzed and additional primers were designed by using the Lasergene 7.0.0 software package (DNAStar, Madison, WI, USA). Gene cassette homology searches were performed by using BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) (*20*).

Ninety-one of the 121 integrons found in this study were sequenced in their entirety. In some cases, when integrons were identified that were of the same size as those previously sequenced, their gene cassettes were identified by PCR using primer pairs designed in this study (Table 2).

Multilocus Sequence Typing

Genetic relatedness of isolates was assessed using multilocus sequence typing (MLST). MLST uses sequences ≈500 bp in length from 7 housekeeping genes to define a sequence type (ST). Isolates with the same alleles at all 7 loci are considered to be genetically indistinguishable by MLST and therefore define an ST. Isolates with the same alleles at 6 loci are considered to be closely related genetically.

MLST was performed using the 7-locus scheme described on the *Salmonella* MLST database (http://web.mpiib-berlin.mpg.de/mlst) (*21–24*). Visualization of PCR products and sequencing of gene fragments was accomplished as described above for integron gene cassettes. Sequences were analyzed using Bionumerics software V 5.10 (Applied Maths, Austin, TX, USA). Alleles and STs were assigned by the *Salmonella* MLST database. All isolates in this study and their associated sequence types have been deposited in the *Salmonella* MLST database.

Definitions of Horizontal Gene Transfer and Clonal Expansion

Horizontal gene transfer was defined as *S. enterica* isolates belonging to different STs (STs that differ at >1 locus) but bearing the same integron. Clonal expansion was defined as *S. enterica* isolates bearing the same integron and belonging to the same ST or STs differing at only 1 locus but occurring in >1 location. Because isolates in this study were collected consecutively over a limited time in each location, and because source was the only epidemiologic information available, we could not determine whether genetically related isolates bearing the same integron in a given location were part of an outbreak or whether the isolates reflected clonal expansion beyond an outbreak. Therefore, *S. enterica* isolates collected from 1 location that belonged to the same ST and harbored identical integron structures were considered to be 1 isolate for classification as either horizontal gene transfer or clonal expansion.

Results

ACSSuT Resistance

Of the 1,920 isolates initially screened by antimicrobial drug susceptibility testing, 104 (4.9%) exhibited the ACSSuT resistance phenotype. The proportion of ACSSuT-resistant isolates ranged from 0% in Australia, Argentina, Belgium, and Canada to 19% in Taiwan and South Africa (Table 3).

Integron Detection and Characterization

Of the 104 nontyphoidal *S. enterica* isolates with the ACSSuT resistance phenotype, 90 (86.5%) were positive for the integrase gene and amplified gene cassette regions by PCR with primers for class 1 integron 5′ and 3′ CS regions. Most cassette-positive isolates contained either 1 (n = 61, 68%) or 2 (n = 26, 29%) integrons (online Appendix Table, available from www.cdc.gov/EID/content/15/3/388.htm). Three isolates contained ≥3 integrons. Although 16 different integrons were found in this collection, 19 distinct integron profiles could be identified because of multiple integrons in some isolates (online Appendix Table). Six integrons contained only 1 gene cassette: *aacA4*, *aadA2*, *aadB*, *blaPSE1*, *dfrA7*, or *dfrA15*. Seven integrons contained 2 or 3 gene cassettes (listed in order of cassette occurrence in the individual integron): *aadB/catB3, aac3A-Id/aadA7*, *blaOXA30/aadA1*, *dfrA12/orfF/aadA2*, *dfrA1/orfC*, *dfrA1/aadA1*, and *tnpA/dfrA7*. A 4.0 kb integron containing the cassettes *arr2/cmlA5/blaOXA10/aadA1*was found alone or in combination with an integron containing the single gene cassette *aacA4*. Two other unique large integrons were found in this collection: a 5.8-kb integron with the gene cassettes *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* and a 6.0-kb integron with cassettes *aac(6')-IIc/ereA2/IS1247/aac3/arr/ereA2*.

A total of 121 class 1 integrons were identified. Of these, 91 were fully sequenced (online Appendix Table). With 2 exceptions, genetic drift in gene cassette sequences was not observed. One integron containing the single cassette *aadA2* showed 1 base difference from other *aadA2* gene cassettes found in this study (a T→C transition at position 39 of the gene cassette, resulting in a synonymous change). One *blaOXA30* gene cassette exhibited a point mutation (A→G) at position 31, also a synonymous change. All other gene cassettes within the study showed 100% nucleotide identity to GenBank reported cassettes, except for the *dfrA17* gene cassette identified in the unique 5.8-kb integron in the Stanley isolates from Taiwan (online Appendix Table). This cassette showed 91% sequence identity to a gene cassette found in uncultured bacteria (GenBank accession no. FM179325) and in *E. coli* (GenBank accession no. EU687490).

Most gene cassettes found in this study confer resistance to the aminoglycosides (*aadA1*, *aadA2*, *aadA7*, *aadB*, *aacA4*, *aac*, *aac3A-Id*, *aac(6')-IIc*), trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*, *dfrA15*, *dfrA17*), and β-lactams (*blaPSE1*, *blaOXA10*, *blaOXA30*). Other resistance cassettes included those coding for chloramphenicol resistance (*cmlA, cmlA5, catB3*), erythromycin resistance (*ereA2*), rifampin resistance (*arr2*), and resistance to quaternary ammonium compounds (*qacH*). Because resistance to the aminoglycoside streptomycin and the β-lactam ampicillin were selection criteria for isolates in this study, the predominance of cassettes encoding resistance to those antimicrobial drugs is not unexpected.

Phenotypic resistance to chloramphenicol was also a selection criterion, but only 3 integrons contained gene cassettes for this resistance. None of the integrons identified in this study carried genes coding for tetracycline resistance, although phenotypic resistance to this antimicrobial drug was also an inclusion criterion. Because the SGI1 contains genes for chloramphenicol and tetracycline resistance, which are not located within integrons, tetracycline- and chloramphenicol-resistant isolates that are SGI1 positive most likely contain these genes. Alternatively, resistance to chloramphenicol and tetracycline may be encoded elsewhere on the chromosome or on structures other than integrons in isolates that are not positive for the SGI1.

The SGI1 was identified in 17 isolates (19%) (online Appendix Table). Twelve of the SGI1-positive isolates were serovar Typhimurium, belonged to ST 19, and showed the integron pattern (*blaPSE1*, 1.0 kb and *aadA2*, 1.2 kb) characteristic of serotype Typhimurium phage type DT 104. An additional Typhimurium ST 19 isolate containing 4 integrons, including *blaPSE1* and *aadA2*, was also positive for SGI1. Four serotype Albany isolates ST 292 were positive

Table 3. Source and ACSSuT resistance in a global collection of *Salmonella enterica* isolates*

Source	Total no. isolates	No. (%) ACSSuT-resistant isolates
Argentina	148	0
Australia	146	0
Belgium	66	0
Canada	144	0
Denmark	153	8 (5.2)
Germany	150	1 (0.7)
Italy	156	3 (1.9)
Philippines	67	6 (8.9)
Spain	151	8 (5.3)
South Africa	160	30 (18.8)
Taiwan	150	29 (19.3)
United States/ACHD	179	8 (4.5)
United States/CDC	150	1 (0.7)
Uganda	100	10 (10.0)
Total	1,920	104 (5.4)

*ACSSuT, ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline; ACHD, Allegheny County Health Department; CDC, Centers for Disease Control and Prevention.

for a variant SGI1, which includes the integrons *dfrA1/orfC* and *blaPSE1* (*15*). This result indicates chromosomal location of these integrons in these isolates.

Genetic Relatedness of Integron-bearing Nontyphoidal *S. enterica*

The 90 integron-containing *S. enterica* isolates represented 17 different STs. Thirty-three (37%) Typhimurium isolates belonged to ST 19 or to STs that differ from 19 at only 1 locus. These isolates contained 10 different class 1 integrons, which combined to create 12 integron profiles (online Appendix Table). Eleven (12%) isolates of serovar Isangi belonged to ST 216 or to STs that differ from 216 at 1 or 2 loci. These isolates contained 3 integrons and 4 integron profiles. The remaining isolates represented diverse STs, which differed from each other at 6 or 7 of the MLST loci.

Integron Distribution Across Nontyphoidal *S. enterica* Genetic Lineages

This study identified 5 class 1 integrons that were distributed across different genetic lineages, supporting the role of horizontal gene transfer in the dissemination of antimicrobial drug resistance in nontyphoidal *S. enterica*. The class 1 integron *dfrA12/orfF/aadA2*, which confers resistance to trimethoprim and streptomycin/spectinomycin, was identified in *S. enterica* from 5 different serotypes belonging to 5 STs (Figure, panel A). This integron was geographically widespread, being found in isolates from Europe, the United States, Taiwan, the Philippines, and South Africa. The integron containing the single trimethoprim-encoding *dfrA7* cassette was present in isolates from 2 serotypes, 2 STs and 3 different areas (Figure, panel B). The integron *dfrA1/aadA1* was found in isolates from 3 serotypes, 2 STs, and 2 areas (Figure, panel C).

Isolates of ST 216, 335, 336, and 337 (serovar Isangi) and ST 19 (serovar Typhimurium) from South Africa contained an identical 4.0 kb integron not previously reported in *S. enterica*, with the cassettes *arr2/cmlA5/blaOXA10/aadA1* (Figure, panel D). In some serovar Typhimurium ST 19 isolates, this integron was found in combination with the *aacA4* integron. The presence of a unique integron in genetically unrelated isolates from the same area indicates

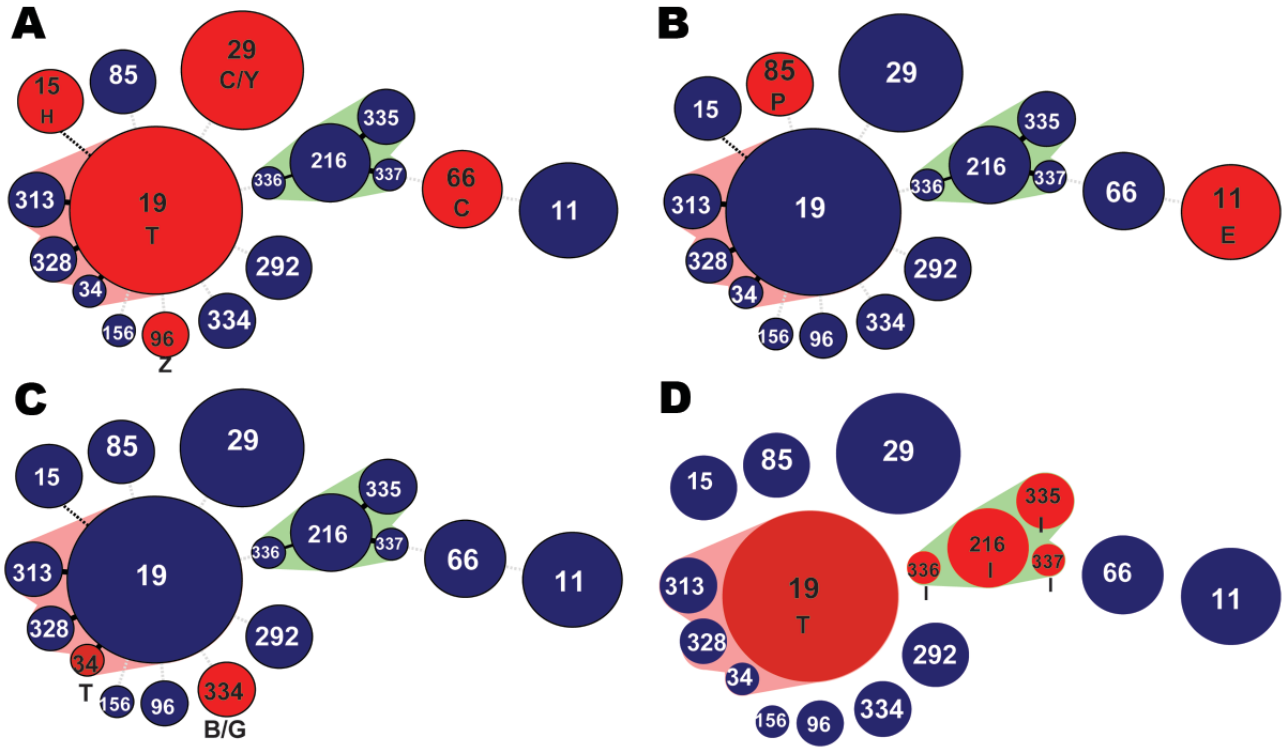

Figure. Minimum spanning trees depicting integron distribution across *Salmonella enterica* genetic lineages. A) dfrA12/orfF/aadA2; B) dfrA7; C) dfrA1/aadA1; D) arr2/blaOXA30/cmlA5/aadA2. Circles represent unique sequence types (STs). Red circles represent the STs that carried the integron involved in horizontal gene transfer. Numbers in circles represent the ST. Circle size reflects number of isolates in each ST. Pink and green shading indicates closely related groups of isolates. Letters refer to serotypes: B, Brandenburg; C, Cholerasuis; E, Enteriditis; H, Heidelberg; G, Goettingen; I, Isangi; P, Paratyphi A; Z, Schwarzengrund; Y, Stanley; T, Typhimurium. Geographic sources of isolates are as follows: Panel A: ST66, serotype C, Taiwan; ST29, serotype C, Taiwan; ST29, serotype Y, Taiwan; ST96, serotype Z, Denmark and Taiwan; ST19, serotype T, US Centers for Disease Control and Prevention and South Africa; ST15, serotype H, Philippines; Panel B: ST11, serotype E, Uganda and South Africa; ST85, serotype P, Denmark; Panel C: ST334, serotype G, Spain; ST334, serotype B, Spain; ST34, serotype T, Germany; Panel D: ST19, serotype T, South Africa; ST216, ST335, ST336, and ST 337, serotype I, South Africa.

that this integron may have been horizontally transferred.

An integron containing the single gene cassette *aacA4* was found alone in isolates of serovar Isangi with ST 335 from Uganda and South Africa. This integron was also found in serovar Typhimurium isolates with ST 19, both alone and in combination with the integron *arr2/cmlA5/blaOXA10/aadA1*. These isolates belong to different genetic lineages, in that they differ at all 7 MLST loci. The occurrence of the same integron in these genetically unrelated isolates further supports a role for horizontal gene transfer in the dissemination of antimicrobial drug resistance in nontyphoidal *S. enterica*.

Evidence for Clonal Expansion of Integron-mediated MDR in *S. enterica*

The serotype Typhimurium phage type DT104 integron pattern, with 2 class 1 integrons of sizes 1.0 and 1.2 kb and bearing the gene cassettes *blaPSE1* and *aadA2*, was found in 12 (13%) serovar Typhimurium isolates of ST 19 (Table 4) and in 1 additional serovar Typhimurium ST 19 isolate, which also contained 2 other integrons. This result is consistent with the hypothesis that a common ancestor has undergone clonal expansion in a number of areas.

Serovar Typhimurium isolates of ST 19 from Taiwan and ST 328 from the Philippines contained 2 class 1 integrons with the cassettes *aadB/catB3* and *blaOXA30/aadA1*. The integron *blaOXA30/aadA1* was also found alone in an isolate of serovar Typhimurium ST 328 from Taiwan and in combination with an integron containing the cassette *aadB* in an isolate of serovar Typhimurium ST 313 from South Africa. ST 313 and ST 328 are closely related to ST 19, differing from it at only 1 locus (ST 313 differs from ST 19 at the MLST locus sucA, ST 328 and ST 19 differ at MLST locus aroC; see online Appendix Table). Therefore, these isolates are all closely related, and their integrons may represent an instance of clonal expansion.

Although the class 1 integron *dfrA12/orfF/aadA2* appears to have been circulated through horizontal gene transfer, this integron has also spread through clonal expansion of nontyphoidal *S. enterica*. This integron appeared in isolates of serovar Schwarzengrund ST 96 in Taiwan and Denmark and in serovar Typhimurium isolates of ST 19 in the United States and South Africa. Similarly, integrons with the single gene cassettes *aacA4* and *dfrA7* also exhibited both horizontal gene transfer and clonal expansion. The *aacA4* integron was found in serovar Isangi isolates of ST 335 in Uganda and South Africa. The *dfrA7* integron was found in serovar Enteritidis isolates of ST 11, also in Uganda and South Africa.

Discussion

To better understand the dissemination of integron-mediated antimicrobial drug resistance, this study characterized the class 1 integrons and genetic lineages associated with 90 multidrug-resistant isolates obtained from a global collection of nontyphoidal *S. enterica*. Integrons found in this collection were diverse in size, gene cassette combination and distribution, and presented evidence for roles of clonal expansion and horizontal gene transfer.

Horizontal gene transfer is an important factor in the dissemination of antimicrobial drug–resistance genes, particularly when those genes are associated with mobile elements such as plasmids, transposons, and integrons (*10*,*11*,*14*). In this study, the widespread distribution of the *dfrA12/orfF/aadA2* integron among several STs and across several distinct regions is an example of horizontal gene transfer, as is the presence of the integrons *dfrA7*, *dfrA1/aadA1*, and *arr2/cmlA5/blaOXA10/aadA1* in different genetic

Table 4. Evidence of clonal expansion among isolates from a global collection of *Salmonella enterica* isolates*

Integron profile	No. isolates	Serotype	Sequence type	Source
blaPSE1, aadA2	4	Typhimurium	19	United States/ACHD
	2	Typhimurium	19	Spain
	3	Typhimurium	19	Italy
	3	Typhimurium	19	South Africa
blaOXA30/aadA1	1	Typhimurium	328	Philippines
	2	Typhimurium	19, 328	Taiwan
	1	Typhimurium	313	South Africa
aadB/catB3	1	Typhimurium	19	Taiwan
	1	Typhimurium	328	Philippines
dfrA12/orfF/aadA2	1	Schwarzengrund	96	Taiwan
	1	Schwarzengrund	96	Denmark
dfrA12/orfF/aadA2	1	Typhimurium	19	United States/CDC
	2	Typhimurium	19	South Africa
aacA4	1	Isangi	335	South Africa
	1	Isangi	335	Uganda
dfrA7	6	Enteritidis	11	Uganda
	2	Enteritidis	11	South Africa

*ACHD, Allegheny County Health Department; CDC, Centers for Disease Control and Prevention.

backgrounds (when assessed by MLST). These integrons are potentially capable of transmitting drug resistance to other *S. enterica* isolates or to other bacteria.

Integrons are widely distributed among bacterial species. The integron found in the greatest number of different genetic lineages in this study, *dfrA12/orfF/aadA2*, has been previously reported in a number of species, including *E. coli* (GenBank accession no. AF335108, unpub. data), *Serratia marcesens* (Genbank accession no. AF284063, unpub. data), and *Salmonella* (*25*). The integron *dfrA1/aadA1* has been documented in *E. coli* from Turkey (*26*) and cited in *E. coli* in GenBank entries from India (GenBank accession no. EF417897, unpub. data) and Kenya (GenBank accession no. EF417897, unpub. data), in *Klebsiella pneumonia* from Poland (GenBank accession no. AY007807, unpub. data), and in *S. enterica* (GenBank accession no. AM746675, unpub. data). Transfer of integrons between different bacterial species has been documented in the clinical setting, which poses a serious threat to containment of nosocomial infections (*27*). The existence of identical integrons in different types of bacteria and the ability of these integrons to be transferred in vivo indicates that many bacteria acquire integrons from a common pool. This is an important consideration in efforts to halt the development and spread of antimicrobial drug resistance.

In this study, clonal expansion of *S. enterica* appears to be responsible for a large fraction of the dissemination of drug resistance integrons. Although several integrons demonstrated evidence of clonal expansion, including *aacA4*, *aadB/catB3*, *blaOXA30/aadA1*, *dfrA7*, and *dfrA12/orfF/aadA2,* the strongest evidence is presented by the prevalence and geographic ubiquity of the serotype Typhimurium DT104 pattern clone, in which the resistance-encoding integrons (*blaPSE1* and *aadA2*) are chromosomally integrated. These integrons are still mobilizable (*28*) but chromosomal location may make them more likely to be disseminated through clonal expansion than through horizontal gene transfer, particularly in the absence of antimicrobial selective pressure.

Our study assessed mechanisms of dissemination of integrons in a collection that is more genetically and geographically diverse than is typical for studies of integrons in *S. enterica*. MLST, used for assessment of genetic relatedness of isolates in this study, is better suited to analysis of global populations than other commonly-used methods, such as pulsed-field gel electrophoresis. In addition, this study focused on the relative contributions of clonal expansion and horizontal gene transfer to the dissemination of class 1 integron borne genes coding for antimicrobial drug resistance, which has not previously been well explored.

Antimicrobial drug resistance is a serious and increasing problem in *S. enterica* and in other gram-negative pathogens, such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, *K. pneumoniae* and *E. coli* (*29*). The genes that code for antimicrobial drug resistance in these pathogens have proven to be remarkably mobile and widely distributed within and between species. The dissemination of integrons bearing antimicrobial drug resistance gene cassettes in *Salmonella* and other bacteria is a complex process that involves both the horizontal transfer of mobile genetic elements and the expansion of particularly fit clones. The combined effect of these mechanisms is that, if integrons confer an adaptive benefit caused by the presence of antimicrobial drug selective pressure or if clones harboring these integrons have increased fitness caused by other factors, then the integrons may disseminate rapidly both geographically and among diverse species. It is important to understand these mechanisms of transmission to develop methods for surveillance and control of antimicrobial drug resistance.

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Coccidioidal Pneumonia, Phoenix, Arizona, USA, 2000–2004

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Community-acquired pneumonia (CAP) often results in severe illness and death. In large, geographically defined areas where *Coccidioides* spp. are endemic, coccidioidomycosis is a recognized cause of CAP, but its frequency has not been studied extensively. To determine the frequency of patients with coccidioidomycosis, we conducted a prospective evaluation of 59 patients with CAP in the Phoenix, Arizona, area. Of 35 for whom paired coccidioidal serologic testing was performed, 6 (17%) had evidence of acute coccidioidomycosis. Coccidioidal pneumonia was more likely than noncoccidioidal CAP to produce rash. The following were not found to be risk factors or reliable predictors of infection: demographic features, underlying medical conditions, duration of time spent in disease-endemic areas, occupational and recreational activities, initial laboratory studies, and chest radiography findings. Coccidioidomycosis is a common cause of CAP in our patient population. In the absence of distinguishing clinical features, coccidioidal pneumonia can be identified only with appropriate laboratory studies.

Coccidioidomycosis is caused by infection with *Coccidioides* spp., which consist of the nearly identical *Coccidioides immitis* and *C. posadasii* that grow in the soils of the desert southwestern United States and in limited areas of Central and South America (*1*). When soil is disrupted, airborne arthroconidia can be inhaled, causing infections in humans and animals. These infections can be asymptomatic or can produce illness of varying severity, from mild, self-limited respiratory infection to severe, life-threatening pneumonia (*2*). In a small percentage of patients, *Coccidioides* spp. may spread beyond the pulmonary tract, most frequently to the cutaneous, osteoarticular, or central nervous systems (*2*).

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The incidence of coccidioidomycosis has increased dramatically from 2.5 cases/100,000 persons in 1996 to 8.4 cases/100,000 persons in 2006 in California (*3*) and from 21 cases/100,000 in 1997 to 91 cases/100,000 in 2006 in Arizona (*4*). Clinicians in disease-endemic areas are usually aware of coccidioidomycosis but often do not consider the diagnosis in patients who initially have respiratory symptoms (*5*). A possible explanation for this oversight may be uncertainty about the frequency of coccidioidomycosis as a cause of acute community-acquired pneumonia (CAP). Valdivia et al. (*5*) reported that 29% of patients with CAP in Tucson, Arizona, had coccidioidomycosis. To determine the frequency of coccidioidal pneumonia in a second sample of the population, we studied patients with CAP in the Phoenix, Arizona, metropolitan area.

Methods

We evaluated patients with acute CAP who were admitted to our hospital or who sought care in the emergency department or ambulatory family practice and internal medicine outpatient clinics of our institution's multispecialty referral practice and primary care practice in the Phoenix area. Patients were enrolled during February 2000–November 2004. This study was reviewed and approved by the Mayo Clinic Institutional Review Board.

Patients were eligible for enrollment if they had acute signs and symptoms of pneumonia (including but not limited to fever, cough, dyspnea, chills, and rash) and radiographic evidence of a pulmonary infiltrate identified by an independent radiologist not on the study team. Patients were excluded if they were <18 years of age, were already receiving oral or parenteral antifungal treatment, had radiographically documented pneumonia predating the current illness but within the past 3 months, were unable to return

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for subsequent serologic tests, or were otherwise unwilling to provide informed consent. Patients were also excluded if the evaluating physician had initially suspected coccidioidomycosis and had already ordered a serologic test for *Coccidioides* spp. as part of the initial clinical evaluation.

After goals and requirements of the study were explained to patients and informed consent was obtained, a pen-and-paper questionnaire was given to each patient for completion. The questionnaire collected information about patients' residence in disease-endemic regions, occupations, daily activities, and previous medical conditions. Within 24–48 hours of enrollment, participants had blood samples collected for serologic tests; these tests were repeated 6–8 weeks later. After all patients completed enrollment, we analyzed the patients' clinical signs and symptoms and results of laboratory studies (including serologic studies, complete blood cell counts, erythrocyte sedimentation rates, eosinophil counts, and culture results, if obtained), chest radiographs, medical treatment, and follow-up data.

Serologic tests for *Coccidioides* spp. were enzyme immunoassay (EIA), immunodiffusion (ID), and complement fixation (CF) tests. Qualitative detection of immunoglobulin (Ig) M and IgG to *Coccidioides* spp. by using EIA was performed by using a commercially available test kit (Meridian Bioscience, Inc., Cincinnati, OH, USA). ID and CF antibody tests were performed at the laboratory of Dr D. Pappagiannis (University of California Medical Center, Davis, CA, USA).

For this study, a case of coccidioidomycosis was defined by the presence of acute signs or symptoms of respiratory infection (e.g., cough, fever, pleuritic or chest pain, or dyspnea) in combination with a radiographically demonstrated pulmonary infiltrate and positive results from paired (initial and follow-up) coccidioidal serologic tests. We considered results of paired serologic tests to be positive if we observed 1) seroconversion (an initial negative serologic result followed by a positive serologic result); 2) an initial positive serologic result followed by an increase in the number of positive qualitative test results among serologic methods (e.g., initial EIA IgM positive, IgG negative, ID negative, CF negative followed by a positive result of ≥1 of the following: EIA IgG, ID, CF); or 3) an increase in serologic titer on the second CF test.

Differences in distributions of dichotomous variables were analyzed by using the χ^2 test or the continuity-adjusted χ^2 test when appropriate. The Fisher exact test was used for comparisons with small sample size. Differences between distributions of continuous variables were analyzed by using the independent *t* test. Data analysis was performed by using the statistical software program SAS version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

Results

During February 2000–November 2004, 62 patients were enrolled in the study, 3 of whom were subsequently excluded from analysis because of incomplete questionnaires and lack of serologic results. Of the remaining 59 patients, 35 completed the requirement for paired coccidioidal serologic testing. For adequate statistical power, we wanted to enroll 175 patients. However, because of the slow accrual of patients, the study ended before we reached this target enrollment. Serologic results for the 59 study participants are summarized in Table 1.

All 35 patients who completed paired serologic testing were white, and 15 (43%) were men (Table 2). For 6 (17%) of the 35 patients (95% confidence interval [CI] 7%–34%), a diagnosis of coccidioidomycosis was based on coccidioidal antibody seroconversion. Patients with coccidioidal infection were more likely than those with noncoccidioidal CAP to have rash ($p = 0.002$). Among 6 patients with coccidioidomycosis, no association was found between coccidioidal infection and patient's sex or race, symptoms other than rash, findings on chest radiographs, or laboratory data. Coccidioidal infection had no association with specific medical conditions, occupations, or recreational activities. Patients who had coccidioidomycosis had lived somewhat less time in the disease-endemic area (mean years of residency 14.6 years, range 2.5–26 years) than had patients whose pneumonia was caused by other factors (mean years of residency 25.2 years, range 0.5–69 years); this difference was not statistically significant.

Of the original 59 patients enrolled, 24 were excluded because they lacked 1 of the 2 blood samples required for paired serologic testing. To ascertain whether bias was introduced by this exclusion, we compared demographics, symptoms, and laboratory and radiographic findings of the excluded patients with those of the 35 patients who remained in the study. The 24 excluded patients were more likely to have unilateral infiltrates (22 [92%] of 24 vs 22 [63%] of 35; $p = 0.01$). No other significant differences were identified.

Discussion

Pulmonary coccidioidomycosis is a febrile respiratory illness with symptoms similar to or identical to those of nonmycotic CAP. Common features include fever, headache, cough, chest pain, dyspnea, and fatigue. This similarity in symptoms makes it difficult to recognize coccidioidal infection in the absence of diagnostic tests. Because most patients with primary coccidioidal pneumonia have spontaneous resolution of signs and symptoms, a patient with undiagnosed coccidioidomycosis who receives antibacterial therapy may appear to respond to treatment. However, a substantial portion of patients with coccidioidal pneumonia

Table 1. Serologic test results for *Coccidioides* spp. in 59 patients, Phoenix, Arizona, USA, 2000–2004*

	First serologic result				Second serologic result				
No. patients	EIA IgM	EIA IgG	ID	CF	EIA IgM	EIA IgG	ID	CF	CF titer
Negative serologic results: completed 2 tests									
11	–	–	–	–	–	–	–	–	
5	ND	ND	–	–	ND	ND	–	–	
3	ND	ND	–	–	–	–	–	–	
6	–	–	ND	ND	–	–	–	–	
1	–	–	–	–	ND	ND	–	–	
1	–	–	ND	ND	–	–	ND	ND	
1	–	–	ND	ND	ND	ND	–	–	
1	–	–	–	–	–	–	ND	ND	
Negative serologic results: completed 1 test									
10	ND	ND	–	–	ND	ND	ND	ND	
4	–	–	ND	ND	ND	ND	ND	ND	
6	–	–	–	–	ND	ND	ND	ND	
Positive serologic results: completed 2 tests									
2	–	–	–	–	+	–	+	–	
1	ND	ND	–	–	+	–	+	+	4
1	ND	ND	–	–	–	+	–	–	
1	ND	ND	–	–	–	–	+	–	
1	–	–	–	–	–	+	+	+	16
Positive serologic results: completed 1 test									
3	+	–	–	–	ND	ND	ND	ND	
1	ND	ND	ND	ND	–	–	–	+	2

*EIA, enzyme immunoassay; Ig, immunoglobulin; ID, immunodiffusion; CF, complement fixation; ND, not done; –, negative test result; +, positive test result.

may have a protracted course and may benefit from specific antifungal treatment (*6*). Two of the authors (J.E.B. and J.D.S.) have observed that medical practitioners in the Phoenix metropolitan area often administer empiric antibacterial treatment to patients with CAP and test for coccidioidomycosis only when treatment fails (unpub. data). This combination of factors is likely to lead to underestimation and underappreciation of the likelihood of coccidioidomycosis as a cause of acute CAP.

In the current study, we diagnosed coccidioidal infection in 6 (17%) of 35 (95% CI 7%–34%) patients in the Phoenix metropolitan area. Similarly, near Tucson, Arizona, coccidioidomycosis had been identified in 16 (29%) of 55 patients (95% CI 6%–44%) who sought treatment for CAP (*5*).The CIs associated with these incidence estimates overlap considerably. If we considered all enrolled patients in the current study who had ≥1 serologic test performed (similar to the methods of the previous study), a similar number of patients (10 [16.9%] of 59) had ≥1 positive result. Therefore, no clear differences between the 2 studies emerge, despite their differences in methods.

Because coccidioidal serologic tests may be insensitive to early infection (*7*), we attempted to maximize identification of coccidioidomycosis by requiring a second specimen from the convalescent phase of the disease. Paired serologic testing was intended to eliminate the potential for false-negative and false-positive results. Of the 59 patients enrolled in our study, 3 had initial positive results for IgM by EIA but negative results for IgG by EIA and negative results by CF and ID. These 3 patients did not return for a second set of serologic tests; thus, their data (and those of several other patients who did not complete paired serologic testing for unknown reasons) were excluded from further analysis. The 3 patients positive for IgM by EIA but negative for IgG by EIA, CF, and ID may have had coccidioidomycosis. However, Crum et al. (*8*) reported that 18% of positive IgM EIA results without other serologic corroboration may be false-positive results, whereas Blair and Currier found no false-positive results in a similar cohort (*9*). In the present study, the second serologic test resulted in subsequent diagnosis of coccidioidal infection in 6 patients for whom the infection had not been identified by the initial serologic evaluation. Although the requirement for paired serologic testing made it exceedingly challenging to recruit participants and complete the study, the need for definitive serologic diagnosis was paramount.

At the time of the initial evaluation, we sought to identify and delineate signs and symptoms, laboratory findings, and characteristics of patients that would help predict coccidioidal CAP. We confirmed that rash, a symptom known to be associated with coccidioidal infection (*8*), was strongly suggestive of coccidioidal infection in patients with CAP. In contrast, Valdivia et al. (*5*) identified myalgia as the only distinguishing clinical characteristic. Perhaps because of the small number of patients

Table 2. Characteristics of 35 CAP patients with or without coccidioidomycosis, Phoenix, Arizona, USA, 2000–2004*

Characteristic	No. patients		p value
	With coccidioidomycosis (n = 6†)	Without coccidioidomycosis (n = 29†)	
Age, y, mean (range)	59.2 (38.0–80.3)	67.8 (61.8–73.8)	0.25
Male sex	2 (33)	13 (45)	0.60
White race	6 (100)	27 (93)	0.50
Location where evaluated			
Hospital	4 (67)	20 (69)	0.99
Emergency department	1 (17)	3 (10)	0.55
Outpatient clinic	1 (17)	6 (21)	0.99
Signs and symptoms			
Cough	4 (67)	27 (93)	0.12
Sputum production	1 (17)	16 (55)	0.18
Dyspnea	6 (100)	18 (62)	0.21
Fever	5 (83)	21 (72)	0.99
Chills	3 (50)	11 (38)	0.98
Night sweats	2 (33)	7 (24)	0.99
Rash	3 (50)	0	0.002
Chest radiograph findings			
Unilateral infiltrates	5 (83)	17 (59)	0.50
Bilateral infiltrates	1 (17)	8 (28)	0.96
Laboratory results			
Leukocyte count, $\times 10^9$ cells/L, mean (range)	15.1‡ (9.1–21.2)	11.0§ (9.1–12.9)	0.07
Absolute eosinophil count, $\times 10^9$ cells/L, mean (range)	2.96¶ (0.18–13)	0.12# (0.0–0.2)	0.32
Empiric coccidioidal treatment prescribed after initial visit	1 (17)	1 (3)	0.76

*CAP, community-acquired pneumonia. Values are no. (%) unless otherwise indicated.
†Unless otherwise indicated.
‡n = 6.
§n = 25.
¶n = 5.
#n = 22.

available for analysis in our study, we found that patterns on chest radiographs or other laboratory findings did not enable us to differentiate between patients with and without coccidioidal infection. There was no statistically significant association between coccidioidal infection and type of occupation or type of outdoor recreation activity. Likewise, we could not identify any statistically significant association between coccidioidomycosis and number of years of residence in the disease-endemic area, as had been noted (*10*).

Several challenges limited enrollment of participants in this study. Most common was the issue of the second (or follow-up) serologic test. Many patients were initially willing to participate, but most were unable or unwilling to return for a second serologic test and were thus not enrolled. Even among those who signed a consent form, completed a questionnaire, and provided an initial blood sample, the difficulty of returning for blood collection 6–8 weeks later was shown by the large number of exclusions (24 of 59) because of a lack of paired samples. Soon after the study started, a second challenge arose among the patients whose physicians had already ordered serologic tests. In our institution, EIA results are reported within 1 day of test submission. Thus, a patient's knowledge of this initial serologic result (whether positive or negative) often led to an unwillingness to participate. Ultimately, we revised the inclusion criteria.

Although a sizeable proportion of patients with CAP had coccidioidomycosis, we almost certainly underestimated its true frequency. We did not systematically attempt to isolate *Coccidioides* spp. from sputum or other respiratory specimens. Moreover, because the sensitivity of available coccidioidal serologic tests is <100%, some patients with coccidioidal CAP may not have been identified. In addition, we did not enroll patients whose clinical evaluation had already included coccidioidal serologic testing. Despite these known preselection biases, we identified coccidioidal infection in 17% of persons with CAP for whom no suspicion of coccidioidomycosis was present initially. We speculate that this finding is an underestimate of the true percentage of coccidioidomycosis in patients with CAP. The limited value of currently available serologic tests makes the diagnosis of coccidioidomycosis difficult, which will no doubt remain so until serologic tests are improved enough to detect early infection.

Our identification of coccidioidal infection in at least 1 of 6 patients who sought treatment for radiologically confirmed CAP in an area where *Coccidioides* spp. are endemic underscores the likelihood that this infection is a common cause of CAP. We believe that coccidioidomycosis should

be strongly considered in the differential diagnosis of all patients with CAP who reside in, or who have recently visited, a disease-endemic area.

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Characterization of Avian Influenza Viruses A (H5N1) from Wild Birds, Hong Kong, 2004–2008

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From January 2004 through June 2008, surveillance of dead wild birds in Hong Kong, People's Republic of China, periodically detected highly pathogenic avian influenza (HPAI) viruses (H5N1) in individual birds from different species. During this period, no viruses of subtype H5N1 were detected in poultry on farms and in markets in Hong Kong despite intensive surveillance. Thus, these findings in wild birds demonstrate the potential for wild birds to disseminate HPAI viruses (H5N1) to areas otherwise free from the viruses. Genetic and antigenic characterization of 47 HPAI (H5N1) viruses isolated from dead wild birds in Hong Kong showed that these isolates belonged to 2 antigenically distinct virus groups: clades 2.3.4 and 2.3.2. Although research has shown that clade 2.3.4 viruses are established in poultry in Asia, the emergence of clade 2.3.2 viruses in nonpasserine birds from Hong Kong, Japan, and Russia raises the possibility that this virus lineage may have become established in wild birds.

Highly pathogenic avian influenza (HPAI) viruses (H5N1) derived from the goose/Guangdong/1/96 (Gs/GD) lineage have spread to more than 60 countries across Eurasia and Africa (*1*–*3*). The unprecedented panzootic caused by the HPAI viruses (H5N1) has been mediated by the movement of poultry and poultry products and, in some instances (e.g., clade 2.2 viruses), by wild bird migration (*4*–*6*). After introduction, the viruses became endemic in some countries, causing repeated poultry outbreaks and spilling over to cause zoonotic infection in humans, thus posing a persistent potential pandemic threat (*7*–*9*). However, in some affected countries with substantial resources (e.g., Japan and South Korea), despite the repeated introduction of subtype H5N1 viruses that have occasionally led to associated outbreaks in poultry, early and aggressive intervention measures prevented these viruses from becoming endemic in poultry, and no human cases were detected (*2*,*10*–*13*).

HPAI viruses (H5N1) were first observed to cause outbreaks of disease in wild and captive birds in Penfold and Kowloon Parks, Hong Kong, in late 2002 and in 2003 (*14*). The Kowloon Park outbreak was concurrent with outbreaks caused by this virus in several live poultry markets and on some chicken farms in Hong Kong (*14*). Measures to improve biosecurity on farms, changes in the poultry marketing system, the introduction of rest days in poultry markets, and vaccination for all poultry entering Hong Kong markets have prevented subsequent HPAI (H5N1) outbreaks in farmed poultry in Hong Kong (*15*). No further cases of infection in live poultry markets were detected from November 2003 through June 2008, when live bird market surveillance detected incursion of a new HPAI (H5N1) virus (*2*).

The avian influenza control program in Hong Kong includes intensive active surveillance of live poultry markets, aviary bird markets, poultry farms, and migratory birds at several wetland sites in Hong Kong (*16*). In addition, avian

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influenza surveillance has been conducted on wild birds found dead (wild birds and caged birds released for ceremonial purposes are collectively referred to as wild birds in this article) (*17*). Until the recent incursion of HPAI virus (H5N1) in June 2008, no viruses of subtype H5N1 had been detected on poultry farms or in markets in Hong Kong since November 2003, although 2 HPAI viruses (H5N1) were detected in chickens smuggled into Hong Kong in 2006 (*18*). However, HPAI viruses (H5N1) have been detected every year in a variety of dead wild birds, including falcons, egrets, herons, and various passerine species (*1*,*4*,*7*,*14*,*18*,*19*).

In this study, we antigenically and genetically characterized all HPAI (H5N1) viruses isolated from the dead bird surveillance program in Hong Kong to gain insights into the evolutionary history and possible transmission pathways of the viruses. Our research shows that viruses isolated each winter from 2004 through 2007 were genetically distinct, belonging to different subtype H5N1 clades. These different clades suggest multiple introductions of HPAI virus (H5N1) reassortments into Hong Kong through wild birds. This study also demonstrates that wild birds can disseminate the HPAI virus (H5N1) and have the potential to seed areas otherwise free from the virus.

Materials and Methods

Virus Isolation and Characterization

Viruses were isolated from specimens obtained from dead wild birds (online Appendix Table, available from www.cdc.gov/EID/content/15/3/402-appT.htm) by inoculating embryonated eggs at the laboratory of the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR Government. Viruses were identified by real time–PCR and by standard hemagglutination-inhibition (HI) tests using a panel of World Organization for Animal Health's Avian Influenza Reference Laboratory antisera (Veterinary Laboratory Agency, Weybridge, UK) as previously described (*14*,*23*,*24*). All virus isolation was conducted in biosafety level 3 facilities. Details of the avian influenza (H5N1) surveillance program in Hong Kong for dead wild birds, including pathologic findings and diagnostic testing, are reported separately (*17*).

Antigenic Analysis

Antigenic characterization of the influenza viruses (H5N1) was carried out by HI assay using 5 ferret polyclonal antisera, as previously described (*24*). The ferret antisera were provided by St Jude Children's Research Hospital (Memphis, TN, USA) (duck/Hunan/101/2004 and muscovy duck/Vietnam/1455/2006) and by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (Anhui/1/2005, Indonesia/5/2005, Indonesia/CDC357/2006, Vietnam/1203/2004, and whooper swan/Mongolia/244/2005). The HI assay started at a serum dilution of 1:40.

Phylogenetic and Molecular Analysis

To understand the evolutionary history of avian influenza viruses (H5N1) isolated from wild birds in Hong Kong, we conducted whole genome sequencing of 29 avian influenza viruses (H5N1) that were isolated from dead wild birds in 2006–2008. All 8 gene segments of these viruses were characterized and phylogenetically analyzed. These data were compared with the virus sequence data for an additional 18 influenza viruses (H5N1) isolated from dead wild birds in Hong Kong in 2004–2008, with virus sequence data for the 2 viruses obtained from chickens smuggled into Hong Kong in 2006, and with all other available sequence data from the NCBI Influenza Virus Resource (*25*).

Viral RNA extraction, cDNA synthesis, PCR, and sequencing were carried out as described previously (*19*). Sequences were assembled and edited with Lasergene version 7.2 (DNASTAR, Madison, WI, USA). Se-Al version 2.0 was used for alignment and residue analysis (http://tree.bio.ed.ac.uk/software/seal). The program MrModeltest version 2.2 was used to determine the appropriate DNA substitution model and rate heterogeneity (*26*). The generated model was used in all subsequent analyses. Neighbor-joining trees were constructed with PAUP* version 4.0b (*27*), and Bayesian analysis was conducted with MrBayes version 3.1.2 (*28*) by using 2 replicates of 1 million generations with 6 chains, sampling every 100 generations. The convergence of chains and the estimation of burn-in were assessed using Tracer version 1.4 (http://beast.bio.ed.ac.uk). Estimates of the phylogenies were calculated by performing 1,000 neighbor-joining bootstrap replicates, and Bayesian posterior probabilities were calculated from the consensus of 18,000 trees after excluding the first 2,000 trees as burn-in. The full-genome sequences of 29 influenza viruses (H5N1) obtained in this study are available from GenBank under accession nos. CY036042–CY036273.

Results

Virus Isolation

From early 2004 through June 2008, most isolates of influenza virus (H5N1) from dead wild birds were detected during the cooler months (i.e., from December to the following February) (online Appendix Table). Almost all positive samples of influenza virus subtype H5N1 were isolated from a variety of dead wild birds, including falcons, egrets, herons, and various passerine species. On 2 occasions, influenza virus (H5N1) was isolated from smuggled chickens (online Appendix Table).

Phylogenetic Analysis

To understand the molecular epidemiology of the viruses isolated from the dead birds, we conducted phylogenetic analysis of the hemagglutinin (HA), neuramindase (NA), and each of the 6 internal gene segments of the viruses, along with the Gs/GD-like HPAI viruses (H5N1) isolated from different regions of Hong Kong. In the HA gene tree, the wild bird viruses fell into 2 main groups, either clade 2.3.2 or 2.3.4, with the exception of 1 virus in clade 9 (Figure). The phylogenetic placement of these viruses corresponds well with the known evolution of the influenza virus subtype H5N1 that has been documented in Asia.

The isolate detected in early 2004 (peregrine falcon/HK/D0028/2004) clustered into clade 9, which includes viruses isolated from both poultry and migratory ducks during 2003–2005 in China (Figure). The 3 viruses in clade 2.3.2 (grey heron/HK/728/2004, grey heron/HK/837/2004, Chinese pond heron/HK/18/2005) that were isolated in late 2004/early 2005 were most closely related to viruses detected in southern China and Vietnam during the same period. However, 29 of 31 viruses isolated in early 2006 and early 2007 were closely related to clade 2.3.4 viruses (represented by Dk/Fujian/1734/2005), corresponding with the time of emergence and predominance of this virus lineage (Figure, online Appendix Table). The wild bird viruses isolated from May 2007 through March 2008 belonged exclusively to clade 2.3.2, with the exception of the clade 2.3.4 virus peregrine falcon/HK/2142/2008. These clade 2.3.2 viruses (H5N1) were most closely related to previous isolates from dead wild birds from Hong Kong (peregrine falcon/HK/5211/2006 and peregrine falcon/HK/1143/2007) and to isolates from Japan and Russia (whooper swan/Hokkaido/1/2008, whooper swan/Akita/1/2008 and Ck/Primorje/1/2008) (Figure).

Phylogenetic analysis of the NA of these isolates showed a similar phylogenetic relationship to that observed for the HA (data not shown). These findings show that influenza viruses (H5N1) detected each winter from 2004 through 2007 were genetically distinct and belonged to different sublineages or clades, suggesting that multiple introductions occurred during the past 4 years.

Phylogenetic analyses of the internal gene complex showed that the viruses from dead wild birds in Hong Kong belonged to different subtype H5N1 genotypes (online Appendix Table). The virus peregrine falcon/HK/D0028/2004 clustered with those genotype Z viruses isolated from poultry in mainland China during the same period, and the 3 viruses in clade 2.3.2 that were isolated in late 2004 and early 2005 belonged to genotype V2 (*22*). The 17 viruses in clade 2.3.4 that were detected in early 2006 belonged to either genotype V (n = 6) or G (n = 10). The viruses isolated from January through June 2007, both clades 2.3.2 and 2.3.4, were mostly genotype V (n = 15), although 3 genotype V1 viruses were also detected (online Appendix Table). Genotypes V1 and V2 are reassortments of genotype V that have incorporated novel PB2 and PB1 genes (*22*).

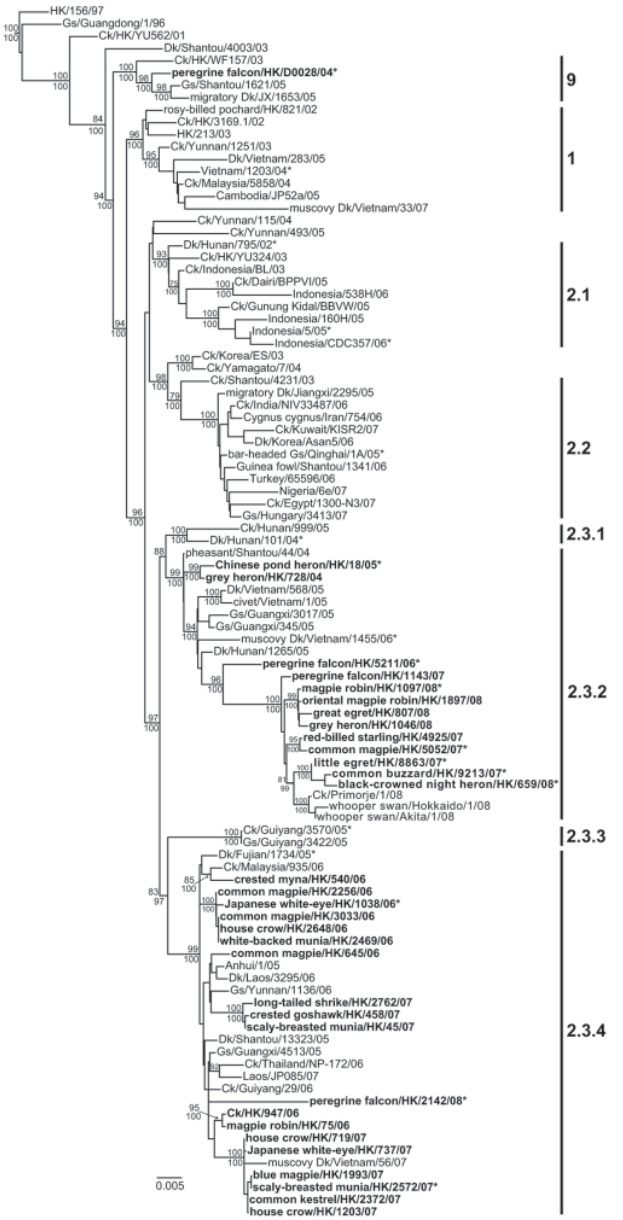

Figure. Phylogenetic relationships of the hemagglutinin genes of representative influenza viruses. Numbers above and below the branch nodes indicate neighbor-joining bootstrap values ≥70% and Bayesian posterior probabilities ≥95%, respectively. Not all supports are shown due to space constraints. Analyses were based on nt 49–1,677 and the tree rooted to duck/Hokkaido/51/1996. Numbers to the right of the figure refer to World Health Organization influenza (H5N1) clade designations (online Appendix Table, available from www.cdc.gov/EID/content/15/3/402-appT.htm). Viruses isolated from wild birds and chickens in Hong Kong during 2004–2008 are in **boldface**. *Indicates viruses included in the antigenic analysis (Table). Scale bar indicates 0.01 nucleotide substitutions per site. Ck, chicken; Dk, duck; Gs, goose; HK, Hong Kong.

Table. Antigenic analysis of influenza viruses A (H5N1) by hemagglutinin inhibition test, Hong Kong, China, 2008*

Virus	Clade†	Ferret antisera titers to: VNM1203‡ (clade 1)	IDN5 (clade 2.1)	CDC357 (clade 2.1)	MNG244 (clade 2.2)	HN101 (clade 2.3.1)	VNM1455 (clade 2.3.2)	Anhui1 (clade 2.3.4)
VNM1203	1	**640**	40	<40	<40	80	40	80
Dk/Hunan/795/2002	2.1	80	640	320	160	160	160	<40
IDN5	2.1	40	**1,280**	640	80	40	160	160
CDC357	2.1	80	2,560	**1,280**	160	80	320	320
BHG/Qinghai/1A/2005	2.2	40	320	160	**320**	160	80	40
HN101	2.3.1	40	640	160	640	**640**	320	80
CPH/HK/18/2005	2.3.2	<40	40	<40	40	80	<40	<40
VNM1455	2.3.2	40	160	160	160	160	**320**	<40
Pfalcon/HK/5211/2006	2.3.2	<40	320	160	40	80	160	<40
Common magpie/HK/5052/2007	2.3.2	<40	320	160	160	80	320	<40
Common buzzard/HK/9213/2007	2.3.2	<40	160	80	80	80	160	<40
Little egret/HK/8863/2007	2.3.2	<40	160	80	160	80	320	<40
BCN heron/HK/659/2008	2.3.2	<40	160	80	80	80	320	<40
Magpie robin/HK/109720/08	2.3.2	<40	320	160	320	160	640	<40
Ck/Guiyang/3570/2005	2.3.3	160	160	40	160	640	160	640
Dk/Fujian/1734/2005	2.3.4	80	160	80	<40	80	40	**640**
JWE/HK/1038/2006	2.3.4	80	320	160	40	640	320	1,280
SB munia/HK/2572/2007	2.3.4	80	80	40	<40	80	<40	640
Pfalcon/HK/2142/2008	2.3.4	80	<40	<40	<40	<40	<40	40
Pfalcon/HK/D0028/2004	9	320	80	40	<40	80	<40	160

*VNM1203, Vietnam/1203/2004; IDN5, Indonesia/5/2005; CDC357, Indonesia/CDC357/2006; MNG244, whooper swan/Mongolia/244/2005; HN101, duck/Hunan/101/2004; VNM1455, muscovy duck/Vietnam/1455/2006; Anhui1, Anhui/1/2005; Dk, duck; BHG, bar-headed goose; CPH, Chinese pond heron; HK, Hong Kong; Pfalcon, peregrine falcon; BCN heron, black-crowned night heron; Ck, chicken; JWE, Japanese white-eye; SB munia, scaly-breasted munia. **Boldface** numbers indicate titers to prototype viruses.
†Clade designations according to the World Health Organization influenza (H5N1) nomenclature system (*21*).
‡Ferret antisera dilution started at 1:40.

Eight viruses isolated from November 2007 through March 2008 also belonged to genotype V, and 2 isolates (little egret/HK/8550/2007 and peregrine falcon/HK/2142/2008) were novel reassortments. The genetic diversity of these viruses confirms the multiple introductions of influenza viruses (H5N1) to Hong Kong.

Two thirds (12/18) of the clade 2.3.2 viruses were isolated from nonpasserine hosts, mostly species of egret, heron, and raptors (online Appendix Table). In contrast, 3 (11%) of 28 viruses in clade 2.3.4 were isolated from nonpasserine hosts, excluding the viruses from the 2 chickens. Although inconclusive, this pattern suggests that clade 2.3.2 viruses may have an adaptation that enables them to infect and cause disease in nonpasserine species more easily than in other bird species.

Molecular Characterization

All 29 viruses characterized were highly pathogenic with variations of the multibasic cleavage site in the HA molecule. However, all clade 2.3.4 viruses had a Gln→Leu substitution at position –9 from the cleavage site (LRERRRK-RG), a factor consistent with previous reports (*18*). The receptor-binding pocket of the HA1 retains amino acid residues 222-Gln and 224-Gly (H5 numbering used throughout) that preferentially bind to α-2,3-NeuAcGal receptors (*29*–*31*). Other amino acid residues relevant to receptor-binding sites were identical to those of HK/156/1997 and Gs/GD-like viruses in most isolates. However, all clade 2.3.2 viruses characterized had an HA Ser129Leu substitution, a factor previously observed in both clade 1 and 2 viruses (*8*,*32*). The clade 2.3.2 virus grey heron/HK/3088/2007 also had a Lys212Arg substitution (*30*).

In the NA amino acid sequences, all isolates characterized had 274-His, indicating sensitivity to oseltamivir (*33*). One virus (common buzzard/HK/9213/2007) had a Ser31Asn substitution in the M2 protein, a change that may confer resistance to the adamantanes and that has been present in all Clade 1 viruses characterized to date. This substitution has also been sporadically detected in other H5N1 lineages (*34*). No amantadine-resistant mutations were observed in the remaining isolates. None of these viruses have the Lys627 residue commonly found in Qinghai-like (clade 2.2) viruses (*6*).

Antigenic Analysis

Two of the clade 2.3.4 representative viruses (Japanese white-eye/HK/1038/2006 and scaly-breasted munia/HK/2572/2007) showed good reactivity against the clade 2.3.4 antiserum, but peregrine falcon/HK/2142/2008 was markedly less reactive with a ≥4-fold reduction in titer (Table). Also, peregrine falcon/HK/2142/2008 was poorly reactive against all ferret antisera tested. The pattern of re-

activity of the clade 2.3.2 viruses from dead wild birds was similar to that of the homologous virus (muscovy duck/Vietnam/1455/2006) (Table).

Discussion

Genetic and antigenic characterization of HPAI wild bird viruses (H5N1) suggests that they are closely related to viruses isolated in Asia during the same time (*1*,*7*,*18*). During this period, an intensive avian influenza (H5N1) surveillance program was conducted concurrently on poultry farms and at markets in Hong Kong, and no subtype H5N1 viruses were detected from late 2003 until June 2008, when it was detected in fecal droppings in retail poultry markets (*2*). Thus, the repeated finding of influenza virus (H5N1) from dead wild birds in the absence of local poultry infection demonstrates the potential of wild birds to disperse the virus over at least moderate distances (i.e., tens or hundreds of kilometers).

The present study also demonstrates the role of the Hong Kong SAR as a sentinel for detecting emerging infectious diseases in Asia. It further demonstrates that surveillance of avian influenza virus (H5N1) in dead wild birds can play a key role as an early warning system for the introduction of this virus, a factor consistent with experience elsewhere (e.g., in Germany, United Kingdom, Russia). A similar strategy of conducting surveillance on wild birds would be useful for other regions in monitoring for these viruses that have the potential to infect a wide range of hosts, including humans (*2*,*35*).

Viruses isolated from January through March 2007 were, with 1 exception, clade 2.3.4 viruses, and were mostly isolated from passerine birds. From 2005 through 2007, clade 2.3.4 viruses became the dominant virus detected in live poultry markets in southern China and were detected in outbreaks of disease in poultry in Laos, Malaysia, Thailand, and northern Vietnam (*2*,*19*,*36*). However, the emergence of clade 2.3.2 viruses as the only viruses detected in wild birds, both passerine and nonpasserine, in the winter of 2007/2008 in Hong Kong is notable. Whether the detection of this clade reflects a dominance of this virus within poultry flocks in the wider region is unknown because little recent genetic data on influenza viruses (H5N1) are available from poultry in the region. Alternatively, the clade 2.3.2 is possibly adapted to wild birds, just as the clade 2.2 viruses appear to be (*37*). Phylogenetically similar clade 2.3.2 viruses of subtype H5N1 have been recently isolated from dead wild swans (whooper swan/Hokkaido/1/2008 and whooper swan/Akita/1/2008) in Japan and from chicken in Russia (Ck/Primorje/1/2008).

The establishment of another influenza virus (H5N1) lineage in wild birds, if indeed this establishment has occurred, has potentially far reaching consequences with the possibility of the long range spread of clade 2.3.2 viruses in a manner similar to the spread of clade 2.2 viruses (*2*,*6*,*37*). This potential for spread, along with the fact that some clade 2.3.2 viruses are antigenically distant from current avian influenza vaccine candidates, highlights why a clade 2.3.2 virus, common magpie/HK/5052/2007, has been recently recommended as an avian influenza (H5N1) vaccine candidate by the World Health Organization (*38*). These developments indicate a need for more intensive surveillance in the region and may also have implications for vaccination programs for poultry.

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Prevalence and Seasonality of Influenza-like Illness in Children, Nicaragua, 2005–2007

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Although information about seasonality and prevalence of influenza is crucial for development of effective prevention and control strategies, limited data exist on the epidemiology of influenza in tropical countries. To better understand influenza in Nicaragua, we performed a prospective 2-year cohort study of influenza-like illness (ILI) involving 4,276 children, 2–11 years of age, in Managua, during April 2005–April 2007. One peak of ILI activity occurred during 2005, in June–July; 2 peaks occurred during 2006, in June–July and November–December. The rate of ILI was 34.8/100 person-years. A household risk factor survey administered to a subset (61%) of participants identified the following risk factors: young age, asthma, and increasing person density in the household. Influenza virus circulation was confirmed during each ILI peak by laboratory testing of a subset of samples. Our findings demonstrate a high rate of ILI, with seasonal peaks, in children in Nicaragua.

Influenza is a major health threat throughout the world, causing substantial illness and death each year (*1*). In temperate regions, the epidemiology and prevalence of influenza have been the topic of extensive investigation. However, data on the epidemiology of influenza in tropical countries are limited (*2*,*3*). Information concerning seasonality and prevalence is crucial for development of effective regional and global seasonal influenza prevention strategies as well as pandemic influenza control measures (*3*,*4*). This scarcity of data can be attributed to limited laboratory resources and capabilities, as well as to the lower priority given to influenza compared with other infectious diseases in these regions. Furthermore, among the small body of evidence that does exist about influenza in the tropics, insufficient data have been reported on its seasonality. Several studies have reported no defined influenza season (*5*–*7*), some have reported that influenza seasonality coincides with the rainy season (*8*–*10*), and others have reported 1 or 2 seasonal peaks of influenza activity per year (*11*–*13*).

In the absence of laboratory confirmation, potential influenza cases can be identified with a clinical definition of influenza, influenza-like illness (ILI). However, multiple respiratory viruses, including respiratory syncytial virus, parainfluenza viruses, adenovirus, and rhinovirus, can cause similar signs and symptoms (*14*). Among these viruses, only respiratory syncytial virus causes seasonal epidemic peaks in children. Because respiratory syncytial virus is most common in children ≤5 years of age, limiting the analysis to children >5 years of age can increase the probability that a peak in ILI is due to influenza virus (*15*,*16*). In addition, ILI has been shown to be more specific for influenza when influenza virus is circulating in an area (*17*). Finally, a more specific clinical definition can be used to increase the likelihood that ILI cases are influenza. Despite the absence of 1 commonly accepted definition for high-probability ILI, using a definition of fever ≥38.2°C with cough has been found to have a high positive predictive value (83%) for laboratory-confirmed influenza (*18*). To study influenza in Nicaragua, a developing country in Central America, we conducted a cohort study to evaluate the prevalence and seasonal pattern of ILI and its associated risk factors among children.

Materials and Methods

Study Site

Managua is the capital of Nicaragua and the largest city in the country; its estimated population is 1.4 million. The Health Center Sócrates Flores Vivas (HCSFV), a public pri-

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mary care facility located in District II of Managua, served as the study site. HCSFV provides medical care for the ≈62,500 persons who reside in the surrounding catchment area.

Study Population

We used information collected through the Nicaraguan Pediatric Dengue Cohort Study, a prospective cohort study established in August 2004 to study pediatric dengue infection. Recruitment of children 2–9 years of age was conducted by door-to-door visits in neighborhoods served by HCSFV. Children were ineligible to participate if their parents or guardians reported a history of any disease or treatment that might suppress the immune system, such as HIV/AIDS or treatment for cancer. To maintain the age structure of the cohort, during July and August of each year, additional participants 2 years of age were enrolled. Age range by the end of the 2-year study was 2–11 years. All participants were provided medical treatment and tests by study physicians at HCSFV free of charge, 24 hours per day, 365 days per year. A study ambulance was available at all times to transfer any child requiring hospitalization or emergency services not available at HCSFV. To determine the percentage of children who did not regularly attend HCSFV when ill and to encourage attendance, home visits were conducted at least 1 time per year. All children were contacted by study medical personnel each year during July or August; if the child could not be located after at least 3 attempts, the child was considered lost to follow-up.

Our analysis covered 2 years, from April 16, 2005, through April 15, 2007. As a part of the study enrollment process, study personnel used questionnaire forms that asked questions in a systematic way to gather demographic information and medical history. Subsequently, a household risk-factor questionnaire was administered at home visits and at HCSFV; children with completed household risk-factor questionnaires at the beginning or during the first few months of the time period analyzed in this study were selected as the convenience sample. The study was approved by the institutional review boards at the University of California at Berkeley and the Nicaraguan Ministry of Health. Informed consent was obtained from the parent or legal guardian of each participant, and participants ≥6 years of age completed assent procedures.

Surveillance and Case Definition

Parents or guardians agreed to bring participants to HCSFV at the first sign of fever and for all medical appointments. Medical information from all visits was recorded on forms for systematic data collection at the time of consultation. The Centers for Disease Control and Prevention (CDC) definition of influenza-like illness was used: fever ≥37.8°C at presentation with a cough and/or sore throat. ILI episodes were considered to be the same episode if they occurred within a week of each other, if they had the same date of fever onset, or if the physician denoted them as a continuing illness. High-probability ILI was defined as a fever ≥38.2°C at presentation with a cough (*18*). To eliminate the younger ages in which respiratory syncytial virus, a common seasonal respiratory virus, is often prevalent, a portion of the analysis was restricted to children ≥6 years of age (*15*,*16*).

Laboratory Methods

Paired serum samples were collected for a subset of participants during each peak of ILI activity (11 paired samples from the 2005 peak, 15 from each of the 2 peaks in 2006, and 10 from nonpeak periods of each year); the first sample was collected at the initial visit, and the second was collected 2–4 weeks later. The hemagglutination inhibition test was performed by using the standardized reagents and protocols provided by the World Health Organization. Results were considered positive for influenza if a ≥4-fold rise in hemagglutination inhibiting antibody titer was noted between acute- and convalescent-phase samples. During December 2006–January 2007, a total of 51 nasal and throat swab samples were collected and analyzed by reverse transcriptase–PCR (RT-PCR). Swabs were collected from children who had fever or a history of fever and cough and/or sore throat and symptoms for ≤5 days. RNA was extracted from 140 μL of viral transport medium containing the swabs by using the QIAamp Viral RNA Isolation Kit (QIAGEN, Valencia, CA, USA) and amplified by using the Access RT-PCR System (Promega, Madison, WI, USA); primers were directed to influenza A and influenza B viruses according to the standard operating procedure of CDC's Influenza Branch.

Statistical Methods

Total person-time was determined by the amount of time that a participant was enrolled in the cohort. For those lost to follow-up, person-time was determined by adding the known person-time between enrollment or the beginning of the study period and last contact with the study to half the amount of time between the last contact with the participant and the official loss date. Incidence was calculated as the number of ILI episodes divided by the person-time multiplied by 100. A Poisson distribution was used to calculate 95% confidence intervals (CIs) for the incidence rates. Weekly incidence was graphed and smoothed by using Lowess (*19*) with a 3-week moving average. Incidence rate ratios (IRRs) were used as the measure of relative risk. General estimating equations with a Poisson distribution were used to estimate IRRs. General estimating equations were chosen to account for the longitudinal nature of the study; most participants contributed 2 person-years of time. To check the assumption that the ILI events were uncor-

related, the models were also run with a negative binomial distribution; however, the results did not significantly differ from those of the models with a Poisson distribution, and therefore the Poisson distribution was used in all models. Robust standard errors with an exchangeable correlation structure were used to estimate the 95% CIs for IRRs. Model selection was performed by using a backward stepwise procedure. The χ^2 test was used to compare proportions. All analysis was conducted using STATA version 9.2 (STATA Corp, College Station, TX, USA).

Results

Demographic Characteristics

A total of 4,276 children contributed 7,449 person-years of time to the study. Of these, 3,240 (75.8%) children contributed 2 full years of time, 555 (13%) 2-year-old children were enrolled during yearly maintenance enrollment, 118 (2.8%) children were withdrawn from the study, and 363 (8.5%) were lost to follow-up. Children were withdrawn from the study if their parents requested it, if they moved from the study area, or if they did not follow study procedures. Cohort characteristics are summarized in Table 1. Attendance at HCSFV was high; 83.3% of participants had ≥1 medical visit, and 94.1% of those with fever visited HCSFV by the fourth day after fever onset. At the time of home visits, only 1.9% of participants reported having sought medical care outside of the cohort.

Incidence of ILI

A total of 2,596 episodes of ILI yielded an incidence rate of 34.8 episodes per 100 person-years (95% CI 33.5–36.2). Decreasing incidence was noted for each 1-year increase in age (Figure 1). Rates for boys and girls did not differ significantly (Table 2). Of the participants, 38% had had ≥1 episode of ILI; of those with ILI, the mean number of ILI episodes per child was 1.6 (range 1–8). Among children with ILI, the rate of hospital transfer for evaluation and possible admission was 14 per 1,000 person-years.

ILI episodes occurred with marked seasonality; they peaked during June–July in both years and again during November–December of the second year (Figure 2, panel A). The same pattern was present when a high probability of influenza definition was used (fever ≥38.2°C with cough) and when the analysis was restricted to children ≥6 years of age (Figure 2, panels B and C). Analysis of a subset of paired serum samples under each peak showed that all 3 peaks were likely due, at least in part, to influenza; 64% of the June 2005 peak samples were positive for influenza A virus (H3N2), 27% of the June 2006 peak samples were positive for influenza B virus, and 20% of the November–December 2006 peak samples were positive for influenza A virus (H1N1) and 7% for influenza B. All samples from nonpeak times were negative for influenza A and B viruses. RT-PCR of 51 samples from the November–December 2006 peak confirmed the hemagglutination inhibition results: 10 (20%) were positive for influenza A virus. Conversely, no influenza B virus-positive samples were identified among the samples collected in June 2005, and no influenza A virus (H3N2)–positive samples were detected in November–December 2006.

Demographic and Medical Risk Factors

Demographic and medical risk factor information was available for all cohort participants. Young age was the strongest predictor of ILI; risk decreased with each increasing year of age (Table 3). Having received a prior diagnosis of asthma was a strong predictor of ILI; risk was 1.79 times higher for children with asthma (95% CI 1.56–2.06) than for other children, after adjusting for age and sex. No

Table 1. Baseline characteristics of cohort of children 2–11 years of age, Managua, Nicaragua, 2005–2007

Characteristic	All cohort participants, no. (%), n = 4,276	Participants who completed household survey, no. (%), n = 2,615	p value*
Sex			0.731
F	2,114 (49.4)	1,304 (49.9)	
M	2,162 (50.6)	1,311(50.1)	
Age, y			<0.001
2	755 (17.7)	566 (21.6)	
3	473 (11.0)	330 (12.6)	
4	539 (12.6)	348 (13.3)	
5	504 (11.8)	317 (12.1)	
6	453 (10.6)	253 (9.7)	
7	462 (10.8)	263 (10.1)	
8	460 (10.8)	227 (8.7)	
9	413 (9.7)	207 (7.9)	
10	217 (5.1)	104 (4.0)	
Asthma			0.001
Yes	259 (6.1)	213 (8.1)	
No	4,276 (94.0)	2,402 (91.8)	

*Determined by χ^2 test.

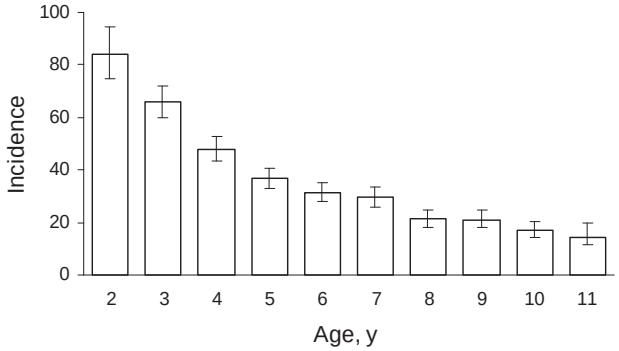

Figure 1. Age-stratified incidence (cases/100 person-years) of influenza-like illness in cohort of children 2–11 years of age in Nicaragua. Error bars indicate SEM.

significant difference in the risk for ILI was noted between boys and girls.

Household Risk Factors

Information concerning socioeconomic and household risk factors (hereafter referred to as household factors) was available for 2,615 (61%) participants (Table 1). The cohort and the subset of children sampled for household factors were similar with regard to sex distribution but differed slightly in age distribution and asthma status. Neighborhood geographic distribution of the entire cohort and of those with household risk factor data did not differ significantly (p = 0.98). Socioeconomic information on those surveyed is shown in Table 4. Household factors collected and included in the model were number of people living in the house, person density (determined by number of people living in the household divided by the number of rooms), mother's literacy status, mother's educational level, type of floor, and presence of a toilet or latrine. Electricity and running water in the house were not included in the model because >99% of the cohort had access to each (at least some of the time). The final model contained person density in the household, mother's literacy status, type of floor, and the child's age, asthma status, and sex (Table 5). A trend of increasing risk for ILI was noted with increasing person density in the household; homes with 3.0–4.9 persons per room had an RR of 1.07 (95% CI 0.96–1.20), and those with ≥5 persons per room had an RR of 1.18 (95% CI 1.04–1.34) compared with households with <3 persons per room. Having a literate mother was protective against ILI (RR 0.79; 95% CI 0.64–0.98). Although not retained in the final model, in the univariate analysis the mother's educational level displayed a U-shaped pattern; risk decreased until completion of primary school and then increased with completion of secondary school or college.

Discussion

We have documented a substantial amount of illness and the seasonal variation of ILI in a large cohort of children in Nicaragua, a tropical developing country. A high level of ILI (34.8 episodes/100 person-years) was found for all

Table 2. Incidence of influenza-like illness in cohort of children 2–11 years of age, Managua, Nicaragua, 2005–2007*

Characteristic	Person-years, %†	ILI episodes‡	Incidence/100 person-years	95% CI
All participants	7,449.4	2,596	34.8	33.5–36.2
Year				
2005–2006§	3,704.5	1,106	29.9	28.1–31.7
2006–2007¶	3,745.0	1,490	39.8	37.8–41.9
Sex				
M	3,778.7	1,280	33.9	32.1–35.8
F	3,670.7	1,316	35.9	34.0–37.8
Age, y				
2	339.2	285	84.0	74.8–94.4
3	721.8	475	65.8	60.2–72.0
4	871.1	418	48.0	43.6–52.8
5	931.6	342	36.7	33.0–40.8
6	916.3	287	31.3	27.9–35.2
7	854.2	252	29.5	26.1–33.4
8	852.7	181	21.2	18.3–24.6
9	815.0	172	21.1	18.2–24.5
10	709.5	122	17.2	14.4–20.5
11	438.0	62	14.2	11.0–18.2
Asthma				
Yes	447.8	322	71.9	64.5–80.2
No	7,001.7	2,274	32.5	31.2–33.8

*ILI, influenza-like illness; CI, confidence interval.
†Person-years were determined by dividing the total number of person-weeks by 52.
‡ILI was defined as an acute fever ≥37.8°C with cough or sore throat.
§April 16, 2005–April 15, 2006.
¶April 16, 2006–April 15, 2007.

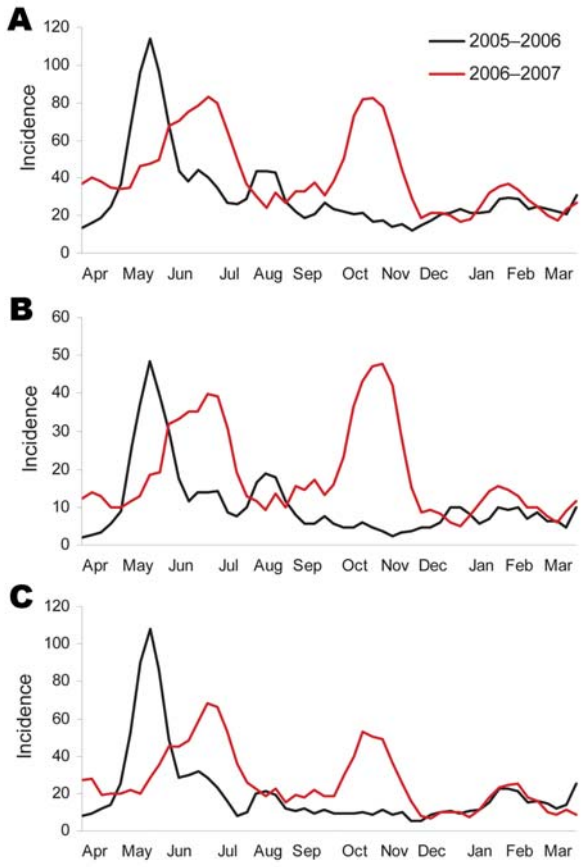

Figure 2. Incidence (cases/100 person-years) of influenza-like illness (ILI) in a cohort of children in Nicaragua, showing seasonal peaks, April 16, 2005–April 15, 2006, and April 16, 2006–April 15, 2007. A) Incidence of ILI episodes per calendar week. B) Incidence of high-probability ILI episodes per calendar week. C) Incidence of ILI in children 6–12 years of age per calendar week. All curves were smoothed by Lowess (*19*) by using a 3-week moving average.

age groups. Incidence was highest for those 2 years of age (84.0 episodes/100 person-years) and decreased with each age increase of 1 year. Furthermore, a seasonal pattern in ILI activity was noted; a peak occurred during June–July in each of the 2 years of the study. Additionally, in the second year, a second peak of ILI was documented during November–December; thus, Nicaragua may experience 2 peaks of influenza activity in some years. The hypothesis that the observed peaks of ILI are due to influenza is supported by the seasonal pattern of high-probability ILI, ILI in children older than 6 years (who are less likely to have respiratory syncytial virus infections) (*15*), and laboratory results.

Risk factor analysis showed that young age and asthma status were strong risk factors for ILI, consistent with what has been found in other studies (*20*,*21*). Person-density in the house was positively associated with risk for ILI. Although children in households with 3.0–4.9 residents per room had an elevated risk that did not achieve statistical significance, those in households with >5 residents per room displayed a significantly increased risk for ILI. In a study of acute respiratory infection in children in Greenland, nighttime crowding (i.e., sharing a room at night) was significantly associated with the risk for acute respiratory infections, but general crowding (i.e., number of persons/room) as measured in this study, was not (*22*). Several studies in developing countries have yielded mixed results concerning the association between household crowding and acute respiratory infection, but most report no association (*23*). Our study found that maternal literacy was protective for ILI but that the mother's education level was not significantly associated. Although not significant in the final model, the mother's education level showed a U-shaped relationship, perhaps because of a higher likelihood that

Table 3. Risk factors for influenza-like illness in cohort of 4,276 children 2–11 years of age, Managua, Nicaragua, 2005–2007*

Characteristic	Crude RR†	95% CI	Adjusted RR†‡	95% CI
Sex				
M	0.94	0.85–1.04	0.95	0.87–1.04
F	Ref		Ref	
Age, y				
2	5.94	4.49–7.85	5.53	4.19–7.32
3	4.67	3.56–6.12	4.39	3.35–5.75
4	3.46	2.63–4.54	3.29	2.51–4.32
5	2.67	2.03–3.53	2.58	1.96–3.41
6	2.30	1.74–3.03	2.24	1.70–2.95
7	2.13	1.61–2.83	2.09	1.58–2.77
8	1.52	1.12–2.04	1.49	1.11–2.01
9	1.51	1.13–2.03	1.50	1.12–2.01
10	1.23	0.91–1.67	1.23	0.91–1.66
11	Ref		Ref	
Asthma				
Yes	2.23	1.92–2.59	1.79	1.56–2.06
No	Ref		Ref	

*RR, relative risk; CI, confidence interval; Ref, reference.
†The measure of RR used is the incidence rate ratio.
‡Multivariate model included sex, age, and asthma status.

Table 4. Household characteristics of participants in cohort of children 2–11 years of age, Managua, Nicaragua, 2005–2007

Characteristic	Participants with household data, no. (%), n = 2,615
Persons/room	
<3	1,254 (47.6)
3–4	917 (35.1)
≥5	444 (17.0)
Mother literate	
Yes	2,436 (94.4)
No	146 (5.6)
Mother's education level	
None	158 (6.1)
Some primary	421 (16.1)
Completed primary	387 (14.9)
Some secondary	1,045 (40.2)
Completed secondary	361 (13.9)
College	225 (8.7)
Type of floor	
Dirt	552 (21.1)
Concrete or other	2,063 (78.9)
Electricity	
Yes	2,605 (99.9)
No	3 (0.1)
Sanitation	
None	17 (0.7)
Latrine	307 (11.7)
Flushing toilet	2,291 (87.6)
Access to potable water	
Yes	2,582 (99.4)
No	16 (0.6)

mothers with a higher education level work outside of the home, necessitating outside childcare. Certainly, this association and others need to be examined further. In particular, including details about childcare (e.g., whether the child is cared for outside the home and the type of facility) and socioeconomic factors, will be informative. We have refined the household questionnaire and will administer it on a yearly basis.

Strengths of this study include the large size of the cohort, its prospective nature, and high compliance with study procedures. In addition, the study had a high rate of follow-up; only 11.3% of the children were either withdrawn or lost to follow-up. Children who were censored from the analysis do not appear to have differed from those who completed the 2-year period (data not shown); thus, we do not believe that our results are biased due to censoring.

One major limitation is that this study used a syndromic definition for ILI because specimens to determine causative agent were not generally available; because the cohort was not initially established to study respiratory diseases, respiratory samples were not collected for most of the study period. However, influenza during all 3 peaks of ILI activity was confirmed by laboratory testing of a subset of samples. Additionally, the study relied on enhanced passive surveillance, and thus we cannot be certain that we captured all episodes of ILI in the cohort. Nonetheless, compliance with study procedures was high; 94% of children visited HCSFV by the fourth day of fever, and only 1.9% reported having sought medical attention by nonstudy medical personnel. However, some mild ILI episodes were likely not detected because participants did not seek medical attention. Because of the passive nature of the surveillance and the requirement for fever, our calculated incidence may underestimate the true incidence of ILI in the cohort. Additionally, this study covered only a 2-year time period, which limits our ability to assess seasonality. Finally, the sample of participants that participated in the household survey did differ somewhat from the general population, likely because of an increased probability of parents of younger children being home in the daytime. However, because the geographic distribution of participants included in the risk factor analysis did not differ significantly from the distribution of the general cohort and because in Nicaragua, neighborhood is strongly associated with socioeconomic status and living conditions, it is likely that the subset for whom risk factor data were available was reasonably representative of the cohort, after age and asthma status were taken into account.

Table 5. Household risk factors for influenza-like illness in a subset of 2,615 children 2–11 years of age, Managua, Nicaragua, 2005–2007*

Characteristic	Crude RR† (95% CI)	Adjusted RR†‡ (95% CI)
Sex		
M	0.96 (0.87–1.07)	0.98 (0.89–1.07)
F	Ref	Ref
Age, y		
2	5.01 (3.63–6.93)	4.83 (3.50–6.66)
3	4.21 (3.08–5.76)	4.07 (2.98–5.56)
4	3.13 (2.29–4.30)	3.05 (2.22–4.18)
5	2.55 (1.85–3.51)	2.51 (1.82–3.45)
6	2.03 (1.47–2.80)	2.01 (1.46–2.78)
7	2.01 (1.45–2.80)	2.00 (1.44–2.78)
8	1.50 (1.06–2.12)	1.49 (1.06–2.10)
9	1.55 (1.10–2.19)	1.54 (1.09–2.17)
10	1.25 (0.88–1.77)	1.24 (0.88–1.76)
11	Ref	Ref
Asthma		
Yes	1.83 (1.57–2.13)	1.51 (1.32–1.75)
No	Ref	Ref
Persons/room		
<3	Ref	Ref
3–4	1.04 (0.93–1.17)	1.07 (0.96–1.20)
≥5	1.14 (1.00–1.31)	1.18 (1.04–1.34)
Mother literate		
Yes	0.80 (0.63–1.01)	0.79 (0.64–0.98)
No	Ref	Ref
Dirt floor		
Yes	0.95 (0.84–1.08)	0.88 (0.78–1.00)
No	Ref	Ref

*RR, relative risk; CI, confidence interval; Ref, reference.
†The measure of RR used is the incidence rate ratio.
‡Multivariate model included sex, age, asthma status, person density in house, mother's literacy, and a dirt floor.

Influenza, in both its epidemic and pandemic forms, is a major health threat in tropical regions, just as it is in temperate regions. The lack of data on the epidemiology of influenza in tropical regions makes it extremely difficult for nations in these areas to plan for and prevent influenza. It also hampers attempts at modeling pandemic influenza and development of appropriate control strategies. Results from this initial study of pediatric ILI in Nicaragua document a high level of disease and demonstrate pronounced seasonal peaks. Risk factors were young age, an asthma diagnosis, and high person-density in the house; a protective factor was having a literate mother.

A prospective study of influenza in the Nicaraguan pediatric cohort in which respiratory samples will be tested for influenza is currently under way. This study should further characterize the epidemiology of influenza and analyze the nucleotide sequence variation and the relationship of influenza viruses circulating in the cohort to those isolated in the Northern and Southern Hemispheres. Further studies, particularly with laboratory-confirmed outcomes, in multiple countries, are needed to confirm the seasonality and level of influenza in the tropics.

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Clinical Risk Factors for Severe *Clostridium difficile*–associated Disease

Timothy J. Henrich, Douglas Krakower, Asaf Bitton, and Deborah S. Yokoe

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

- Identify the criteria used to define severe *Clostridium difficile*–associated disease (CDAD) in the current study
- Specify the prevalence of severe CDAD in the current study
- Identify the clinical risk factors for severe CDAD
- List the laboratory risk factors for severe CDAD

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Identifying patients who are at high risk for severe *Clostridium difficile*–associated disease (CDAD) early in the course of their infection may help clinicians improve outcomes. Therefore, we compared clinical features associated with severe versus nonsevere CDAD by retrospectively reviewing records of hospitalized patients whose fecal assays were positive for *C. difficile* toxin. Of 336 patients, 12.2% had severe disease and 10.1% died from all causes. Regression modeling showed the following to be significantly associated with severe CDAD ($p \leq 0.05$): age >70 years (odds ratio [OR] 3.35), maximum leukocyte count >20,000 cells/mL (OR 2.77), minimum albumin level <2.5 g/dL (OR 3.44), maximum creatinine level >2 mg/dL (OR 2.47), small bowel obstruction or ileus (OR 3.06), and computed tomography scan showing colorectal inflammation (OR 13.54). These clinical and laboratory markers for severe disease may be useful for identifying patients at risk for serious outcomes or death.

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The incidence and severity of *Clostridium difficile*–associated disease (CDAD) is increasing in North America (*1*–*3*) and Europe (*4*,*5*). During the past 10 years in the United States, prevalence, case-fatality rates, total attributable mortality rates, and colectomy rates for persons with CDAD have markedly increased (*6*). Acquisition of *C. difficile* and the development of severe CDAD is associated primarily with healthcare, although community-acquired severe disease among persons previously thought to be at low risk for infection have been reported (*5*,*7*,*8*). Several mechanisms for increased disease severity have been proposed, including emergence of specific strains with genetic polymorphisms that encode higher levels of bacterial toxins A and B and the production of a binary toxin (*3*,*9*,*10*). The Centers for Disease Control and Prevention has reported outbreaks of CDAD associated with the new BI/NAP1 strain in 40 of 50 US states, although the association

between BI/NAP1 and severe disease was not consistent among all facilities (*11*).

Host factors are also likely to be predictors of illness and death. For example, in an elderly population, leukocytosis, hypoalbuminemia, and nasogastric tube feedings were associated with high mortality rates from CDAD (*12*). Severity of underlying illness, as measured by an increased Horn score, has only moderate association with severe CDAD (*13*). Exposure to specific antimicrobial drugs, notably fluoroquinolones, clindamycin, and cephalosporins, has been associated with severe CDAD in some studies (*2*,*14*) but not others (*13*). Overall, previous studies have identified few clinical characteristics that consistently predict severe CDAD.

Identifying patients who are at high risk for severe CDAD early in the course of their infection might help clinicians improve patient outcomes, but predictors are not well known. Genetic subtyping, binary toxin assays, and culture of isolates are currently not widely accessible, which makes integrating knowledge of emerging bacterial factors into patient management difficult. To elucidate patient and clinical factors associated with severe CDAD, we conducted a 1-year retrospective study of Brigham and Women's Hospital patients who had had positive *C. difficile* toxin results.

Methods

Study Population

We performed a retrospective chart review of electronic medical records for all patients who had had a positive fecal result for *C. difficile* toxin from June 2005 through May 2006, a period of increased incidence of severe CDAD in this hospital. During June–April 2006, CDAD was diagnosed by cytotoxic assay for cytopathic effects in cell culture. During May 2006, our laboratory changed to a toxin A and B ELISA. Before assay replacement, samples were tested with both techniques and results were comparable (A. Onderdonk, pers. comm., 2008).

All inpatients ≥18 years of age who had had a positive fecal *C. difficile* toxin result were included in the study. Ambulatory and emergency department patients were not included.

Study Variables and Data Collection

Study variables included those identified as risk factors for development of CDAD, those associated with disease severity in previous studies, or those that logically predisposed patients to other severe disease outcomes. We collected patient demographic, historic, radiographic, and laboratory information.

Demographic variables included age, gender, hospital service (i.e., medical, surgical, obstetric, or gynecologic), date of positive *C. difficile* toxin sample, and length of hospitalization. We also collected number of antimicrobial drug classes used during hospitalization before positive *C. difficile* assay result, class and number of days used for each antimicrobial drug, and starting date and type of CDAD treatment initiated on or after the day the positive *C. difficile* toxin samples.

Historic data included use of corticosteroids, immunomodulating drugs, chemotherapy for hematologic and solid organ malignancies, proton pump inhibitors, and histamine-2 blockers. All medication data were limited to the 30 days before each patient's first positive *C. difficile* toxin result. Physician-documented medical conditions included cardiovascular disease, diabetes mellitus, chronic kidney disease, past or current need for hemodialysis (not including intensive care unit [ICU] setting), pulmonary disease, hematologic malignancy, solid tumor malignancy, and immunocompromisation (i.e., solid organ or hematopoietic stem cell transplantation, immunoglobulin deficiencies, use of immunosuppressive agents, and severe autoimmune syndromes not associated with other malignancy).

Laboratory data were collected for a 7-day interval spanning 4 days before and 2 days after the day of submission of the first *C. difficile*–positive fecal specimen. This interval was chosen to account for variability in the promptness of *C. difficile* testing and initiation of treatment among healthcare providers. We recorded maximum leukocyte, serum glucose, creatinine, alanine aminotranferase levels, and minimum serum albumin concentrations.

Other variables were clinical or radiographic evidence of a small bowel obstruction or ileus in addition to abdominal and pelvic computed tomography (CT) scans with abnormal findings (colitis, pericolonic stranding, and abnormal rectal findings) documented anytime during hospitalization before laboratory diagnosis of CDAD. We also included skilled-nursing home or rehabilitation stays within 60 days before laboratory diagnosis of *C. difficile* infection, acute-care hospitalization within 30 days before diagnosis, date of death, date and number of admissions to ICU, number of surgical procedures, and enteral or total parenteral nutrition within 30 days before diagnosis of CDAD.

Definition of Severe CDAD

Patients were defined as having severe CDAD if they met at least 1 of the following criteria: 1) death within 30 days after onset of symptoms or positive assay in which *C. difficile* infection was a major contributor; 2) ≥1 ICU admissions in which *C. difficile* infection was a major contributor; 3) colectomy or other surgery directly attributed to *C. difficile*; or 4) intestinal perforation in the presence of *C. difficile* infection. To minimize subjectivity, cases were reviewed independently by 2 study personnel directly involved with data collection and extraction and were count-

ed as severe only if both reviewers agreed. A third investigator, who was not involved with data collection, reviewed each case and acted as a tie-breaker.

Statistical Methods

The analysis was conducted in 3 stages. First, all-cause deaths and incidence of severe CDAD and death directly related to CDAD were explored in relation to age group. Second, univariate analyses were used to identify significant differences in variables for patients with severe and nonsevere disease. Chi-square testing with continuity correction was used to compare intergroup variation between nonparametric variables. Fisher exact tests were used if expected counts were <5. Mann-Whitney tests of ranked data were used to compare ordinal/parametric variables given the size discrepancy between the severe– and nonsevere–disease cohorts and to adjust for potential deviation from a normal distribution. Third, 2 logistic regression models were created. The first model was designed to evaluate independent associations between disease severity and antimicrobial drug use, demographics, and significant clinical variables identified from univariate analyses (prior nursing home/rehabilitation stays or acute-care hospitalizations, immunocompromisation, small bowel obstruction or ileus, and abnormal radiographic findings). The second model was designed to assess independent associations of laboratory variables with CDAD severity. Clinical variables likely to influence these laboratory values, such as hemodialysis, steroid use, and chemotherapy use, were included in this model. We used 2 models, rather than combining all variables into 1 model, because the small number of severe CDAD cases relative to total number of CDAD cases and large number of variables and covariates with potential collinearity in a combined model would decrease the power to detect statistical significance. Odds ratios (ORs) and 95% confidence intervals were calculated for each variable in the regression models (SPSS version 10; SPSS Inc., Chicago, IL, USA).

Results

Study Population

For the study interval, we identified 336 patients and 373 hospitalizations. However, to minimize the underestimation of variance among our sample population, we analyzed data from only 1 admission per patient (initial hospitalization), for a total of 336 hospitalizations.

The all-cause crude mortality rate during initial admissions was 10.1%. Most (82%) CDAD patients were >50 years of age; crude mortality rate in this group was 12.0%. For patients <50 years of age, crude mortality rate (1.7%) was markedly lower; for patients >70 years of age, crude mortality rate was highest (15.4%) (Table 1).

Table 1. All-cause deaths of inpatients with laboratory-confirmed CDAD, June 2005–May 2006*

Age group, y	Total no. patients	No. deaths	% Case-fatality†
18–50	60	1	1.7
51–60	70	7	10.0
61–70	76	6	7.6
71–80	83	12	14.5
81–90	40	7	17.5
>90	7	1	14.3
Total	336	34	10.1

*CDAD, *Clostridium difficile*–associated disease.
†Percentage of deaths within age group.

The study definition for severe CDAD was met by 41 (12.2%) patients. Incidence of severe CDAD among all patients with CDAD was markedly higher in patients >70 years of age (p = 0.001). Of all patients, 21 (6.3%) died as a result of CDAD according to physician impression from chart review; none was <50 years of age (Table 2). Proportion of severe CDAD cases among patients with CDAD on these services did not differ significantly according to service (p = 0.18): 64% medical, 33% surgical, and 3% obstetric or gynecologic. Numbers of days from admission to laboratory diagnosis of CDAD patients with or without severe CDAD were similar (6.6 vs. 8.2; p = 0.13), as were lengths of hospitalization (18.3 vs. 18.2; p = 0.70).

Univariate Analysis

Table 3 lists variables (except antimicrobial drug use) and laboratory values included in univariate analysis. Mean age of patients was 64 years. Patients with severe CDAD were significantly older (mean age 71 years) than those without severe disease (mean age 63 years); p = 0.001, Mann-Whitney test of ranked data. Proportion of male and female patients with or without severe CDAD did not differ significantly.

Other variables that did not differ significantly between patients with or without severe CDAD were underlying medical illness, malignancy, use of nonantimicrobial medications (including steroids and chemotherapy), and enteral or parenteral feeding (Table 3). CDAD was significantly less severe in patients who were immunocompromised or receiving immunosuppressive medications than in those who were not immunocompromised (OR 0.22, p = 0.044).

Other variables associated with severe disease included small bowel obstruction or ileus (OR 3.33, p = 0.014), abdominal CT results suggestive of colorectal pathologic changes (OR 13.09, p<0.001), acute-care hospitalization within 30 days before CDAD laboratory diagnosis (OR 2.12, p = 0.036), and rehabilitation or skilled-nursing facility stay within the 60 days before CDAD diagnosis (OR 2.17, p = 0.043). Maximum leukocyte count was significantly higher for patients with severe CDAD, and significantly more patients with severe disease had a maximum

Table 2. Severe CDAD and death as a result of CDAD, by age group, June 2005–May 2006*

Age group, y	Total no. patients	Severe CDAD, no. (%)	Deaths from CDAD, no. (%)
18–50	60	3 (5.0)	0 (0)
51–60	70	8 (11.4)	5 (7.1)
61–70	76	4 (5.2)	1 (1.3)
71–80	83	13 (15.7)	8 (9.6)
81–90	40	11 (27.5)	6 (15.0)
>90	7	2 (28.6)	1 (14.3)
Total	336	41 (12.2)	21 (6.3)

*CDAD, *Clostridium difficile*–associated disease.

leukocyte count >20,000 cells/μL, minimum albumin level <2.5 g/dL, maximum glucose level >150 mg/dL, and serum creatinine level >2 mg/dL (Table 3).

Some exposure to antimicrobial drugs during the 30 days before laboratory diagnosis of CDAD was noted for ≈85% of patients. Exposure to, or number of, antimicrobial drugs did not differ significantly among patients with or without severe CDAD. In the severe and nonsevere CDAD cohorts, 85.4% of patients had used any antimicrobial drugs (p = 1.0). In addition, no significant differences were found between exposure to any of the following groups of antimicrobial drugs for patients with severe or nonsevere CDAD: fluoroquinolones; penicillin derivatives with or without β-lactamase inhibitor; aminoglycosides; clindamycin; first-generation cephalosporins; second- through fourth-generation cephalosporins; carbapenems; trimethoprim/sulfamethoxazole; intravenous vancomycin; systemic antifungal drugs. Use of oral or intravenous metronidazole before laboratory diagnosis of CDAD (p = 0.860) did not differ significantly.

Antimicrobial drugs (including oral and rectal vancomycin and metronidazole) for CDAD were given to 291 (86.7%) patients during their hospitalization. A higher percentage of patients with severe CDAD than without CDAD were treated with oral vancomycin (OR 8.27, p<0.001), rectal vancomycin (OR 20.35, p<0.001), or intravenous

Table 3. Univariate analysis results for 336 patients with and without severe CDAD, June 2005–May 2006*

Variable	All patients, %†	Severe CDAD, % (n = 41)‡	Nonsevere CDAD, % (n = 295)	OR§	p value
Age >70 y	38.7	63.4	35.3	3.18	0.001¶
Female	48.2	51.2	47.8	1.15	0.807
Chemotherapy use§	16.1	9.8	16.9	0.53	0.343
Corticosteroid use§	25.6	31.7	24.7	1.41	0.444
Proton pump inhibitor use§	63.7	61.0	64.1	0.88	0.832
H2 blocker use	32.1	34.1	31.9	1.11	0.909
Enteral feeding	21.7	26.8	21.0	1.38	0.520
Parenteral feeding	3.3	2.4	3.4	0.71	1.000
Cardiovascular disease	41.7	53.7	40.0	1.74	0.135
Pulmonary disease	19.3	24.4	18.6	1.41	0.508
Diabetes	22.6	22.0	22.7	0.96	1.000
Renal disease	22.0	19.5	22.4	0.84	0.831
Hemodialysis	6.0	4.9	6.1	0.79	1.000
Immunocompromised	17.3	4.9	19.0	0.22	0.044¶
Malignancy	46.9	39.0	47.1	0.72	0.420
Small bowel obstruction or ileus	8.3	19.5	6.8	3.33	0.014¶
Abnormal abdominal CT scan	28.3	78.0	21.4	13.09	<0.001¶
Prior hospitalization	39.9	56.1	37.6	2.12	0.036¶
SNF/rehabilitation stay	22.9	36.6	21.1	2.17	0.043¶
Max glucose level >150 mg/dL	49.1	70.7	46.1	2.83	0.005¶
ALT >40 U/L	23.1	28.2	22.3	1.37	0.540
Min albumin level <2.5 g/dL	27.7	59.0	22.8	4.89	<0.001¶
Max creatinine level >2 mg/dL	22.0	41.5	19.3	2.96	0.003¶
Max leukocyte count >20,000/μL	28.3	53.7	24.7	3.52	<0.001¶
Mean max leukocyte count × 10^3/μL	17.6	25.8	16.5	–	<0.001¶

*CDAD, *Clostridium difficile*–associated disease; OR, odds ratio; H2, histamine-2; CT, computed tomography; SNF, skilled-nursing facility; max, maximum; ALT, alanine aminotransferase; min, minimum.
†Total of 336 patients were included in analysis except for ALT (n = 286) and albumin (n = 295).
‡n = 39 for ALT and albumin.
§OR for severe CDAD in patients with positive *C. difficile* assay results; calculated for binary variables only (by χ^2 or Fisher exact test) with exception of mean leukocyte count (significance calculated using Mann-Whitney test of ranked data).
¶Statistically significant at α = 0.05.

Table 4. Binary logistic regression model to identify variables independently associated with severe CDAD, 336 patients*

Variable	OR	95% CI
Age >70 y	3.35†	1.48–7.57
Female	0.93	0.42–2.03
Antimicrobial-drug use	1.76	0.60–5.21
Malignancy	0.73	0.33–1.65
Immunocompromised	0.38	0.07–1.96
Small bowel obstruction or ileus	3.06‡	1.00–9.39
Abnormal abdominal CT scan	13.54†	5.72–32.07
Prior hospitalization	1.39	0.61–3.18
SNF/rehabilitation stay	1.11	0.46–2.68

*CDAD, *Clostridium difficile*–associated disease; OR, odds ratio; CI, confidence interval; CT, computed tomography; SNF, skilled-nursing facility. Constant included in analysis.
†p<0.05.
‡p = 0.05.

metronidazole (OR 4.2, p<0.001) on or after the day of laboratory diagnosis of *C. difficile* infection. There was no significant difference in frequency of severe outcomes among patients treated with or without oral metronidazole (OR 1.02, p = 1.0). All patients with severe CDAD were treated with at least 1 antimicrobial drug with activity against *C. difficile*.

Regression Analyses

The following variables from the logistic regression model to identify covariates independently associated with severe CDAD were significant (p≤0.05): age >70 years, ileus or small bowel obstruction, and abnormal abdominal CT image (Table 4). The following variables were not significantly associated with development of severe CDAD when adjusted for covariates: immunocompromisation status, prior acute-care hospitalization, and stay in a skilled-nursing facility. The independent association of laboratory values with severe CDAD was also investigated by using a single binary logistic regression model covariate adjusted with factors that would logically or historically influence each value. Maximum leukocyte count >20,000 cells/μL, maximum creatinine level >2 mg/dL, and minimum albumin level <2.5 g/dL were all independently associated with severe CDAD (p<0.05; Table 5).

Discussion

As would be expected in a general hospitalized population, advanced age was associated with all-cause mortality rates. Similarly, the odds of severe CDAD and death attributable to CDAD increased with age, especially for patients >70 years of age. Advanced age is known to be associated with CDAD, but whether age influences the severity of disease outcomes is in conflict in different publications (*3*,*6*,*12*). For example, a study of 72 hospitalized patients with endoscopically proven pseudomembranous colitis showed advanced age to be associated with higher mortality rates, but age of patients who died of or survived after pseudomembranous colitis did not differ significantly (*12*). In contrast, Loo et al. noted a clear increase in the 30-day mortality rate in CDAD patients >80 years of age (*3*). In our study, no patient 18–50 years of age died as a result of CDAD, and advanced age was a significant risk factor for illness and death among patients with CDAD. Unlike age, gender was not associated with severe CDAD; this finding is similar to those of studies that investigated the role of gender on development, recurrence, or severity of CDAD (*12*,*15*). However, a large US study based on International Classification of Diseases, 9th revision, codes showed that CDAD case-fatality rate was higher for men than for women (*6*).

Because patients with CDAD are older and have more concurrent illness than the general population, effect of residence in long-term and acute-care facilities on the development and course of CDAD has generated interest (*13*,*16*–*18*). Our univariate analysis showed each of these variables to be associated with severe CDAD. However, when adjusted for age and concurrent illness, prior hospitalizations at long-term and acute-care facilities were not significantly associated with severe CDAD. On the basis of our regression model results, it is likely that the significance of prior hospitalizations (noted with univariate analysis) was the result of the more advanced age of patients who had had prior acute- or skilled-nursing facility hospitalizations and that age was the clinically important variable.

We found no association between malignancy or chemotherapy and severe CDAD. In contrast, Duberkke et al. found that 57% of patients who had undergone allogenic stem cell transplant had severe CDAD, although severity of disease was based on grade of diarrhea and colitis (*19*). The reason for the absence of association between immunosuppression due to malignancy or chemotherapy and severe CDAD found in our study is unclear, but it is possible that

Table 5. Binary logistic regression model to identify independent associations of laboratory values and pertinent variables with severe CDAD, 285 patients*

Variable	OR	95% CI
Age >70 y	3.24†	1.42–7.38
Female	1.16	0.51–2.62
Antimicrobial-drug use	1.04	0.35–3.12
Malignancy	0.90	0.37–2.18
Chemotherapy	1.02	0.27–3.92
Steroid use	1.13	0.48–2.68
Hemodialysis	0.5	0.8–3.01
Max leukocyte count >20,000/μL	2.77†	1.28–6.0
Max glucose level >150 mg/dL	1.46	0.63–3.43
ALT >40 U/L	1.47	0.58–3.69
Min albumin level <2.5 g/dL	3.44†	1.56–7.57
Max creatinine level >2 mg/dL	2.47†	1.04–5.88

*CDAD, *Clostridium difficile*–associated disease; OR, odds ratio; CI, confidence interval; max, maximum; ALT, alanine aminotransferase; min, minimum. Constant included in analysis.
†p<0.05.

at this institution, which has a large oncology and solid-organ transplantation population, clinicians are more likely to order laboratory testing, implement precautions, and empirically initiate treatment earlier for immunosuppressed patients with suspected CDAD, thus avoiding severe sequelae. In addition, immunosuppressive medications have been associated with higher mortality rates in patients with CDAD who do or do not have fulminant colitis, but our univariate analysis results suggested that immunosuppressive comorbid conditions or use of immunomodulating agents (other than chemotherapy for malignancy) were protective against severe CDAD (*1*,*15*,*20*). However, this protective association was no longer noted when we adjusted for other factors.

Antimicrobial-drug use has been studied extensively with regard to development of CDAD and, to a lesser extent, severity and recurrence of disease (*3*,*12*,*14*,*21*–*25*). We found no association between severe CDAD and total number of antimicrobial drugs used, class of antimicrobial drug, and duration of exposure. In particular, use of antimicrobial drugs that are commonly associated with CDAD, including clindamycin and fluoroquinolones, did not differ among patients in whom severe CDAD did and did not develop. According to findings of previous studies, it is probable that our study population's exposure to antimicrobial drugs was a risk factor for CDAD. However, antimicrobial-drug exposure did not appear to predispose patients with CDAD to severe disease. Recent data also suggest that patients who continue to receive antimicrobial-drug therapy without activity against CDAD while being treated for CDAD have a higher likelihood of CDAD treatment failure (*26*). Our study did not take into account whether patients continued to receive antimicrobial-drug therapy after laboratory diagnosis of CDAD. Antimicrobial-drug stewardship, however, has been shown to be useful in reducing CDAD rates (*27*).

Our univariate model showed aggressive treatment regimens for *C. difficile*, such as intravenous metronidazole and oral or rectal vancomycin, to be associated with worse outcomes. This finding is likely the result of our standard hospital practice to upgrade treatment of the sickest patients from oral metronidazole to oral vancomycin, intravenous metronidazole, rectal vancomycin, or a combination of these, so that exposure to these antimicrobial drugs was more likely to have been a surrogate marker of severe disease.

Laboratory markers such as leukocytosis, increased creatinine levels, and decreased albumin or globulin levels may correlate with poor outcome for patients with CDAD. Studies have yielded variable results, although multiple studies have shown that markedly increased leukocyte counts correlate with more severe disease (*1*,*12*,*15*,*20*). Similarly, our logistic regression model showed the following variables to be significantly correlated with severe CDAD: maximum leukocyte count >20,000/μL, minimum serum albumin level <2.5 g/dL, and maximum serum creatinine level >2 mg/dL. These laboratory values were adjusted for potential effects of concurrent underlying clinical conditions or treatments, such as hemodialysis, and use of steroids or chemotherapy. We also found some radiographic abnormalities to be associated with severe disease. On the basis of the results of our analyses, laboratory and imaging abnormalities may be useful for predictive modeling of severe outcomes from CDAD.

One major limitation of this study was our dependence on the date of laboratory diagnosis of CDAD to define disease onset. Clinical signs and symptoms (e.g., diarrhea, bloody feces, or abdominal pain) may have developed before the patient was tested for CDAD, and the date of laboratory diagnosis likely reflected the timing of physicians' clinical suspicion for *C. difficile* infection rather than exact onset of symptoms. Our inability to reliably assess the presence of diarrhea may also have resulted in inclusion of some patients colonized with but not clinically ill from *C. difficile*. In addition, we found that several patients had been treated with oral vancomycin before laboratory diagnosis of CDAD and that severe disease was more likely to develop in patients treated before laboratory diagnosis. These patients were likely treated on grounds of clinical suspicion and may have had more aggressive onset and worse clinical markers for disease before laboratory diagnosis. We also did not evaluate whether cessation or continuation of antimicrobial drugs other than metronidazole and oral vancomycin affected progression to severe *C. difficile* infection.

As discussed previously, maximum and minimum laboratory values were collected for the time interval spanning the 4 days before and 2 days after the day of submission of the first *C. difficile*–positive specimen. We included the 2 days after laboratory diagnosis to account for variability in the timing of recognition and response to positive *C. difficile* assay results, but this fairly broad time interval limits to some extent our ability to evaluate the diagnostic utility of these laboratory values. Abnormal creatinine levels and leukocyte counts during this 7-day interval, for example, may have reflected the natural history and course of severe disease and skewed values higher for patients with severe CDAD. Finally, growing evidence indicates that BI-NAP 0127 causes more severe CDAD, and in our population bacterial subtype was likely an unrepresented predictor of severe disease.

The results of our study suggest that readily available clinical data, such as age and basic laboratory and radiology data, are correlated with severe CDAD outcomes. These findings suggest that clinicians may be able to gauge the risk for severe outcomes without individual genotyp-

ing. This ability is likely to be valuable in community as well as tertiary-care settings. Use of these markers for early identification of patients at high risk for severe disease may facilitate rapid implementation of aggressive medical and surgical CDAD therapy.

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Capacity of Thailand to Contain an Emerging Influenza Pandemic

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Southeast Asia will likely be the epicenter of the next influenza pandemic. To determine whether health system resources in Thailand are sufficient to contain an emerging pandemic, we mapped health system resources in 76 provinces. We used 3 prepandemic scenarios of clustered cases and determined resource needs, availability, and gaps. We extended this analysis to a scenario of a modest pandemic and assumed that the same standards of clinical care would be required. We found that gaps exist in many resource categories, even under scenarios in which few cases occur. Such gaps are likely to be profound if a severe pandemic occurs. These gaps exist in infrastructure, personnel and materials, and surveillance capacity. Policy makers must determine whether such resource gaps can realistically be closed, ideally before a pandemic occurs. Alternatively, explicit assumptions must be made regarding allocation of scarce resources, standards of care, and priority setting during a pandemic.

The World Health Organization (WHO) has highlighted how the Asia-Pacific region has been an important center of emerging diseases such as severe acute respiratory syndrome (SARS) and avian influenza. Since 2003 (as of September 10, 2008), 15 countries have experienced human cases of infection with influenza virus A subtype H5N1 (*1*), and subtype H5N1 infection is now endemic in poultry in several countries. The H5N1 subtype continues to pose an important public health threat in both the short term and the long term. Southeast Asia remains a likely region from which future emerging infectious diseases, including the next influenza pandemic, are likely to emerge (*2*,*3*).

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In a resolution issued in April 2005, WHO expressed concern about the general lack of global preparedness for pandemic influenza (*4*). Since then, considerable international efforts have been expended, and substantial resources have been committed to controlling avian influenza and preparing for pandemic influenza (*5*). Because the question is not whether a pandemic will occur but rather when (*6*), policy makers have been urged to take action in preparedness planning, including making national preparedness strategies operational (*5*,*7*,*8*). However, despite efforts to support preparedness, no universally accepted, organized method of evaluating preparedness exists, and concerns have been raised that implementation of many national strategic plans may be unrealistic (*9*,*10*). Several approaches have been adopted to evaluate preparedness, including assessments of national strategic plans (*9*,*11*), desk-top simulations (*12*), full-scale field exercises, case studies with site visits to assess health systems (*8*), and mathematical modeling exercises (*13*,*14*). All have particular strengths and weaknesses. Most of these approaches, although linked to national strategic and operational plans, have not included assessments of capacity to respond (that is, of available resources at each site and the potential to mobilize these resources). Without determining capacity to respond, the feasibility of effectively and efficiently implementing plans in a time of crisis remains highly uncertain.

In this article, we define and quantify, at the province level, the health system resources likely to be drawn upon in the event of WHO prepandemic phases 4 and 5 in Thailand, a relatively well-developed, middle-income, Southeast Asian country at high risk for being the epicenter of the next pandemic. We estimate gaps in resources, given several prepandemic influenza scenarios. These scenarios were previously developed by policy makers and have been used extensively in tabletop exercises in most provinces throughout the country. Our aim was to determine the chal-

lenges still remaining in preparing the country to effectively meet and contain the danger of an emergent pandemic. We addressed the challenge of mitigation in the event of a modest pandemic scenario, but, in agreement with national strategic policy, we assumed no diminution of standards of care or rationing of clinical services. Although the ability to maintain such levels of care is unlikely in reality, no national policies explicitly acknowledge this possibility; thus, this research draws on scenarios and assumptions currently guiding policy making.

Methods

Resource Mapping

The health system in Thailand is organized through 12 health regions. These regions include 76 provinces (in this article, we consider Bangkok a province). The provinces comprise 784 administrative districts; Bangkok has an additional 50 administrative districts. We mapped the presence of resources across Thailand's provinces. We developed a survey instrument to determine resources likely to be drawn upon at the province level if human-to-human spread of a novel influenza virus occurs. The survey instrument was developed in a stepwise manner. First, we reviewed the case notes about all human cases of avian influenza that have occurred in Thailand since 2004 and determined the resources used to manage the cases. Second, we conducted a literature review of resources used in managing influenza and SARS, and we then expanded the list of resources determined from the case notes review list. Third, we reviewed the resource list with experts in communicable disease control at national institutions and, through these discussions, modified the list.

A survey instrument was developed from the resource list and pilot tested in Kanchanaburi Province among healthcare personnel from several public health and healthcare institutions at the local, district, and province levels. Minor modifications and clarifications were made to the survey instrument as a result.

The survey instrument addressed resource needs across 4 topics of interest: surveillance, case investigation, case treatment, and prevention of spread of disease in the community. Thirty-nine resources were assessed. Data on infrastructure, personnel, and materials were collected. Province data sources were derived from the following institutional settings, which were identified through national routine health system data sources: district hospitals; subdistrict health centers; district public health offices; regional, provincial, and higher level health institutions; private healthcare facilities; and university healthcare facilities.

The survey instrument was sent to representatives of each of the 75 provinces and Bangkok in July 2007. These province representatives sent questionnaires to institutions at lower organizational levels. Duplication of data was avoided by coordinating data collection through designated institutional respondents. Those who did not respond were reminded by letter and phone calls 2 months after they had received the questionnaires.

Scenarios

Building on simulation exercises conducted in Thailand and on transmission dynamics in the published literature, we assumed 3 scenarios (*14*). The scenarios were previously developed by Thailand's Department of Disease Control and made explicit assumptions about attack rates, illness, and mortality rates (*15*,*16*). As of July 2007, 66 (88%) provinces and 468 (60%) districts had conducted tabletop exercises that drew on these scenarios. Of note, these scenarios were static; that is, cases and contacts (i.e., opportunities for spread) occurred simultaneously. We assumed that the current policy focus in Thailand is on containment, rather than on mitigation. Our interest was in determining the resource gaps in WHO phases 4 and 5 (localized and substantial clusters, respectively). We did not analyze the processes of mobilizing resources or the associated logistical challenges.

Scenario 1, WHO Phase 4

This scenario assumed human-to-human transmission from case-patients to caregivers. It involved 2 patients with confirmed influenza, 3 health personnel with confirmed mild influenza, and 10 persons who were close contacts of the patients.

Scenario 2, WHO Phase 5

This scenario assumed human-to-human transmission in localized clusters. It involved 5 patients with confirmed influenza and 75 contact persons.

Scenario 3, WHO Phase 5

This scenario assumed human-to-human transmission that resulted in a substantial number of cases. One cluster of human-to-human influenza cases was identified in each of 5 districts of the province. Each cluster consisted of 5 patients with confirmed influenza (25 in total) and 375 contact persons across the province.

Resource Needs

We determined resource needs at the province level for each of the 3 above-mentioned scenarios. Resource needs were determined through retrospective analyses of case notes and discussions with clinicians and surveillance personnel intimately involved in managing earlier cases of avian influenza in persons in Thailand. For case-patients and their contacts, infrastructural, personnel, and material needs were determined. Thus, for the outbreaks, we

assumed that needs were the resources used, multiplied by the number of case-patients or by the total number of contacts of the case-patients and the contacts generated respectively through different scenarios. We assumed that resource needs for any case-patient would be the same as for subsequent case-patients (that is, that resource needs are linearly related to the numbers of case-patients and their contacts as an outbreak develops).

Province Resource Gaps

We determined resource gaps at the province level for each scenario and defined influenza-specific resources. Some resources such as oseltamivir are used specifically for treatment of influenza. We assumed, therefore, that some resources were dedicated influenza resources. For oseltamivir use, we assumed that case-patients would receive treatment and that their contacts would be given prophylaxis. Other resources were nonspecific for influenza. For example, physicians would still be needed to provide essential healthcare services. We assumed, on the basis of other reports (*17*,*18*), that because resources would still be demanded by essential health services, 12% of non–influenza-dedicated resources would be available to support influenza control. That is, 88% of resources would still be dedicated to essential services. We assumed that available beds in negative-pressure rooms would be needed first, then isolation beds, then single-occupancy rooms, and so forth. We assumed that care for case-patients would be provided in hospitals and that care for contacts would be provided in the community. Some resources, such as hospital beds, cannot be shared between provinces. We assumed that other resources would not be shared between provinces in a timely manner (an unpublished qualitative analysis of the mobilization of resources showed that mobilization of resources through formal agreements is ill defined and has been difficult to achieve during simulation exercises; P. Chompook, unpub. data).

Dynamic Timeline Analysis

Although scenarios used in tabletop simulation exercises across Thailand to date have been static, in reality, WHO phases 4 and 5 are likely to emerge over several days and weeks. In a secondary analysis, we determined the needs and gaps for resources if we assumed that cases would emerge in a manner predicted by published transmission dynamics scenarios (*19*). We assumed that case-patients would need to be hospitalized for 7 days and that treatment with antiviral drugs would be provided to case-patients and contacts in accordance with recommendations (*20*).

National Resource Gaps under WHO Phase 6

National strategic policy regarding pandemic influenza makes no explicit acknowledgment that standards of care will decrease or that allocation of scarce resources will, of necessity, demand rationing. We determined national gaps in resources under mild pandemic conditions by assuming that scenario 3 would develop evenly and simultaneously across all provinces (that is, early pandemic WHO phase 6). We first assumed perfect mobilization of resources such that provinces with excess resource capacity effectively and efficiently supported provinces with gaps. Resource gaps described under this scenario were determined by the summation of surplus and gaps in resources from all provinces. Also, under the same WHO phase 6 scenario, we assumed inadequate (imperfect) mobilization of resources across provincial borders such that resources remained within provinces. Resource gaps under this scenario were derived from the summation of gaps only from provinces where estimated resource shortfalls occur.

Results

Data were collected from respondents at the region, province, and district levels. Data from 73 (96%) provinces were made available through respondents in 765 districts (765/834, 92%). Full data from all province institutions were provided from 53 (70%) provinces. Data from Bangkok were provided solely by public hospitals.

To determine total availability of provincial and national resources and account for missing data, we estimated the resource availability in districts where data were unavailable and extrapolated these estimations. We assumed that districts with similar numbers of hospital beds would have the same quantity of other resources available. The Ministry of Health determines bed quotas, and data were derived from routine data sources.

The average quantity of province resources is listed in Table 1. The estimated average province resources are the result of extrapolation and correction when data points were missing. Because few data points were missing, the estimates were very similar to the averages derived from hard data. The estimated resources were further analyzed to determine resource gaps. Substantial differences in resource availability exist across provinces. We found no correlation of resources with gross provincial product (a measure of a province's economic well-being) or with province poultry density. We found, however, correlations between some resources (for example, healthcare personnel, hospital beds, and ventilator equipment) and both population size and density (Table 1).

The differences in resource availability across provinces are illustrated through 7 selected resources (Figures 1, 2). These selected resources offer insights into the geographic variations in preparedness in relation to surveillance capacity (surveillance and rapid response team [SRRT] personnel), case investigation capacity (SRRT, internal medicine doctors), case-patient treatment capacity (oseltamivir treat-

ment courses, respirators, critical care nurses), and capacity to prevent spread of disease in the community (negative-pressure rooms, isolation rooms, surgical masks).

Gaps in resources existed in some provinces under scenario 1 (and thus for subsequent scenarios). These resource gaps include infrastructure, personnel, and materials and potentially limit capacity in all 4 control areas (surveillance capacity, case-investigation capacity, case-treatment capacity, and capacity to prevent spread of disease in the community) (online Technical Appendix, available from www.cdc.gov/EID/content/15/3/423-Techapp.pdf). If care for case-patients is limited to negative-pressure rooms or isolation beds, then bed availability is likely to be problematic, even with a small numbers of

Table 1. Average available resources and estimated average resources needed for pandemic influenza control at province level and correlation of selected province data with province resources, 76 provinces, Thailand*

Selected resources	Province resources available† (range)	Province resources needed†	Correlation with province resources			
			Population	Population density	GPP	Poultry density
Hospitals‡	14 (3–36)	15	0.129	–0.113	0.008	0.531
Health centers§	133 (21–403)	143	0.327	–0.270	–0.143	0.511
Infrastructure (no. beds)						
Negative-pressure rooms (single bed)	13 (1–38)	13	0.482	–0.006	–0.096	0.331
Isolation beds	9 (1–117)	9	**0.725¶**	**0.812**	0.261	–0.045
Single-occupancy room beds	158 (24–2,942)	158	**0.785**	**0.880**	0.206	–0.029
ICU beds	37 (4–605)	37	**0.817**	**0.842**	0.172	–0.044
General medicine beds	134 (6–1,301)	134	**0.818**	**0.833**	0.180	–0.004
Other beds (OB/GYN, surgical, etc.)	1,066 (90–4,377)	1,184	**0.763**	0.575	0.160	0.190
Child beds	80 (21–814)	80	**0.848**	**0.832**	0.187	–0.047
Personnel						
SRRT personnel	202 (50–604)	223	0.580	–0.013	–0.100	0.325
Internal medicine doctors	43 (1–670)	44	**0.817**	**0.834**	0.180	0.005
Pediatricians	25 (1–336)	25	**0.828**	**0.841**	0.216	0.021
Radiologists	6 (0–117)	6	**0.805**	**0.791**	0.159	–0.010
Pathologists	9 (0–111)	9	0.617	0.571	0.114	0.331
Other physicians#	241 (32–2,229)	251	**0.806**	**0.791**	0.160	0.004
Critical care nurses	34 (0–535)	34	**0.766**	**0.833**	0.202	0.024
General nurses	1,219 (176–9,831)	1,284	**0.919**	**0.832**	0.187	0.091
Health officer in health center§	322 (72–977)	345	0.363	–0.265	–0.209	0.444
Village health volunteer§	10,424 (1,500–49,597)	11,006	0.442	–0.218	–0.296	0.411
Materials						
Ambulances	25 (8–79)	28	0.619	0.235	0.091	0.333
Patient transportation vehicles	96 (24–324)	104	0.521	–0.019	–0.123	0.259
Portable radiography machine	10 (3–100)	11	0.599	0.547	0.147	0.064
Adult (Bird's and volume) respirator	90 (8–1,076)	96	**0.850**	**0.803**	0.228	0.082
Children's volume respirator	24 (0–212)	25	0.596	0.514	0.175	0.165
Vital sign machine	280 (14–1,723)	302	0.560	0.250	–0.037	0.182
Oximeter	70 (4–813)	74	**0.810**	**0.770**	0.132	0.025
Disposable gowns	1,328 (93–17,249)	1,377	**0.737**	**0.717**	0.181	0.054
N95 masks	6,681 (1,247–27,721)	7,181	0.517	0.304	0.021	0.108
Surgical masks	16,031 (673–211,411)	16,440	0.349	0.472	–0.013	–0.080
Plastic face shields	541 (52–4,366)	567	0.349	0.092	–0.046	0.005
Goggles	919 (204–6,220)	961	0.643	0.550	0.199	0.044
Surgical gloves	64,757 (605–731,117)	66,201	0.583	0.456	–0.015	0.118
Surgical hats	9,558 (390–234,955)	9,861	**0.843**	**0.865**	0.178	0.100
Rapid test kit for influenza	544 (62–3,005)	576	0.366	0.267	–0.021	0.111
Swab bags	630 (0–10,901)	669	0.228	–0.028	–0.021	–0.001
Oseltamivir tablets	14,525 (1,290–60,110)	14,854	0.175	0.065	0.028	–0.072
Viral transport media	231 (35–818)	249	0.539	0.283	–0.014	0.159
Body bags	129 (0–1,050)	145	0.432	0.551	0.138	0.097
Lime (10-kg bags)	67 (0–1,008)	71	0.225	–0.051	–0.048	0.048
Chlorine (50-kg bags)	211 (0–10,121)	206	–0.071	0.079	0.067	–0.065
Sodium hypochlorite (1 L)	1,570 (0–50,190)	1,540	0.061	0.085	0.223	0.048

*GPP, gross provincial product; SRRT, surveillance and rapid response team; ICU, intensive care unit; OB/GYN, obstetricians/gynecologists. Data sources: Population and population density data are from the Department of Provincial Administration, 2007; GPP is from 2005 data from the National Economic and Social Development Board; poultry density was determined from the number of chickens and ducks in each province in 2006 from the Information and Statistics Group, Information Technology Centre, Department of Livestock Development, Bangkok, Thailand.
†Average. Missing district-level data are estimated.
‡Excludes private hospitals in Bangkok.
§Excludes data from Bangkok.
¶**Boldface** indicates that correlation is significant at the 0.01 level (2-tailed).
#General practitioners, surgeons, OB/GYN, etc.

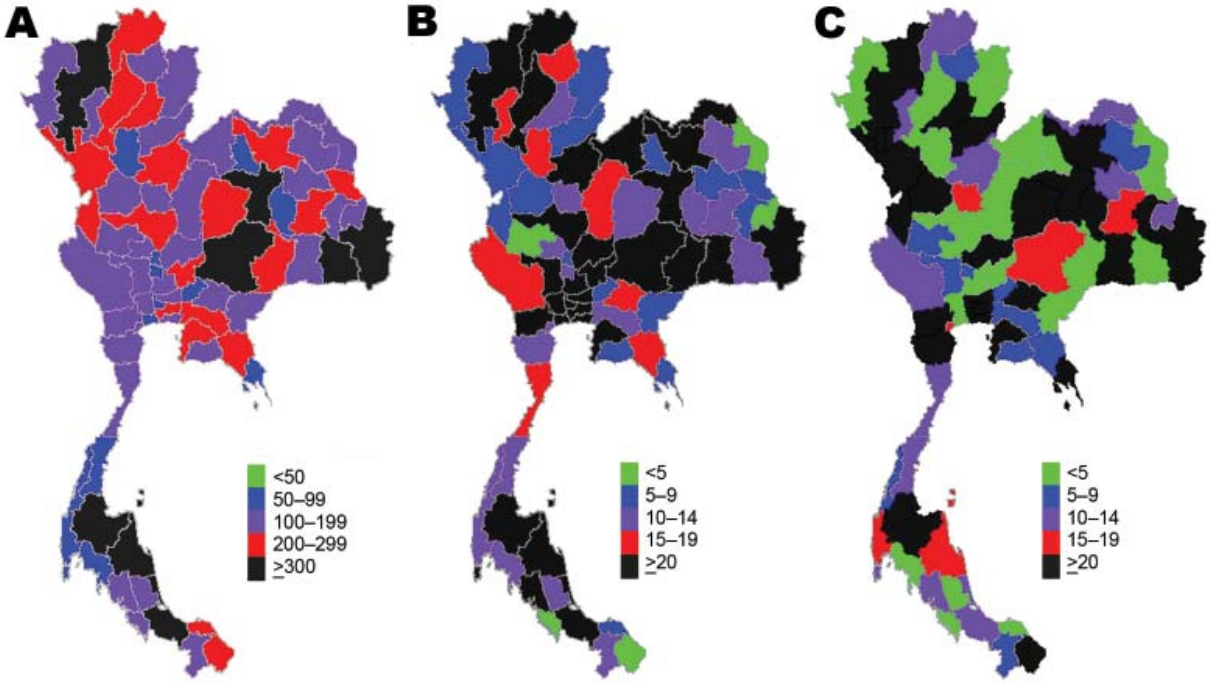

Figure 1. Density of selected health system resources available for pandemic influenza across provinces, Thailand. A) Surveillance and rapid response team personnel; B) internal medicine physicians; C) critical care nurses.

cases. However, if beds dedicated to wider use are made available, then shortfalls are unlikely when limited cases occur. Most resource gaps are linked to critical care and include lack of trained personnel and respirators. For example, by scenario 3, 92% of provinces will have insufficient negative-pressure rooms to respond effectively to case-patients, and a severe shortage of critical care nurses will occur. However, if isolation beds are used, the proportion of provinces with insufficient resources falls to ≈75%, and if single occupancy rooms are also used, bed capacity across the country is sufficient. As the number of case-patients and contacts increases through scenarios 2 and 3, the number of provinces with gaps in resources grows. The geographic distribution of resource gaps varies, depending on resource and scenario (Figures 3–5; online Technical Appendix).

The need for 4 selected resources changed over time, assuming the epidemic curve has the usual shape (Figure 6). The gap in available resources was limited to only a few days for respiratory support. For beds, likewise, when

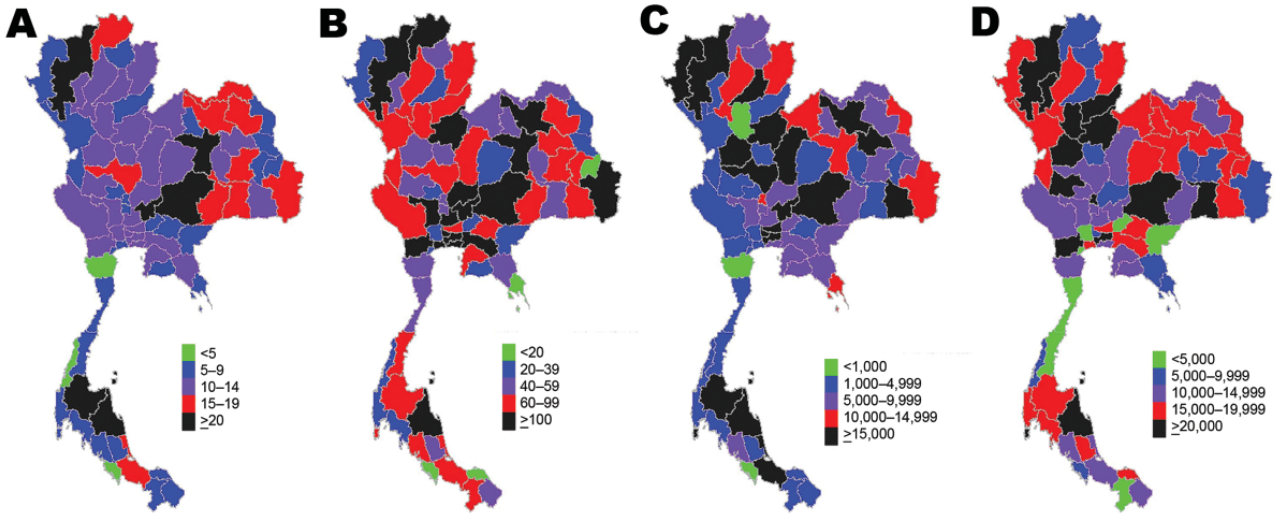

Figure 2. Density of selected health system resources available for pandemic influenza across provinces, Thailand. A) Negative-pressure rooms; B) adult respirators; C) surgical masks; D) oseltamivir tablets.

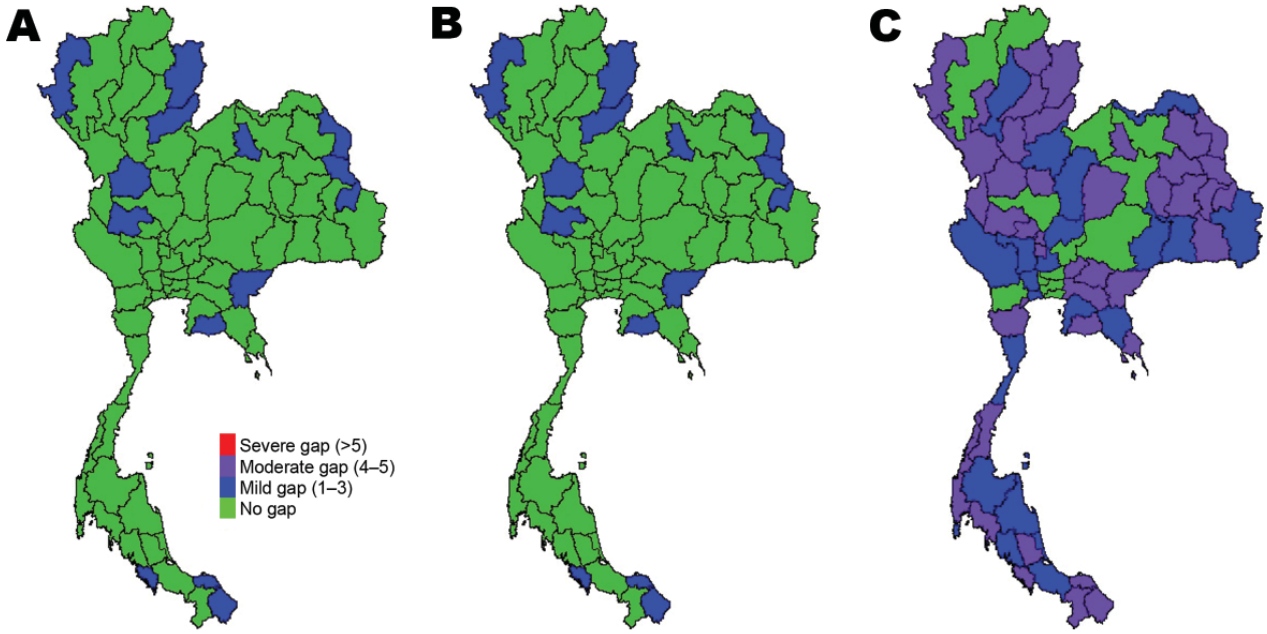

Figure 3. Gaps in health system resources (internal medicine physicians) likely to occur for 3 scenarios of prepandemic influenza across provinces, Thailand. A) Scenario 1; B) scenario 2; C) scenario 3.

small numbers of cases occur and case-patients are cared for in negative-pressure rooms or isolation rooms, shortages are likely to arise for only a few days. Sufficient stocks of oseltamivir are currently held at the provincial level to meet the needs of a few case-patients and their contacts.

Resource gaps exist on the national level if scenario 3 occurs simultaneously in all provinces across Thailand (Table 2). Such an event represents WHO phase 6, that is, sustained human-to-human transmission, albeit on a relatively small scale. Despite this small scale, national

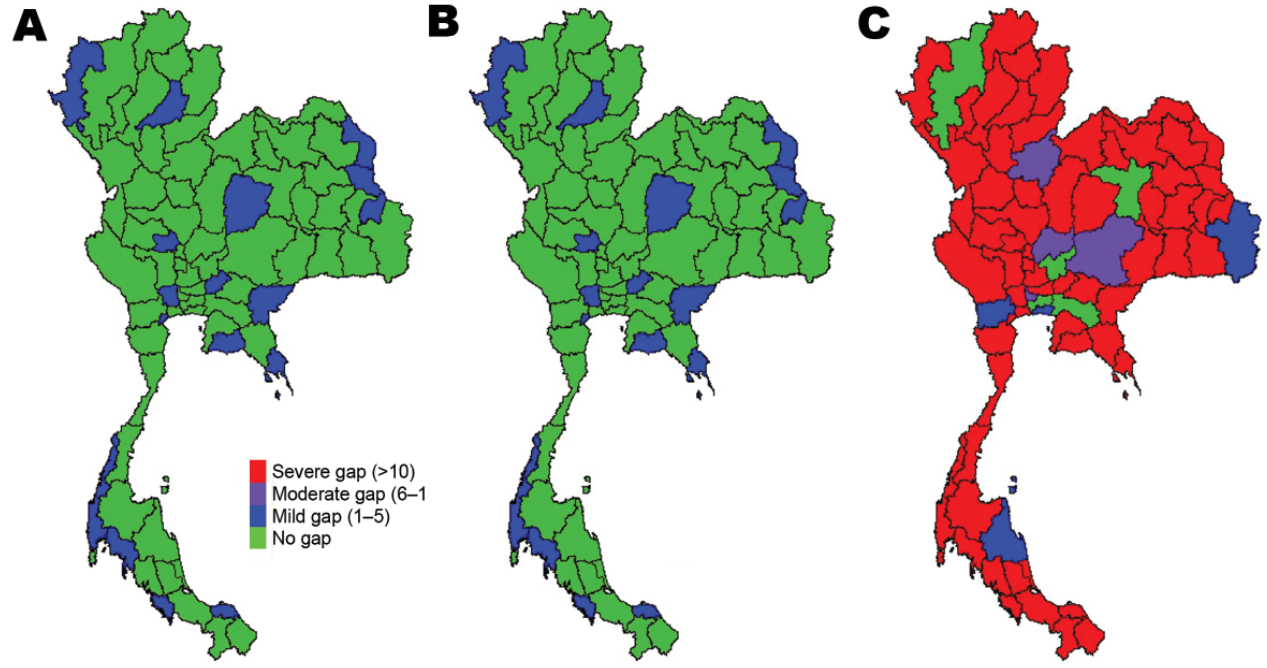

Figure 4. Gaps in health system resources (adult respirators) likely to occur for 3 scenarios of prepandemic influenza across provinces, Thailand. A) Scenario 1; B) scenario 2; C) scenario 3.

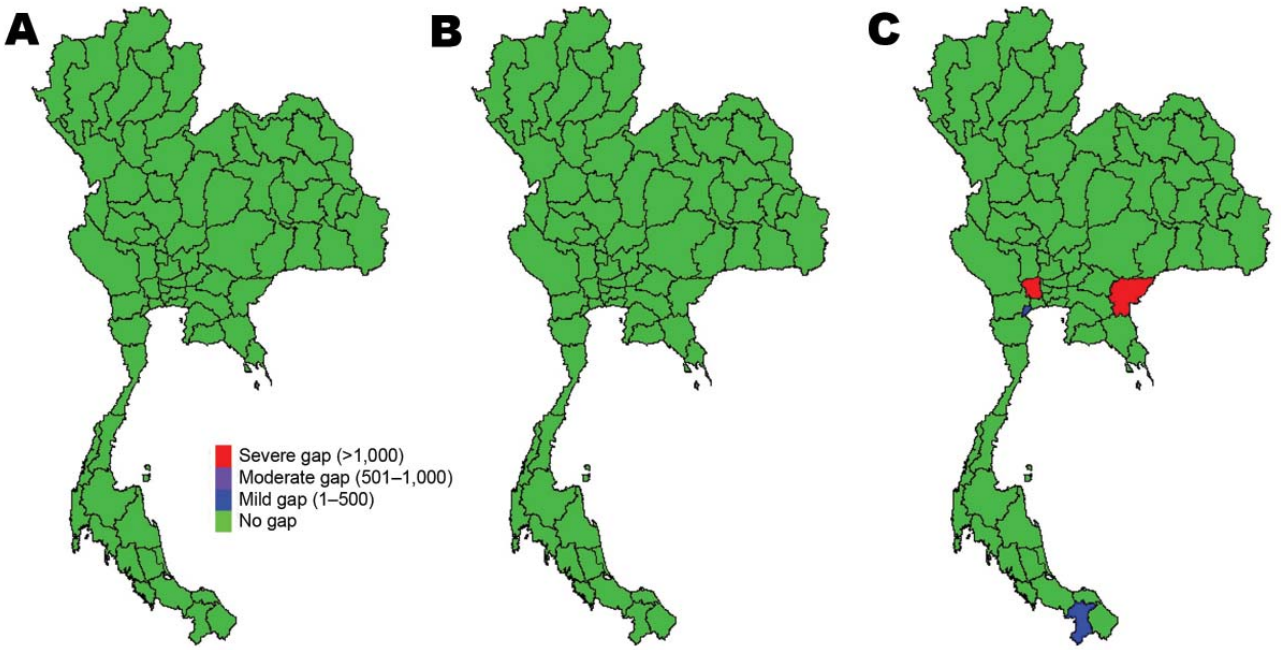

Figure 5. Gaps in health system resources (oseltamivir tablets) likely to occur for 3 scenarios of prepandemic influenza across provinces, Thailand. A) Scenario 1; B) scenario 2; C) scenario 3.

resource gaps are substantial if the same standard of clinical care is maintained when fewer cases arise. For some critical resources, such as internal medicine physicians and oseltamivir tablets, the problem is mitigated if we assume perfectly effective, timely, and efficient movement of resources from provinces with surplus capacity to provinces with gaps. Some resources, however, are limited in number (such as critical care nurses), and even effective redistribution may make little difference in outcome.

Discussion

We showed that Thailand is likely to have some resource gaps in responding to clusters of cases in an emergent influenza pandemic and that these gaps vary across different provinces. These gaps are, however, likely to occur over a limited duration if the cases occur over several weeks and the numbers of cases are limited. As the number of cases increases, however, provincial and national capacity is likely to be tested in certain ways if clinical care and surveillance are expected to remain at a similar standard as when cases are limited. The results of such a scenario are similar for some countries of western Europe (*21*). Although policy makers will, in all likelihood, need to consider issues of rationing and priority setting explicitly in national strategic planning, resources in Thailand are substantial overall, although geographic distribution likely poses logistical challenges. In the event of a modest outbreak of pandemic influenza (WHO phase 6) similar to these locally developed scenarios, Thailand, a relatively affluent country in Southeast Asia, might encounter relatively modest gaps in available resources. However, if a pandemic is substantial in terms of the severity of illness and proportion of deaths, resources are likely to be insufficient, and policy makers will have to consider whether such resource gaps can be closed in reality. This conclusion has important policy implications and raises several questions. Should most resources and planning be focused explicitly on early containment potentially at the expense of mitigation, particularly in developing countries? How, to whom, and where should the deployment of scarce resources be planned (*22*)? A further issue raised is how realistic simulation exercises are and whether they effectively inform preparedness planning. Investment to address gaps in resources can be focused where the most important gaps exist. Some gaps, however, for example, in clinical and nursing staff, will take time and considerable investment to fill. We show at the province level where resource gaps are most profound and thus where future investment might be focused.

The variations of resources correlate strongly with both the population size and the population density of a province. Historically, healthcare resources have been distributed on the basis of provincial population size and not according to poverty indices. To date, risk assessments related to pandemic influenza have not informed planning and deployment of resources. This circumstance has potentially important implications for future preparedness planning. Provinces with relatively less dense populations, in which

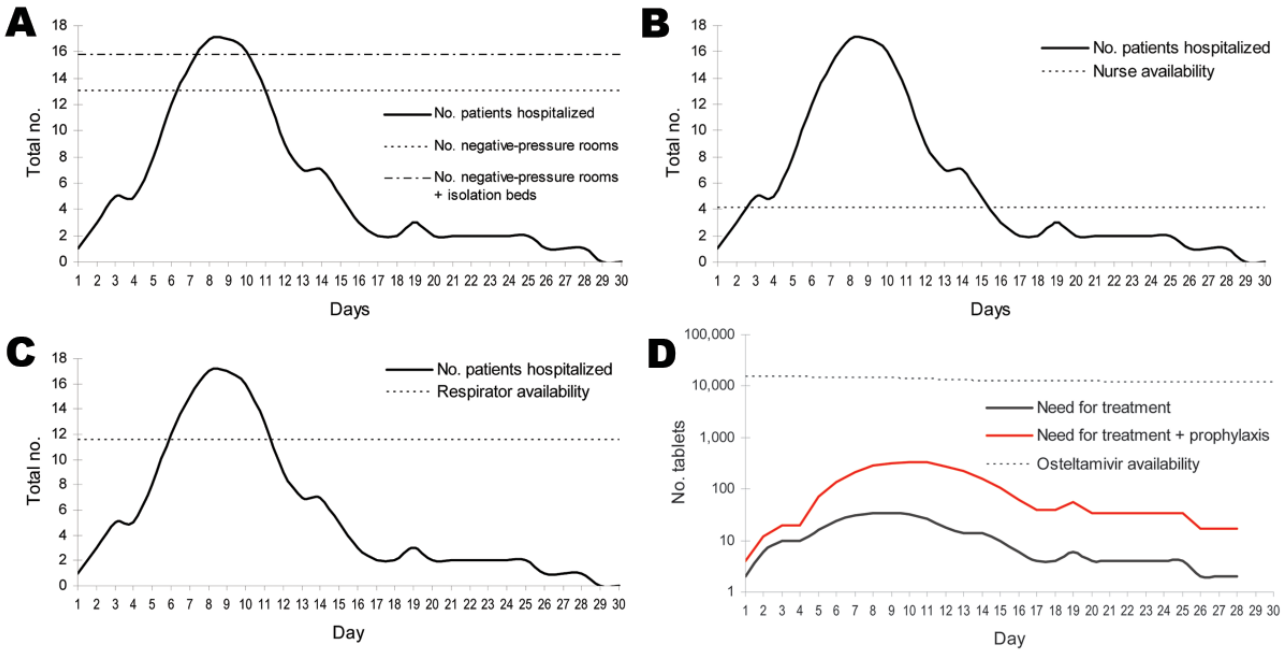

Figure 6. Projected demand and gaps in selected health system resources in Thailand, assuming prepandemic containment. A) Hospital beds; B) critical care nurses; C) adult respirators; D) oseltamivir tablets.

communication facilities are stretched to their limit and access to services is already likely to be problematic, may be further challenged by their relatively fewer resources (*23*). Focused investment in resources may be needed if the response to an emergent pandemic is to be equitable. Variations exist also among provinces in their resource capacity for surveillance, case investigation, case-patient treatment, and control of community spread.

Responding effectively and in a timely manner to gaps we have highlighted will be a managerial challenge. Moreover, using available resources most effectively and efficiently on a national scale also demands considerable managerial and administrative capacity (issues we did not examine). The timely mobilization of most resources remains to be planned.

This study has several limitations. First, our survey focused on the narrow clinical response and ignored the capacity of management, administrative systems, financial systems, and communications, capacities that are likely to be needed to efficiently mobilize resources (*24*). Second, we assumed that the relationship between resource need and case-patients is linear, and we estimated gaps on the basis of assumptions that care for case-patients as the pandemic unfolds will draw upon similarly characterized resources as in earlier phases. This assumed relationship is unlikely to occur, and care for case-patients is likely to be different from our study assumptions. However, few strategic plans explicitly acknowledge this change in resource use and thus do not plan for it (*9*). Third, our scenarios, although based on tabletop exercises conducted across the country, are limited in terms of anticipated case-patients and their contacts. Even though we extended our scenario to a modest pandemic, an alternative real-life scenario under which large numbers of cases occur is likely to test the health system profoundly. Fourth, we assumed that resource sharing between provinces would be limited on the basis of an analysis of formal strategic arrangements. Fifth, some data points were missing. Although the missing data were few, and corrections were possible, some of these data were from Bangkok. In Bangkok, any determination of the city's overall resources is a challenge because of the many private autonomous healthcare facilities and their lack of systematic integration into the public health system. This factor means that our interpretation of Bangkok's capacity to respond should be considered with caution. Bangkok has 65 private hospitals with >50 beds (14,000 beds in total), and these institutions were excluded from our survey. The challenge of coordinating the city's resources in the event of a pandemic is substantial. Without an understanding of what and where those resources are, their management will be much more challenging. This lack of knowledge would have profound implications for Thailand because Bangkok is a city of 10 million persons, the economic powerhouse of the country, and a hub for transportation and communications with the rest of Thailand. The missing information also has important implications for the global control of a pandemic because of Bangkok's role as a major international transport hub. If emergent pandemic influenza can-

not be controlled in Bangkok, the world will be affected. The same lack of complete information would apply to other major cities where complex health systems exist.

We have shown that the health system resources available to Thailand are likely to be sufficient to respond to emergent pandemic influenza if the pandemic is modest and occurs in a manner similar to the assumptions informing Thailand's simulation exercises. Other countries in the region, which is acknowledged to be at high risk for being at the epicenter of the next global pandemic of influenza (*25*), are likely to have fewer resources than Thailand (*3*). We are currently investigating the capacity of Thailand and neighboring countries to respond to more profound pandemic influenza scenarios. Policy makers in the region may need to reflect on where health system resources in the region might best be positioned and further expanded; what scenario assumptions are used to inform preparedness planning; whether containment, mitigation, or both, should be the focus of attention; and whether provinces with the largest probable gaps should be supported further

Table 2. National resource gaps for pandemic influenza control if perfect mobilization and imperfect mobilization in WHO phase 6, assuming scenario 3 occurs simultaneously in all provinces, Thailand*

Selected resources	National gaps	
	Assuming perfect mobilization	Assuming imperfect mobilization
Infrastructure (beds), assuming care limited to these		
Negative-pressure rooms (single bed)	–1,015	–1,052
Negative-pressure rooms (single bed) + isolation beds	–225	–517
Negative-pressure rooms (single bed) + Isolation beds + single-occupancy room beds	0	0
Negative-pressure rooms (single bed) + isolation beds + single-occupancy room beds + ICU beds	0	0
Negative-pressure rooms (single bed) + Isolation beds + single-occupancy room beds + ICU beds + general medicine beds	0	0
Negative-pressure rooms (single bed) + Isolation beds + single-occupancy room beds + ICU beds + general medicine beds + other beds (OB/GYN, surgical, etc.)	0	0
Children's beds	NA	NA
Personnel		
SRRT personnel	0	0
Internal medicine physicians	–40	–195
Pediatricians	NA	NA
Radiologists	0	–5
Pathologists	0	–9
Other physicians (general practitioners, surgeons, OB/GYN, etc.)	0	0
Critical care nurses	–1,640	–1,679
General nurses	0	0
Health officer in health center†	0	0
Village health volunteers†	0	0
Materials		
Ambulances	0	0
Patient transportation vehicles	0	0
Portable radiography machines	0	0
Adult (Bird's and volume) respirator	–1,023	–1,166
Children's volume respirator	NA	NA
Vital sign machine	0	–365
Oximeter	–1,221	–1,317
Disposable gowns	–16,6041	–166,041
N95 masks		
Surgical masks	–59,063	–120,186
Plastic face shields	0	–668
Goggles	0	0
Surgical gloves	0	–39,242
Surgical hats	–88,665	–119,239
Rapid test kit for influenza	0	0
Swab bags	0	–59
Oseltamivir tablets	0	–3,717
Viral transport media	0	0
Body bags	0	–373
Lime (10-kg bags)	0	–716
Chlorine (50-kg bags)	0	–18
Sodium hypochlorite (1 L)	0	–216

*WHO, World Health Organization; ICU, intensive care unit; OB/GYN, obstetricians/gynecologists; SRRT, surveillance and rapid response; NA, not applicable: no child cases in 3 scenarios. 0 means that there was no shortfall in the resource item. Scenario 3 assumed human-to-human transmission resulting in a substantial number of cases.
†Excludes data from Bangkok.

in strengthening response capacity. Policy makers should also consider how the capacity of the private healthcare sector can, if a public health crisis occurs, be drawn upon in a timely and effective manner. In the event of a major pandemic, difficult decisions regarding the use of scarce resources will need to be made, and explicit planning ahead for the pandemic is advised.

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Epidemiology of Bluetongue Virus Serotype 8, Germany

Franz J. Conraths, Jörn M. Gethmann, Christoph Staubach, Thomas C. Mettenleiter, Martin Beer, and Bernd Hoffmann

In Germany, bluetongue disease had not been reported before 2006. During August 2006–August 2008, >24,000 bluetongue virus serotype 8 infections were reported, most (20,635) in 2007. In 2006 and 2007, respectively, case-fatality rates were 6.4% and 13.1% for cattle and 37.5% and 41.5% for sheep. Vaccination in 2008 decreased cases.

Bluetongue disease (BT) is an infectious, but noncontagious, viral infection of ruminants that is transmitted by *Culicoides* spp. biting midges. It can cause massive losses in farmed ruminants, particularly sheep. BT had never been reported in Europe north of the Alps before August 2006, when outbreaks almost simultaneously occurred in Belgium, France, Germany, and the Netherlands (*1*–*4*). We examined the epidemiology of this outbreak in Germany, where reporting of BT cases through the national animal disease notification system (*5*) is mandatory. In this article, an outbreak is defined as the occurrence of BT in cattle, sheep, or goats on a farm involving at least 1 infected animal; a case is defined as a single infected animal. Wild ruminants infected with BT were always considered cases.

The Study

In Germany, cattle were tested for BTV before trade or if disease was suspected from clinical signs. Diagnoses were made by serologic testing with commercial test kits (Pourquier ELISA Bluetongue Serum, Institut Pourquier, Montpellier, France; Bluetongue Virus Antibody Test Kit, VMRD, Pullman, WA, USA; ID Screen Blue Tongue Competition ELISA Kit, ID Vet, Montpellier, France; INGEZIM BTV, INGENASA, Madrid, Spain) or real-time reverse transcription–PCR (RT-PCR) (*6*).

Because no real-time RT-PCR for the sensitive detection of BTV serotype 8 (BTV-8) was available in Europe in 2006, we developed and validated such an assay. For the detection of BTV-8 genome, 0.8-μM primers (BTV8-NS1-1F [5′-AAT GGG ATG TGT GTC AAA CAA AAT-3′]; BTV8-NS1-1R [5′-CAA CTA ATT TAT ACG CTT TCG CC-3′]) and a 0.2-μM probe (BTV8-NS1-1FAM [5′-FAM-CTC CTC CGC ATC GGT CGC CGC-TAMRA-3′]) were used in a QuantiTect Probe RT-PCR kit (QIAGEN, Hilden, Germany). The method was then transferred to and implemented at the regional laboratories of the German federal states responsible for BT screening; the results were confirmed by proficiency testing. Since the summer of 2007, real-time RT-PCR assays for all known 24 serotypes of BTV (*3*) have also been used for BTV genome detection. Samples with inconclusive results in the differential pan-BTV and BTV-8 tests were referred to the national reference laboratory, which confirmed the exclusive presence of BTV-8 during the study period (August 2006–August 2008).

In 2006, the disease was diagnosed on 571 cattle farms and in 309 sheep flocks, 3 other bovines, 6 red deer, 3 mouflons, and 1 roe deer in the federal states of North Rhine-Westphalia, Rhineland-Palatinate, Hesse, Lower Saxony, and Saarland (Figure 1, panel A). The core region of the epidemic in Germany was in North Rhine-Westphalia, adjacent to the affected areas in Belgium, the Netherlands, and Luxembourg. The disease was first detected in late August in calendar week 34 (37 cases), peaked mid October in calendar week 42 (154 cases), and decreased slowly until the end of the year (Figure 2). Apparently, the infection overwintered in the region (*6*) and flared up again in 2007, spreading over most of Germany during the summer and autumn of 2007 and resulting in 20,624 new outbreaks (Figure 1, panel B). During 2007, BT was detected on 12,638 cattle farms and in 23 other individual bovines, 7,790 sheep flocks, 115 goat herds, 34 fallow deer, 11 mouflons, 10 red deer, and 3 roe deer. The first case of 2007 was detected in early June, calendar week 23. The number of new cases started to rise constantly from the end of July (week 30), peaked at 3,001 new cases in mid September (week 37), and subsequently decreased slowly until the end of year. The number of affected animals was higher for sheep in the first months of the BT season in 2007; the number of affected cattle dominated the late phase (Figure 2).

In 2006, at least 48,364 cattle, 9,781 sheep, and 56 goats were exposed to BTV on affected premises (Table). Of these animals, 1,131 cattle (2.34%) and 590 sheep (6.03%) were found to be infected; 72 cattle and 221 sheep died. The case-fatality rate was much higher for sheep (37.5%) than for cattle (6.4%). These calculations are based on the assumption that all BT cases were reported. Because the infections caused only mild disease or remained even clinically inapparent in some animals, especially cattle, underreporting is likely and the case-fatality rate in cattle may be slightly overestimated.

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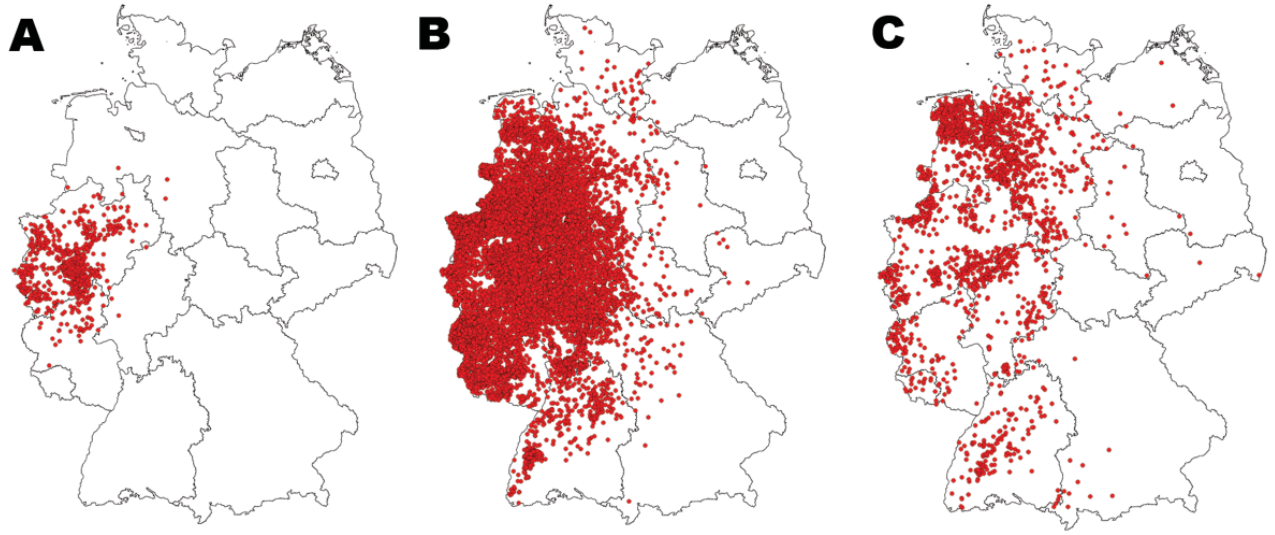

Figure 1. Maps showing outbreaks of bluetongue disease among all affected species in Germany in A) 2006, B) 2007, and C) 2008 (through August 31). Red dots indicate confirmed cases/outbreaks.

In 2007, due to the spread of the disease, the exposed population rose to at least 1,317,111 cattle, 503,282 sheep, and 3,346 goats on affected farms. The numbers of infected animals on these farms amounted to 26,772 cattle, 32,116 sheep, and 209 goats in 20,624 new outbreaks. While mortality rates remained relatively low, as in 2006, the case-fatality proportion rose to 13.1% in cattle and 41.5% in sheep.

In 2008, BT incidence decreased considerably under the influence of a mass vaccination campaign that started in May and June 2008 (Figure 2), before the 2008 vector season. In 2008, a total of 1,070 new cases (PCR-positive, sampled after May 1, 2008; i.e., infection was acquired during the current transmission season) were reported. They were found mainly in 2 regions in northwestern Lower Saxony and western Baden-Württemberg (Figure 1, panel C), where the vaccination campaign started relatively late because of an initially limited supply of BTV-8-vaccine.

Conclusions

The number of BTV-8 infections in Germany that peaked during the summer and autumn of 2007 showed that even a limited BTV-8 outbreak can dramatically spread within a few months after detection of the first cases. After the initial incursion and limited spread in 2006, BTV-8 overwintered, resulting in efficient spread of BTV-8 during 2007 and severe consequences for cattle and sheep farmers. The case-fatality rate was ≈3× higher for sheep than for cattle (37.5% vs. 6.4% in 2006 and 41.5% vs. 13.1% in 2007). These findings illustrate that BTV-8 was more pathogenic for sheep than for cattle. It must be stressed, however, that the virus caused clinical disease and death in cattle, although other serotypes cause clinical disease and deaths primarily in sheep.

As a result of the vaccination campaign, the number of new cases reported in 2008 (through August 2008) was substantially lower than in 2007, demonstrating that conva-

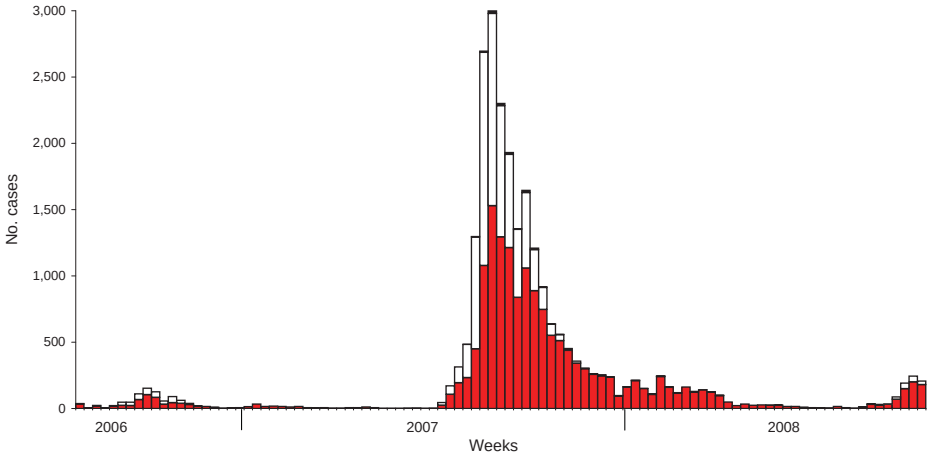

Figure 2. Number of new cases/outbreaks of bluetongue disease per calendar week in cattle (red), sheep (white), and goats (black), Germany.

Table. Animals affected by bluetongue virus serotype 8, Germany, 2006 and 2007

Animals	Total no. animals	No. diseased animals	No. deaths	Morbidity rate, %	Mortality rate, %	Case-fatality rate, %
Cattle						
2006	48,364	1,131	72	2.34	0.15	6.37
2007	1,317,111	26,772	3,512	2.03	0.27	13.12
Sheep						
2006	9,781	590	221	6.03	2.26	37.46
2007	503,282	32,116	13,324	6.38	2.65	41.49
Goats						
2006	56	0	0	0	0	0
2007	3,346	209	54	6.25	1.61	25.84

lescent animals are protected from reinfection and that vaccination was successful. In addition, restriction of animal movement from protection and surveillance zones appears reasonable, although the ability of restricted movement to decrease virus spread is limited, particularly during the vector season.

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Evaluation of Commercially Available Anti–Dengue Virus Immunoglobulin M Tests

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Anti–dengue virus immunoglobulin M kits were evaluated. Test sensitivities were 21%–99% and specificities were 77%–98% compared with reference ELISAs. False-positive results were found for patients with malaria or past dengue infections. Three ELISAs showing strong agreement with reference ELISAs will be included in the World Health Organization Bulk Procurement Scheme.

An estimated 2.5–3 billion persons live in tropical and subtropical regions where dengue virus (DENV) is transmitted (*1*–*3*). Absence of inexpensive and accurate tests to diagnose dengue makes case management, surveillance, and outbreak investigation difficult. During infection, immunoglobulin (Ig) M against DENV can often be detected ≈5 days after onset of fever (*4*–*6*). First-time (primary) DENV infections typically have a stronger and more specific IgM response than subsequent (secondary) infections, for which the IgM response is low compared with a strong IgG response. These patterns underscore the need for evaluating the performance of commercially available tests, especially for diagnosis of secondary DENV infections (*7*–*10*).

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The Study

To provide independent evaluation of dengue diagnostic tests, the United Nations International Children's Emergency Fund/United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases and the Pediatric Dengue Vaccine Initiative established a network of 7 laboratories based on criteria related to dengue expertise of the principal investigator, and type, capacity, management of the laboratory. The laboratories contributed serum specimens for the evaluation panel and conducted the evaluation. The 7 laboratories were located at Mahidol University (Bangkok, Thailand), Cho Quan Hospital (Ho Chi Minh City, Vietnam), Institut Pasteur (Phnom Penh, Cambodia), University of Malaya (Kuala Lumpur, Malaysia), Centers for Disease Control and Prevention (CDC) (San Juan, PR, USA), Instituto Medicina Tropical Pedro Kouri (Havana,

Table 1. Panels used for evaluation of 350 serum samples from patients with positive and negative results for IgM to DENV*

Evaluation panel	No. samples
DENV IgM positive	
From primary infections	27
From secondary infections	154
Total positive	181†
DENV IgM negative	
DENV positive/DENV IgM negative	19
DENV IgG positive	7
Related flavivirus IgM positive	
West Nile virus positive	25
Yellow fever virus positive	4
Related flavivirus IgG positive	
West Nile virus positive	1
Yellow fever virus positive	10
St. Louis encephalitis virus positive	2
Japanese encephalitis virus positive	10
Febrile illness	
Lyme disease IgG positive	9
Malaria	31
New World hantavirus IgM positive	7
Systemic conditions	
Rheumatoid factor	6
Systemic lupus erythematosus	2
Healthy persons‡	
Negative	36
Total negative	169

*DENV, dengue virus; Ig, immunoglobulin.
†No. serum samples identified as serotype specific: 26 DENV-1, 19 DENV-2, 13 DENV-3, and 7 DENV-4. Serotype was not identified for 116 samples.
‡From areas where dengue is not endemic.

Cuba), and Instituto Nacional Enfermedades Virales Humanas Dr. Julio I. Maiztegui (Buenos Aires, Argentina). Laboratories at Mahidol University and CDC acted as reference laboratories by providing samples for proficiency testing among laboratories and for assembling and validating the evaluation panel.

The evaluation panel consisted of 350 well-characterized serum specimens (Table 1). Specimens positive for IgM against DENV were obtained from patients with primary and secondary infections and represented all 4 DENV serotypes. IgM levels were determined by reference standard ELISAs used by CDC and the Armed Forces Research Institute of Medical Science (Bangkok, Thailand) (*6*,*7*). Positive samples were selected based on optical density (OD) and were weighted toward low and medium ODs. Negative control samples included serum samples from healthy persons in areas where dengue is not endemic and from patients with other flavivirus infections, febrile illness of other causes, or systemic conditions. Results were confirmed as negative for IgM antibodies against DENV by using predetermined reference standards. Additionally, 20 anti-DENV IgM-negative specimens were obtained from SeraCare Diagnostics (Milford, MA, USA). Panel specimens were coded, heat-inactivated, aliquoted, and lyophilized; 1 aliquot was retested by the reference laboratories after reconstitution.

Letters of interest and the evaluation protocol were sent to 20 dengue kit manufacturers. Six companies agreed to participate and provided 4 rapid diagnostic tests (RDTs) and 5 microplate ELISAs. Test characteristics are summarized in Table 2. Price per test ranged from US $3 to $15.

Laboratories evaluated the kits for sensitivity and specificity by using the evaluation panel. For each test, kappa coefficient values were determined to assess agreement of mean sensitivity and specificity of each test with the reference standard. A test of homogeneity was used to determine extent of agreement of results among sites.

Table 2. Characteristics of 9 tests used for detection of IgM against dengue virus*

ELISAs					
Test name	Dengue IgM capture	Pathozyme dengue M	Pathozyme dengue M capture	Dengue fever virus IgM capture DxSelect	Dengue IgM capture
Company, location	Panbio Diagnostics, Windsor, Queensland, Australia	Omega Diagnostics, Alva, UK	Omega Diagnostics	Focus Diagnostics, Cypress, CA, USA	Standard Diagnostics, Kyonggi-do, South Korea
Detection method	IgM capture	Indirect IgM detection	IgM capture	IgM capture	IgM capture
Format	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells
No. tests/package	96	96	96	96	96
Antigen	Recombinant DENV 1–4	Purified DENV 2	DENV 1–4	DENV 1–4	DENV 1–4
Sample volume, μL	10	10	20	10	10
Total incubation time	130 min at 37°C	120 min at 37°C	110 min at 37°C	240 min at room temperature	130 min at 37°C
Storage conditions, °C	2–30	2–8	2–8	2–8	2–8

Rapid diagnostic tests				
Test name	Dengue duo cassette	Hapalyse dengue-M PA kit	Dengucheck WB	SD dengue IgG/IgM
Company, location	Panbio Diagnostics	Pentax, Tokyo, Japan	Zephyr Biomedicals, Panaji, India	Standard Diagnostics
Assay principle	Lateral flow	Particle agglutination	Lateral flow	Lateral flow
Target antibody	IgM and IgG	IgM	IgM and IgG	IgM and IgG
Format	Cassette	12 strips of 8 wells	Cassette	Cassette
No. tests/package	25	96	25	25
Antigen	Recombinant DENV 1–4	DENV 1–4	Recombinant DENV (serotype not specified)	Recombinant DENV 1–4 envelope protein
Specimen type	Serum, plasma, or whole blood	Serum or plasma	Serum, plasma, or whole blood	Serum or plasma
Volume of sample required, μL	10	1	5	5
Duration of test, min	15	90	15	15–20
Storage conditions, °C	2–30	2–8	4–30	1–30
Additional equipment required	No	Yes (e.g., micropipette)	No	No

*Ig, immunoglobulin; DENV, dengue virus.

Mean sensitivities of ELISAs were 61.5%–99.0%, and specificities were 79.9%–97.8% (Figure 1, panels A and B). Tests from Panbio Diagnostics (Windsor, Queensland, Australia), Focus Diagnostics (Cypress, CA, USA), and Standard Diagnostics (Kyonggi-do, South Korea) showed significantly higher mean sensitivities (99.0%, 95% confidence interval [CI] 98.4%–99.5%; 98.6%, 95% CI 98.0%–99.2%; and 97.6%, 95% CI 96.8%–98.4%, respectively) than 2 tests from Omega Diagnostics (Alva, UK) (62.3% and 61.5%; p<0.0001 for all comparisons). The Omega Pathozyme Capture test showed significantly higher mean specificity (97.8%, 95% CI 97.0%–98.6%) than the other ELISAs (79.9%–86.6%; p≤0.02 for all comparisons). The Focus, Panbio, and Standard ELISAs showed strong agreement with the reference standard (kappa values 0.81–0.85). Kappa values for Omega kits were below the acceptable range (0.46 and 0.59). Site-to-site variation for ELISAs was not significant (homogeneity >0.05).

Mean sensitivities of RDTs were 20.5%–97.7%, and specificities were 76.6%–90.6% (Figure 1, panels C and D). None had an acceptable kappa value for overall performance compared with reference methods. The Pentax (Tokyo, Japan) test had significantly higher mean sensitivity (97.7%, 95% CI 96.9%–98.5%) than all other RDTs (p<0.0001 for all comparisons), but lowest mean specificity (76.6%, 95% CI 74.1%–79.0%; p<0.0001 for all comparisons) and high false-positive rates for malaria and anti-DENV IgG specimens (Figure 2). Panbio and Standard tests showed high mean specificities (90.6%, 95% CI 88.9%–92.3%, and 90.0%, 95% CI 88%.3–91.7%) with different mean sensitivities (77.8%, 95% CI 75.5%–80.1%, and 60.9%, 95% CI 58.2%–63.6%).

Conclusions

This laboratory-based evaluation used a serum panel to determine the ability of 9 commercially available anti-

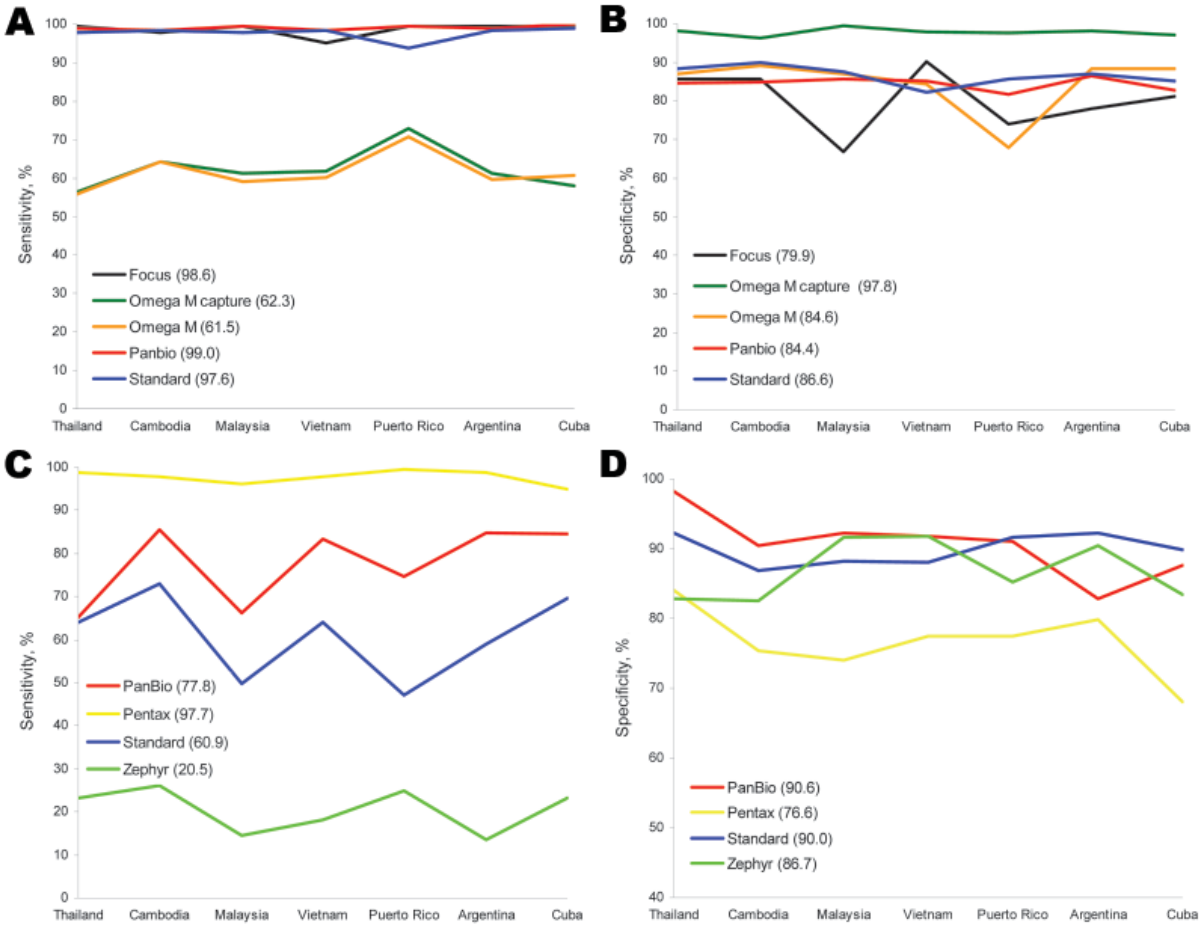

Figure 1. A) Sensitivity and B) specificity of 5 microplate ELISAs used at laboratories in 7 countries for detecting immunoglobulin (Ig) M against dengue virus compared with reference solid-phase IgM antibody-capture ELISAs used by the Centers for Disease Control and Prevention (Atlanta, GA, USA) and the Armed Forces Research Institute of Medical Science (Bangkok, Thailand). Mean sensitivities and specificities for the 5 tests are shown in parentheses. C) Sensitivity and D) specificity of 4 rapid diagnostic tests used at laboratories in 7 countries for detecting IgM against dengue virus compared with solid-phase IgM antibody-capture ELISAs. Mean sensitivities and specificities for the 4 tests are shown in parentheses.

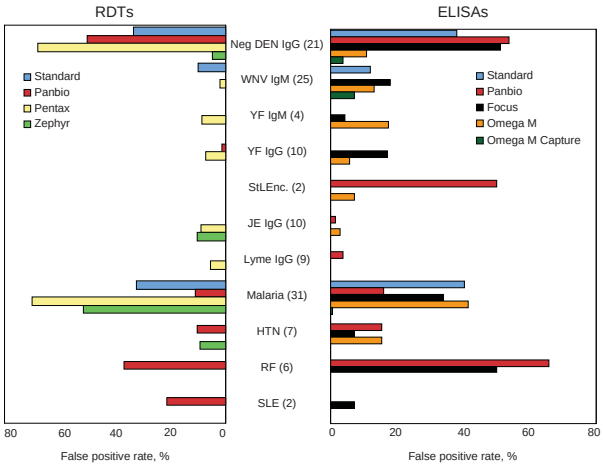

Figure 2. Percentages of false-positive results for 4 rapid diagnostic tests (RDTs) (left panel) and 5 microplate ELISAs (right panel). Numbers of samples tested are shown in parentheses. Neg, negative; DEN, dengue; IgG, immunoglobulin G; WNV West Nile virus; YF, yellow fever; StLEnc, St. Louis encephalitis; JE, Japanese encephalitis; Lyme, Lyme disease; HTN, New World hantavirus; RF, rheumatoid factor; SLE, systemic lupus erythematosus.

DENV IgM tests to detect low levels of IgM and to determine specificity against pathogens that often cocirculate with DENV. Field trials are needed to determine the performance and utility of these tests in a local context.

Of the 5 ELISA kits evaluated, 3 (Focus, Panbio, and Standard) showed strong agreement with reference standard results and were consistent across all evaluation sites. Of concern are false-positive results shown by some tests on sera that were anti-DENV IgM negative but malaria positive, anti-DENV IgG positive, or rheumatoid factor positive. The laboratory at Mahidol University also tested the kits against 12 serum samples from patients with leptospirosis. The Panbio ELISA showed cross-reactivity with 58% of these samples, and the Focus ELISA showed cross-reactivity with 25%. Further studies are needed to elucidate the cause of this cross-reactivity.

Technicians were asked to score tests' user-friendliness. All RDTs scored higher than ELISAs, and the Panbio RDT scored highest.

Limitations of anti-DENV IgM tests include their inability to identify the infecting DENV type and potential antibody cross-reactivity with other flaviviruses (*11*,*12*). However, cross-reactivity to related viruses did not appear to be a problem with these tests. IgM tests can be useful for surveillance and support diagnosis of DENV infection in conjunction with clinical symptoms, medical history, and other epidemiologic information (*13*). Because IgM persists for ≥60 days, IgM assays should not be used in dengue-endemic countries as confirmatory tests for current illness. Presence of IgM indicates that a dengue infection has occurred in the past 2–3 months.

This evaluation has several limitations. Test performance was compared with reference laboratory assay results, which may be less sensitive than commercial assays, leading to some results being misclassified as false positive. Specificity of these tests may be higher in a field setting than in this evaluation because not all potential causes of false-positive results would be present. The panel consisted of a high proportion of specimens from persons with secondary DENV infections. Thus, the panel was weighted toward lower anti-DENV IgM levels. However, this feature reflects the situation in most dengue-endemic countries. Thus, tests that performed well against this panel could be expected to perform well in these diagnostic settings. We could not comprehensively evaluate whether the kits could detect primary infections with all 4 DENVs because all DENV types were not represented in the panel.

Data from this evaluation have been provided to the manufacturers and WHO member states. On the basis of these results, ELISAs from Focus, Panbio, and Standard Diagnostics will be included in the WHO Bulk Procurement Scheme. Technical discussions are ongoing to determine how tests might be improved to accelerate availability of useful methods for dengue case management, surveillance, and disease control.

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Detection of Newly Described Astrovirus MLB1 in Stool Samples from Children

Stacy R. Finkbeiner, Binh-Minh Le, Lori R. Holtz, Gregory A. Storch, and David Wang

The prevalence of the recently identified astrovirus MLB1 in a cohort of children with diarrhea in St. Louis, Missouri, USA, was defined by reverse transcription–PCR. Of 254 stool specimens collected in 2008, 4 were positive for astrovirus MLB1. These results show that astrovirus MLB1 is circulating in North America.

Astroviruses infect a variety of hosts, including humans, turkeys, chicken, cattle, sheep, dogs, cats, deer, ducks, and bats (*1*,*2*). The 8 known human serotypes are genetically closely related. Astroviruses typically cause diarrhea in their hosts; in humans, symptoms usually last 2–4 days (*3*). Children <2 years of age, elderly persons, or otherwise immunocompromised persons are most commonly affected (*3*). Epidemiologic studies suggest human astroviruses 1–8 are responsible for up to ≈10% of cases of acute, nonbacterial diarrhea in children (*4*–*8*).

Recently, a highly divergent astrovirus, referred to as astrovirus MLB1 (AstV-MLB1), was identified in the stool of a 3-year-old boy in Australia (*9*). The entire genome of this novel virus was subsequently sequenced and characterized (*10*). No published reports have described AstV-MLB1 outside of the index case. In this study, we determined the prevalence of this novel virus by reverse transcription–PCR (RT-PCR) screening of stool samples collected at the St. Louis Children's Hospital in St. Louis, Missouri.

The Study

Pediatric stool specimens sent for bacterial culture to the clinical microbiology laboratory at the St. Louis Children's Hospital were analyzed for AstV-MLB1. The Human Research Protection Office of Washington University in St. Louis approved this study. Samples were collected during January through May 2008. Stools were diluted in phosphate-buffered saline at a 1:6 ratio (wt/vol), and total nucleic acid was extracted from 200 μL of each stool suspension by the MagNAPure LC Automated Nucleic Acid Extraction System (Roche, Indianapolis, IN, USA).

Previously described astrovirus primers Mon269 and Mon270 (*11*) frequently have been used for detecting human astrovirus serotypes 1–8 in clinical stool specimens. However, the extensive divergence of AstV-MLB1 to the known human astroviruses rendered these primers unable to amplify AstV-MLB1 (data not shown). Because AstV-MLB1 might represent a new grouping of astroviruses that could include multiple subtypes, we designed primers to conserved regions of the AstV-MLB1 genome to maximize the likelihood of detecting any AstV-MLB1 variant viruses or even other novel astroviruses.

We identified conserved regions by using multiple sequence alignments of AstV-MLB1 amino acid sequences to all fully sequenced astrovirus genomes (Figure 1, panels A and B). The corresponding nucleotide sequences for these regions were then aligned to define the most highly conserved regions (Figure 1, panels C and D). Two regions within open reading frame (ORF) 1b were identified that yielded primers SF0073 (5′-GATTGGACTCGATTTGATGG-3′) and SF0076 (5′-CTGGCTTAACCCACATTCC-3′), which are predicted to generate an ≈409-bp product. Control experiments validated this primer pair could detect AstV-MLB1, as well as human astrovirus 1 (online Appendix Figure, available from www.cdc.gov/EID/content/15/3/441-appF.htm). Given that some of the canonical human astroviruses are identical in the primer binding sites, these data suggest that at least some of the canonical human astroviruses can be detected by the primer pair SF0073/SF0076. In theory, under appropriate experimental conditions, these primers also may be able to detect all other known human and animal astroviruses, although that remains to be experimentally tested. These primers were used with the QIAGEN One-Step RT-PCR Kit (QIAGEN, Valencia, CA, USA) by using the following cycling conditions: 30 min RT step, 94ºC for 10 min, followed by 40 cycles of 94ºC for 30 s, 52ºC for 30 s, and 72ºC for 50 s.

Samples that tested positive with primers SF0073 and SF0076 were then tested in a second round of screening with 2 different primer sets in parallel to determine whether the samples contained canonical human astrovirus serotypes 1–8 or AstV-MLB1. The previously reported Mon269 (5′-CAACTCAGGAAACAGGGTGT-3′) and Mon270 (5′-TCAGATGCATTGTCATTGGT-3′) primers, which generate a 449-bp amplicon, were used to detect canonical human astroviruses (*11*). Another set of primers, SF0053 (5′-CTGTAGCTCGTGTTAGTCTTAACA-3′) and SF0061 (5′-GTTCATTGGCACCATCAGAAC-3′), was designed to exclusively detect AstV-MLB1 and produce a 402-bp PCR product. These primers target a region of the capsid gene. The second round of screening with both sets of primer pairs was performed as described

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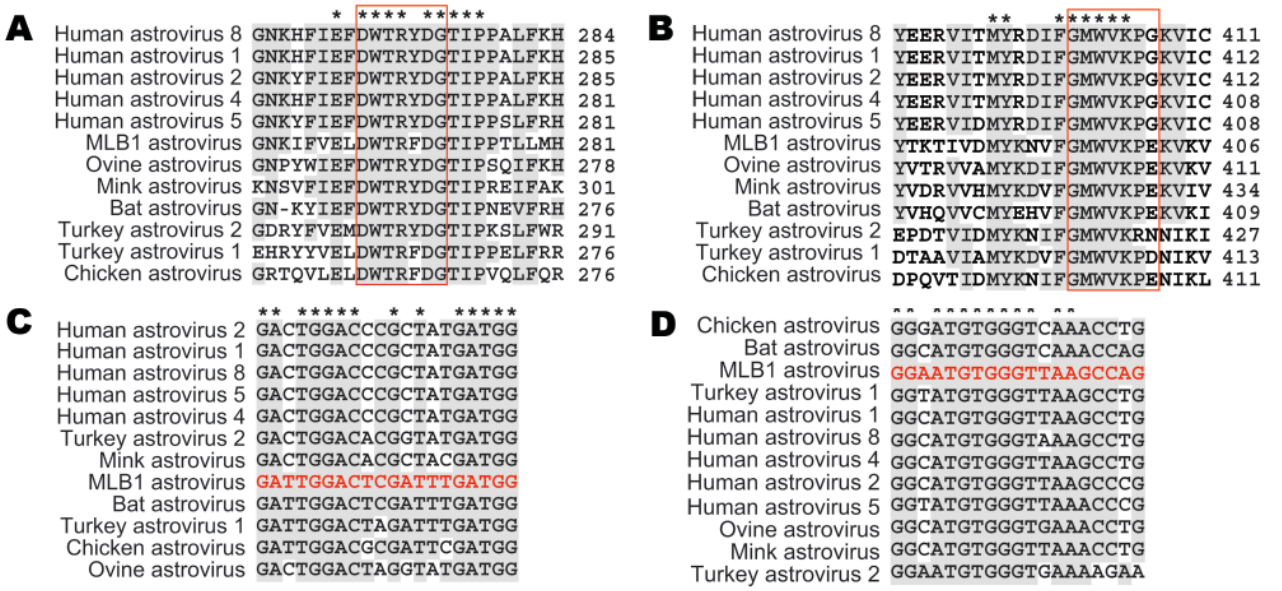

Figure 1. Astrovirus open reading frame (ORF) 1b alignments for design of pan-astrovirus primers. Astrovirus RNA polymerase sequences (ORF1b) were aligned at the amino acid level to define the conserved regions used for the design of primers SF0073 (A) and SF0076 (B). The numbers to the right of the sequences indicate the position of the last amino acid within each ORF1b sequence. Red boxes represent the specific regions that were reverse translated into the corresponding nucleic acid sequences used for the design of SF0073 (C) and SF0076 (D). Red sequences shown in the nucleotide alignments are the actual primer sequences. Asterisks indicate nucleotide identity.

above except that an annealing temperature of 56ºC was used.

Of 254 stool specimens screened, 9 (3.5%) tested positive in the initial round of screening that used the newly designed pan-astrovirus primers, SF0073 and SF0076. Secondary screening showed that 5 (2% of all samples) were canonical human astroviruses. This probably underestimates the prevalence of the astrovirus serotype 1–8 in the cohort because the initial screening primers were biased toward detection of AstV-MLB1. The remaining 4 (1.6% of all samples) were positive for AstV-MLB1 using primers SF0053 and SF0061. For each of the 4 samples positive for AstV-MLB1, 2 additional fragments were generated by RT-PCR for phylogenetic analysis. A 1,228-bp fragment of ORF1a, which encodes the serine protease, and a 920-bp fragment of ORF2, which encodes the capsid proteins, were amplified using AstV-MLB1–specific primers from each of the 4 samples designated WD0016, WD0055, WD0104, and WD0227. The primers used for the ORF1a fragment are SF0080 (5′-AAGGATAGTGCTGGTAAAGTAGTTCAGA-3′) and SF0094 (5′-CAAGAGCCTTATCAACAACGTA-3′) and the primers used for the ORF2 fragment are SF0064 (5′-GTAAGCATGGTTCTTGTGGAC-3′) and SF0098 (5′-TGCATACATTTATGCTGGAAGA-3′). The ORF1a fragments (GenBank accession nos. FJ227120–FJ227123)

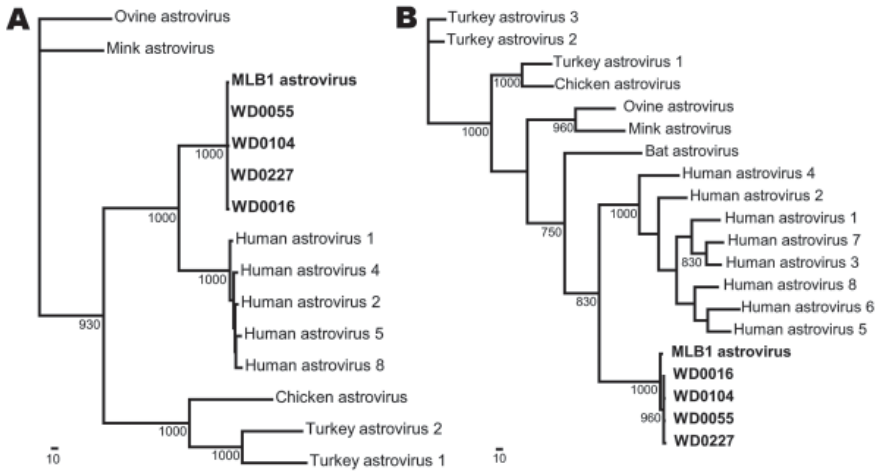

Figure 2. Phylogenetic analysis of astrovirus MLB1 (AstV-MLB1) isolates. A region of the serine protease (A) and the capsid (B) of each virus detected by the AstV-MLB1–specific primers was amplified and sequenced. Multiple sequence alignments were then generated with these sequences and the corresponding regions of known astroviruses using ClustalX (www.clustal.org). PAUP* (Sinauer Associates, Sunderland, MA, USA) was used to generate phylogenetic trees; bootstrap values (>700) from 1,000 replicates are shown. The previously identified AstV-MLB1 isolate (9,10) and the isolates from this study are shown in **boldface**. Scale bars indicate number of amino acid substitutions per site.

Table 1. Similarity of fully sequenced WD0016 genome to AstV-MLB1*

Genome	Nucleotide identity with AstV-MLB1, %		
	ORF1a (serine protease)	ORF1b (RNA polymerase)	ORF2 (capsid)
WD0016	92.6	93.9	91.9

*AstV-MLB1, astrovirus MLB1; ORF, open reading frame.

from these samples all shared ≈92% nt identity to the reference astrovirus MLB1 sequence (GenBank accession no.: FJ222451) and 99% aa identity, indicating that most mutations were synonymous. The ORF2 fragments (GenBank accession nos. FJ227124–FJ227127) shared ≈91%–92% nt identity and 95%–96% aa identity to the reference astrovirus MLB1 sequence. The 4 positive St. Louis samples shared ≈99% nt identity to each other. The ORF1a and ORF2 sequences were aligned to other astroviruses for which full genome sequences were available using ClustalX version 1.83 (www.clustal.org); maximum-parsimony trees were generated using PAUP with 1,000 bootstrap replicates (*12*) (Figure 2). The entire genome of one of the isolates, WD0016 (GenBank accession no. FJ402983), was sequenced and had 92.6% identity overall to that of AstV-MLB1 on the basis of a pairwise nucleotide alignment (Table 1).

Patients with AstV-MLB1–positive stools ranged in age from ≈4 months to 4 years (Table 2). All patients had symptoms of diarrhea at stool collection, except the patient with isolate WD0016, who reported having diarrhea 2 days before stool collection. All specimens were tested for *Escherichia coli*, *Campylobacter* spp., *Yersinia* spp., *Shigella* spp., and *Salmonella* spp. by standard bacterial culture. The WD0227 sample tested positive for *E. coli* O157:H7; the other samples were negative for all bacterial cultures. A pan-viral microarray, the ViroChip (GEO platform GPL 3429; National Center for Biotechnology Information, Bethesda, MD, USA) (*13*), was used to examine whether other viruses were present in the stool of 3 (WD0055, WD0104, and WD0227) of the 4 AstV-MLB1–positive samples for which enough material remained for analysis. WD0055 and WD0104 were negative by array, but WD0227 was positive for rotavirus as determined by the ViroChip.

Conclusions

The newly identified AstV-MLB1 virus was discovered in a stool specimen collected in Melbourne, Victoria, Australia, in 1999. In this study, we describe the detection of AstV-MLB1 in a cohort from St. Louis collected in 2008. This observation provides evidence of AstV-MLB1 outside Australia and suggests that AstV-MLB1 is likely to be globally widespread. In addition, these data demonstrate that AstV-MLB1 is circulating in the human population. The sequence divergence of ≈8% at the nucleotide level between the reference AstV-MLB1 genome and the viruses detected in this study suggests substantial sequence heterogeneity within the AstV-MLB1 group of viruses. Multiple serotypes or subtypes of AstV-MLB1 might exist, as with the canonical human astroviruses. More extensive screening of stool samples with PCR primers targeted toward detection of AstV-MLB1, such as those described here, may provide insight into the true diversity and prevalence of AstV-MLB1–like viruses. Finally, a critical direction for future investigation is determining whether AstV-MLB1, like the canonical astrovirus serotypes 1–8, is a causal agent of human diarrhea, and if so, assessing the extent and severity of disease associated with this virus. Further epidemiologic studies, including both case–control prevalence studies and seroprevalence assays, and efforts to fulfill Koch's postulates should be pursued.

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Table 2. Clinical and demographic characteristics of patients with stool samples positive for astrovirus MLB1

Characteristic	Sample			
	WD0016	WD0055	WD0104	WD0227
Age, m	15	17	4	43
Sex	F	F	M	M
Diarrhea	No*	Yes	Yes	Yes
Other symptoms	Abdominal pain	Vomiting, fever	Fever, seizures, respiratory distress	Fever
Hospitalization	Yes	No	Yes	Yes
Bacterial cultures†	Negative	Negative	Negative	Positive for *E. coli* O157:H7

*Patient had diarrhea 2 days before stool collection but not at collection.
†Tests were conducted for *Escherichia coli*, *Campylobacter* spp., *Shigella* spp., *Salmonella* spp., and *Yersinia* spp.

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Introduction into Nigeria of a Distinct Genotype of Avian Influenza Virus (H5N1)

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Genetic characterization of highly pathogenic avian influenza viruses (H5N1) isolated in July 2008 in Nigeria indicates that a distinct genotype, never before detected in Africa, reached the continent. Phylogenetic analysis showed that the viruses are genetically closely related to European and Middle Eastern influenza A (H5N1) isolates detected in 2007.

In February 2006, highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype was detected in chickens in Kaduna state in northern Nigeria, the first African country reporting a confirmed HPAI (H5N1) outbreak. The infection later spread to 25 of the 36 Nigerian states and to the Federal Capital Territory and persisted for 21 months. Consequently, ≈368,000 domestic birds (mostly chickens; also guinea fowl, turkeys, ducks, geese, and ostriches) were killed by the virus or culled, and a fatal human case was reported. By the end of 2007, the outbreaks appeared to have been controlled by such measures as stamping-out with compensation, restricting movement of poultry and poultry products, improving biosecurity measures, and enhanced surveillance systems. The last reported case of HPAI occurred in the southern state of Anambra and was reported by the Government of Nigeria in October 2007. In addition to the routine avian influenza surveillance program, industrial poultry in the 36 states plus the Federal Capital Territory, and live bird markets have been actively monitored since March 2007, with >13,000 samples collected and analyzed for avian influenza viruses (T. Joannis, pers. comm.).

During the surveillance activities at the live bird markets, new cases of HPAI (H5N1) were detected in July 2008 in the city of Gombe in the northeastern state of Gombe after a 9-month period during which no influenza A virus was identified. In particular, 2 tracheal swabs collected from apparently healthy domestic ducks were submitted to the laboratory for virus isolation in embryonated specific antibody-negative fowl eggs. Allantoic fluid harvested from inoculated eggs showing embryo death tested positive for hemagglutinating agents. RNA extracted from positive allantoic fluid also tested positive by real-time reverse transcription–PCR for type A influenza RNA (*1*) and for the H5 subtype (*2*). Hemagglutination and neuraminidase (NA) inhibition assays with monospecific antiserum (*3*) confirmed the H5N1 subtype. Viruses were designated as A/duck/Nigeria/3724-2/2008 and A/duck/Nigeria/3724-10/2008.

The Study

We obtained the full-length genome sequence for A/duck/Nigeria/3724-2/2008 and the sequence of the hemagglutinin (HA) segment for A/duck/Nigeria/3724-10/2008 (*4*). Sequences of the 8 gene segments of A/duck/Nigeria/3724-2/2008 were submitted to the Global Initiative on Sharing Avian Influenza Data public database (accession nos. EPI161701–EPI161708). The HA segment of the 2 isolates was identical, and the deduced amino acid sequence of the HA cleavage site was characteristic of HPAI (H5N1) (PQGERRRKKR*GLF). The highly pathogenic pathotype was confirmed by the result of the intravenous virus pathogenicity index test (index 2.87) (*3*).

Phylogenetic analysis of the 8 genes was conducted by using MEGA 4 (*5*) with the neighbor-joining method, and the HA and NA tree topology was confirmed by using Bayesian methods (*6*) (Figures 1, 2). Phylogenetic analysis of the HA gene segment showed the viruses fall in clade 2.2, according to the unified nomenclature system (*7*). Unexpectedly, the viruses were grouped separately from the viruses previously detected in Nigeria and in other African countries. They clustered in the sublineage here designated III, together with HPAI (H5N1) viruses isolated in 2007 in Europe and Middle East (Figure 1). Phylogenetic analysis of the NA gene segment of A/duck/Nigeria/3724-2/2008 supported these results (Figure 2).

Sequence analysis of the 8 gene segments of A/duck/Nigeria/3724-2/2008 showed the highest similarity at the nucleotide level with the HPAI (H5N1) virus isolate A/*Cygnus olor*/Czech Republic/10732/2007 (99.3% for HA, 99.8% for NA, 99.7% for nonstructural protein, 99.4% for polymerase basic protein 1 [PB1], 99.7% for polymerase basic protein 2 [PB2], 99.8% for nucleoprotein [NP], 99.3% for polymerase acidic protein [PA], and 100% for matrix [MA] protein) and with the HPAI (H5N1) strains from Romania collected in 2007 (99.3%, only HA gene segment is available). For the HA protein, only 3 amino ac-

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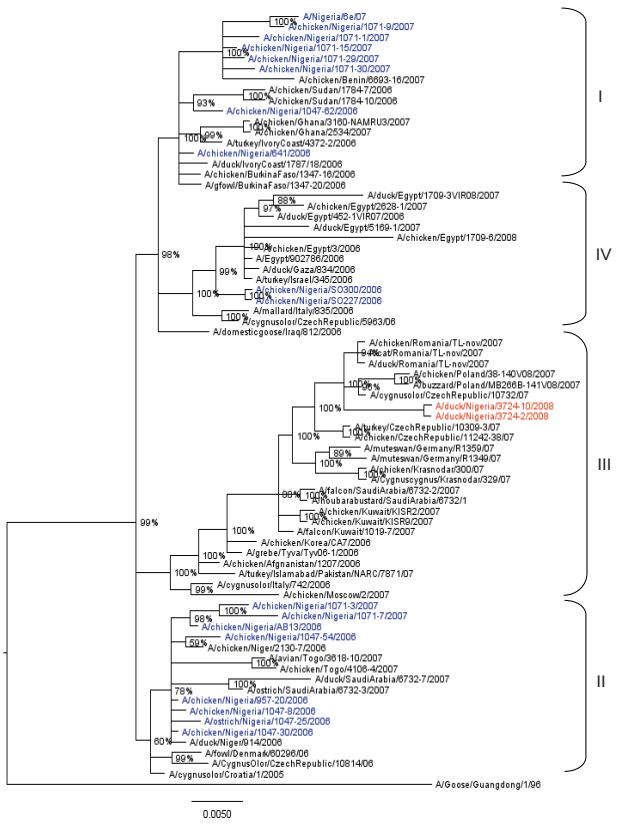

Figure 1. Phylogenetic tree constructed by Bayesian analysis of the hemagglutinin gene segment of representative influenza viruses A (H5N1) from Africa, Europe, and the Middle East. Taxon names of the Nigerian viruses isolated during 2006–2007 are marked in blue, 2008 isolates in red. Posterior probabilities of the clades are indicated above the nodes. Scale bar indicates number of nucleotide substitutions per site.

ids differences were observed between the Nigerian isolate and the strain A/*Cygnus olor*/Czech Republic/10732/2007. Lower similarities (ranging from 96.9% to 98% for the HA gene) were shown with previous isolates from Nigeria. No molecular changes were associated with increased affinity toward α 2,6 linkage sialic acid substrates in the HA receptor-binding domain (*8*) or mutations related to resistance to NA inhibitors and to adamantanes were observed in the HA, NA, and M2 genes of the Nigerian isolate. Analysis of the amino acid sequences of the internal proteins of A/duck/Nigeria/3724-2/2008 virus showed the amino acid lysine at position 627 of the PB2 gene known to be associated with increased virulence of HPAI (H5N1) virus in mice (*9*) and 1 amino acid mutation at position 33 (V33I) of the NP gene, which is described as genetic signature of human influenza A virus (*10*). The PB1-F2 protein had 1 mutation at position 66 (N66S), previously observed only in the Hong Kong 1997 subtype H5N1 viruses and in the 1918 pandemic strain (A/Brevig Mission/18) and is associated to high pathogenic phenotype in mice (*11*).

Conclusions

Since the earliest known progenitor detected in China, A/goose/Guandong/96, numerous lineages of HPAI (H5N1) viruses have emerged (*7*). Introduction and spread of distinct H5N1 genetic lineages were described in several Asian countries and in Europe, such as Germany, Italy, and France (*12*,*13*). Surprisingly, Nigeria is the only country in Africa where HPAI (H5N1) belonging to distinct sublineages have been detected (*4*,*12*,*14*). Previous genetic characterization of HPAI (H5N1) viruses isolated during 2006 and 2007 indicated the cocirculation in Nigeria of 3 distinct sublineages, here designated I, II, and IV (*4*,*14*). Sublineages I and II appeared to be widespread in this country during 2006 and 2007 (*14*), and their extended cocirculation enabled reassortment events between these sublineages. The first reassortant virus was identified in June 2006 (*12*), and in early 2007, an additional reassortant virus was identified in 7 of 22 Nigerian states where infection was found (*4*).

Our results indicate a novel introduction in Nigeria of a virus belonging to sublineage III, a genotype not previously detected in the African continent. Indeed, previous surveil-

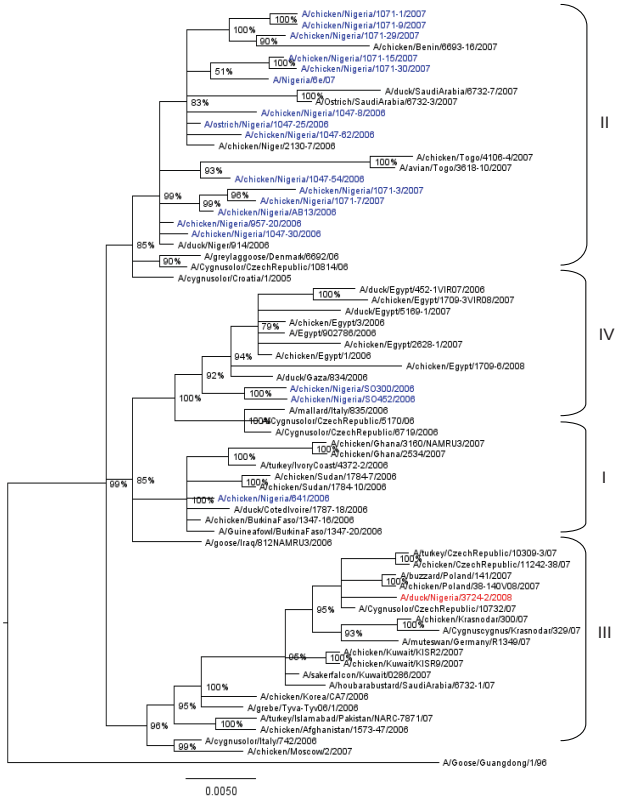

Figure 2. Phylogenetic tree constructed by Bayesian analysis of the neuraminidase gene segment of representative influenza viruses A (H5N1) from Africa, Europe, and the Middle East. Taxon names of the Nigerian viruses isolated during 2006–2007 are marked in blue, 2008 isolate in red. Posterior probabilities of the clades are indicated above the nodes. Scale bar indicates number of nucleotide substitutions per site.

lance efforts (passive and active) since 2006 in Nigeria and other African countries (*4*,*12*,*14*) never showed evidence of the circulation of a virus belonging to this sublineage. Viruses belonging to sublineage III have been detected in domestic and wild birds in 2007 in European, Middle Eastern, and Asian countries such as Germany, Poland, Romania, the Czech Republic, Kuwait, Saudi Arabia, Russia, and Pakistan.

The Nigerian isolate A/duck/Nigeria/3724-2/2008 resulted in a genome almost identical to an isolate from a mute swan living in the wild, namely, A/*Cygnus olor*/Czech Republic/10732/2007 (*15*). This finding, however, does not shed light on how the virus was introduced into Nigeria because neither of the 2 main means of spread, through wild birds or the poultry trade, can be excluded.

The evidence of a mutation in the PB1-F2 gene segment that increases the pathogenicity of the virus in mammalian hosts is of concern. This type of mutation has never been observed in HPAI (H5N1) viruses of clade 2.2; this finding supports the need for a continuous monitoring effort of influenza viruses A (H5N1) viral genotypes and their evolution. Our findings highlight the evolving epidemiology of HPAI (H5N1) viruses and the need for implementation and maintenance of sustainable surveillance programs in countries where infection has been found and in countries where it has not. The outcome of these efforts, however, can be maximized only through prompt reporting to international organizations and international collaboration that leads to timely molecular and antigenic comparisons of isolates.

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Border Disease Virus among Chamois, Spain

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Approximately 3,000 Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) died in northeastern Spain during 2005–2007. Border disease virus infection was identified by reverse transcription–PCR and sequencing analysis. These results implicate this virus as the primary cause of death, similar to findings in the previous epizootic in 2001.

Chamois (genus *Rupicapra*) are goat-like bovids native to the mountain areas of Europe and the Near East; they have also been introduced into New Zealand. In 2001 and 2002 a new pestivirus (family *Flaviviridae*) was associated with an outbreak of a previously unreported disease in Pyrenean chamois (*R. pyrenaica pyrenaica*) at the Alt Pallars-Aran National Hunting Reserve in the Pyrenees in northeastern Spain (Figure 1) (*1*). Molecular characterization assigned this virus to the border disease virus (BDV) cluster, 1 of the 4 main species of the genus *Pestivirus* (*2*,*3*). Later studies showed that the disease has become endemic in the area and could have a serious effect on chamois population dynamics (*4*,*5*).

The Study

In December 2004, an adult male chamois was found alive with respiratory disease at the Cerdanya-Alt Urgell National Hunting Reserve, ≈30 km southeast of a previously affected area (Figure 1). At the beginning of 2005, sudden deaths of chamois were observed in the same area. Dozens of carcasses were found in February and March. Three animals captured alive showed severe clinical signs of respiratory disease. Later, 2 isolated sick chamois found in May and October had mainly clinical signs of cachexia and alopecia, similar to those previously observed in 2001 and 2002 (*1*).

A census conducted in July 2005 corroborated the collapse of the chamois population, which decreased from 563 chamois in 2004 (preoutbreak) to only 81 chamois. Thus, the estimated cumulative rate of decrease in this area would have been 85.6%. In June 2005, the disease spread to the nearby Cadí National Hunting Reserve and private hunting areas (Figure 1), triggering another episode of mass deaths that has lasted for ≈31 months. An area >125,000 hectares of chamois habitat was affected, with the population decreasing from 3,458 chamois in 2004 (preoutbreak) to 1,281 in July 2006 (estimated cumulative rate of decrease 63%).

We studied 68 affected chamois (41 males and 27 females, age range 1–15 years), 6 from Cerdanya-Alt Urgell and 62 from Cadí Reserve. The main clinical signs and lesions of sick animals were cachexia, alopecia, and respiratory disease (Table 1). We performed necropsies on all 68 chamois. Apart from cachexia in most animals, bronchopneumonia was the main macroscopic lesion (in 31 chamois); 4 had diarrhea, 2 had infectious keratocojunctivitis, 2 had an abscess, and 1 had severe fibrinous pleuropericarditis.

Spleen and kidney homogenates were examined for pestivirus nucleic acid by reverse transcription PCR by using panpestivirus primers (Pesti 3 and Pesti D) (*6*,*7*). All chamois, with the exception of 2 animals from the Cadí Reserve, were positive for pestivirus.

Sequence analyses of the 243-bp fragment of the 5′ untranslated region of 9 isolates were performed for phylogenetic analysis by using primers 324 and 326 (*8*). Purified amplicons (Minelute Gel Extraction Kit; QIAGEN, Hilden, Germany) were analyzed with the BigDye Terminator kit version 3.1 and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Warrington, UK). Seven isolates had been sequenced in an investigation of BDV shedding and detection in organs of naturally infected Pyrenean chamois. The phylogenetic tree was constructed by the neighbor-joining method (*9*) by using automatic root location. Bootstrap analysis of 1,000 replicates was performed by creating series of bootstrap samples to test tree branch reliability. Re-

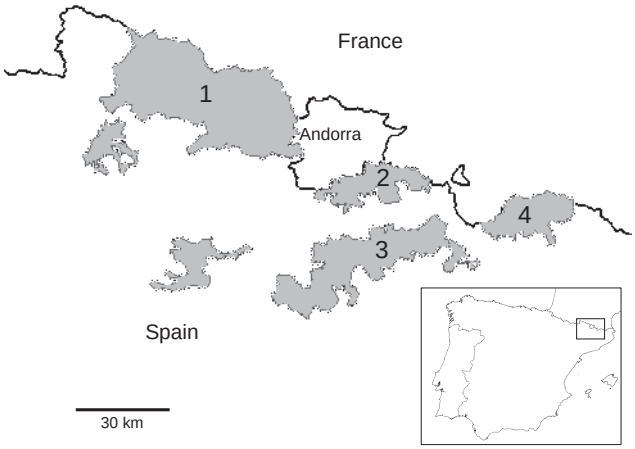

Figure 1. Map of northeastern Spain showing the National Hunting Reserves in Catalonia (shaded areas): 1, Pallars-Aran; 2, Cerdanya-Alt Urgell; 3, Cadí; 4, Freser-Setcases.

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Table 1. Characteristics of 23 ill Pyrenean chamois, Spain, 2005–2007*

Chamois no.	Date	Clinical symptoms	ELISA result for pestivirus	RT-PCR result for pestivirus	Isolate and origin	GenBank accession no.
1	2004 Dec	Pneumonia	–	+	Cerdanya-1	AM905930†
2	2005 Mar	Pneumonia	–	+	Cerdanya-2	AM905931
3	2005 Mar	Pneumonia	–	+	Cerdanya-5	NA
4	2005 Mar	Pneumonia	–	+	Cerdanya-3	AM905932†
5	2005 May	Cachexia, alopecia	–	+	Cerdanya-4	AM905933†
6	2005 Aug	Cachexia, pneumonia	–	+	Cadi-3	AM905920†
7	2005 Aug	Diarrhea, pneumonia	+	+	Cadi-13	NA
8	2005 Sep	Pneumonia, predation	+	+	Cadi-14	NA
9	2005 Sep	Predation	–	–	Cadi-15	NA
10	2005 Oct	Cachexia	–	+	Cadi-2	AM905919†
11	2005 Oct	Trauma	NA	–	Cadi-16	NA
12	2005 Oct	Cachexia, alopecia	–	+	Cerdanya-6	NA
13	2005 Dec	Cachexia, diarrhea	–	+	Cadi-4	AM905921†
14	2006 Feb	Cachexia, alopecia	–	+	Cadi-7	AM905924
15	2006 Feb	Cachexia, alopecia	–	+	Cadi-5	AM905922†
16	2006 Apr	Cachexia, alopecia	–	+	Cadi-10	AM905927
17	2006 Apr	Cachexia	–	+	Cadi-1	AM905918
18	2006 Apr	Cachexia, alopecia	–	+	Cadi-8	AM905925
19	2006 May	Cachexia, alopecia, pneumonia	–	+	Cadi-9	AM905926
20	2006 Jun	Cachexia, alopecia, pneumonia	–	+	Cadi-6	AM905923†
21	2006 Aug	Cachexia, alopecia, pneumonia	–	+	Cadi-11	AM905928
22	2006 Aug	Cachexia, alopecia, pneumonia	–	+	Cadi-12	AM905929
23	2007 Dec	Cachexia, pneumonia	–	+	Cadi-17	NA

*RT-PCR, reverse transcription–PCR; NA, not available.
†Virus sequenced in this study.

sulting sequences showed that chamois were infected with the BDV-4 genotype and that isolates from the 2 outbreaks formed a discrete cluster separated from isolates of the previous outbreak (Figure 2).

Serum samples from 60 chamois were tested for pestivirus-specific antibodies by using an ELISA (Synbiotics, Lyon, France) that detects antibodies against a protein (p80/125) common to all bovine viral diarrhea virus (BVDV) and BDV strains. Only 2 PCR-positive chamois had antibodies, which suggests that most of them could have been persistently infected. In this condition, animals become infected during early pregnancy, are immunotolerant and seronegative, and release large amounts of virus into the environment, thus being the main source of transmission. Retrospectively, serum samples of 78 healthy chamois captured at the Cadí Reserve during 2000–2002 were also tested and showed positive results in 4 (5.1%) chamois.

To confirm ELISA results and determine antibody specificity, serum samples from 6 ELISA-positive chamois were tested by using a comparative virus neutralization test. Viral strains tested were BVDV-1 strain NADL, BVDV-2 strain atypical, BDV strain Spain 97 (*10*), BDV strain Moredun, BDV strain 137/4, and BDV strain CADI-6 (chamois). Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses in all cultures. Titers $\geq$10 were considered positive. Viral replication was monitored by immunoperoxidase monolayer assay with a polyclonal pestivirus-specific antibody. The comparative virus neutralization test confirmed ELISA results in all 6 chamois. Higher titers to BDV Cadi-6 were observed in the 2 pestivirus RT-PCR–positive chamois. Higher titers to BDV Spain 97 were also observed in most healthy animals, which suggest infection with strains of ovine origin (Table 2).

Conclusions

BDV infection in chamois in the Cerdanya-Alt Urgell Reserve could have been the result of the spread of the disease reported in the Alt Pallars-Aran Reserve in 2001 and 2002. The extreme severity of the disease in the Cerdanya-Alt Urgell and Cadí Reserves is unprecedented in pestivirus infections in wild ruminants (*11*). Clinical and pathologic findings suggest that pneumonia was a major contributing factor for the high mortality rate observed at the Cerdanya-Alt Urgell Reserve. Bronchopneumonia is frequently found in chamois (*12*), but this problem may have been magnified by immunosuppressive effects of coincident BDV infection. The severity of the outbreak may have been affected

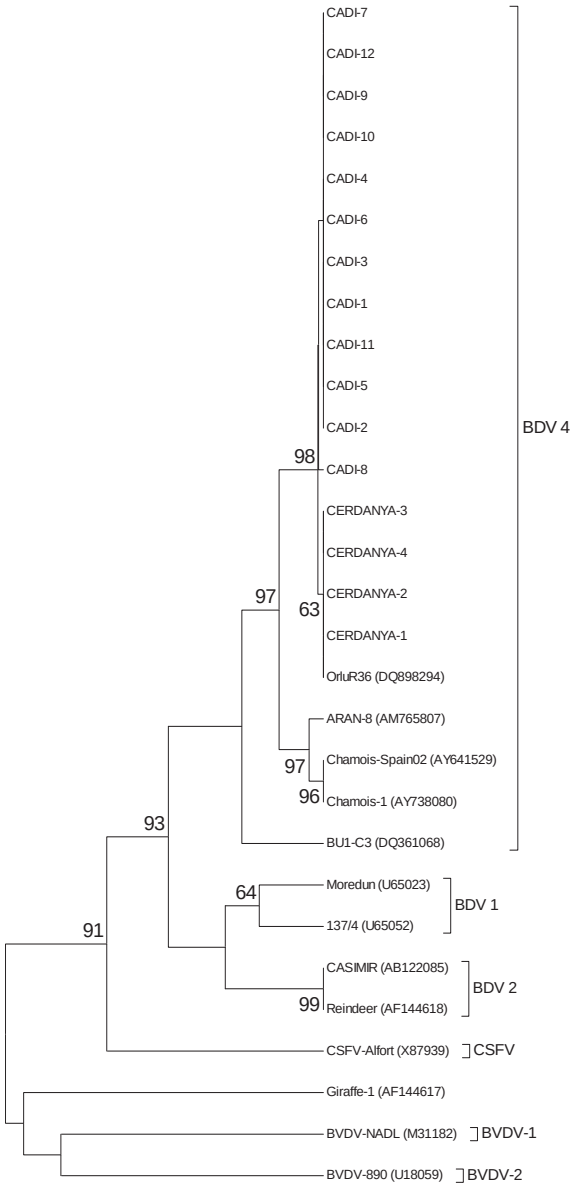

Figure 2. Unrooted neighbor-joining phylogenetic tree based on the 5′ untranslated region sequence among pestiviruses isolated from chamois, Spain. Chamois strains were enclosed in a differentiated group into border disease virus 4 (BDV-4). Numbers on the branches indicate percentage bootstrap values of 1,000 replicates. Numbers on the right in parentheses indicate GenBank accession numbers. CSFV, classical swine fever virus; BVDV, bovine viral diarrhea virus.

by lack of immunity at the population level. Data from the Cadí Reserve before the outbreak showed low antibody seroprevalence. In comparison, in the Freser-Setcases National Hunting Reserve, ≈8 km from Cadí, seroprevalence was 71% in 2003. In this reserve, the same virus was identified in a healthy chamois in September 2006 and in an isolated diseased chamois in June 2007. However, to date no epidemics have been reported.

After these 2 outbreaks in Cerdanya-Alt Urgell and Cadí Reserves, the remaining population may have acquired immunity against the infection, as was the case after the first outbreak in the Alt Pallars-Aran National Hunting Reserve (*4*). The recovery rate in the Cerdanya-Alt Urgell and Cadí chamois populations has not been as fast as expected. In July 2007, the census in these 2 populations identified 153 and 1,616 chamois, respectively. In July 2008, only 165 and 1,661 chamois, respectively, were identified.

These results implicate BDV infection as the primary cause of death in chamois, as previously reported in 2001 and 2002. Further experimental studies are ongoing to fulfill criteria needed for a specific microorganism to be identified as the cause of this disease. Additional studies are needed to determine whether this infection will have a negative effect on the population dynamics of Pyrenean chamois and epidemiologic relationships between chamois and sheep with respect to infection with different BDV strains.

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Table 2. Virus neutralization titers against 6 pestivirus strains in serum samples from 6 chamois, Spain, 2005–2007*

Chamois no.	BDV Spain 97	BDV Cadi-6	BDV 137/4	BDV Moredun	BVDV-1 NADL	BVDV-2 atypical
1 (PCR positive)	20	160	0	0	0	0
2 (PCR positive)	0	160	0	0	0	0
3 (healthy)	320	160	160	40	40	0
4 (healthy)	320	80	80	40	40	0
5 (healthy)	0	0	0	40	0	10
6 (healthy)	80	80	40	0	0	0

*BDV, border disease virus; BVDV, bovine viral diarrhea virus.

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Methicillin-Resistant *Staphylococcus aureus* in Poultry

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has been detected in several species and animal-derived products. To determine whether MRSA is present in poultry, we sampled 50 laying hens and 75 broiler chickens. MRSA was found in some broiler chickens but no laying hens. In all samples, *spa* type t1456 was found.

Staphylococcus aureus is a well-known pathogen of humans and animals. Methicillin resistance in this bacterial species represents a threat to human health. Originally, methicillin-resistant *S. aureus* (MRSA) was a nosocomial pathogen, but in the 1990s, MRSA spread into communities worldwide.

Recently, pigs were shown to be a major reservoir for MRSA multilocus sequence type 398 (ST398). Because this sequence type has also been isolated from other animal species, it is referred to as livestock-associated MRSA (*1*). It has also shown potential for zoonotic transmission (*2*). Among ST398 isolates, a variation in *spa* types has been found (*3*). MRSA has been isolated from raw chicken meat or carcasses in Korea (*4*,*5*) and Japan (*6*); however, these strains were human-associated and not the livestock-associated strains. Thus, the possibility of human contamination of poultry carcasses by slaughterhouse employees cannot be ruled out. We investigated whether livestock-associated MRSA is present in commercial broiler chickens and laying hens.

The Study

In 2007, from randomly selected farms in Belgium, we sampled 5 laying hens from each of 10 farms and 5 broiler chickens from each of 14 farms. One broiler farm was sampled twice (4 months apart, from different flocks in the same house, leaving 1 production round unsampled). Samples were taken from the cloaca and nasal cavity of these 50 laying hens and 75 broiler chickens.

Samples were first incubated in a brain–heart infusion broth supplemented with nalidixic acid and colistin, each at a concentration of 10 μg/mL. After overnight incubation at 37°C, 1 μL of this broth was streaked onto an MRSA ChromID plate (bioMérieux, Marcy l'Etoile, France) and incubated for 24–48 h at 37°C. To differentiate phenotypes of *S. aureus*, we purified colonies that showed typical growth on MRSA Chrom-ID plates by transferring them to modified Baird-Parker agar (*7*) and Columbia blood agar (Oxoid, Hampshire, UK) for DNAse and catalase testing.

The phenotypically identified MRSA strains were then confirmed by 16S rRNA-*mecA*-*nuc* triplex PCR as previously described (*8*). For all strains, the spa type was determined, and for 3 strains, multilocus sequence typing (MLST) was performed as described (*9*). Relatedness with other *spa* types from porcine origin (*10*) was determined by using Ridom SpaServer software version 1.3 (Ridom GmbH; Würzburg, Germany; www.ridom.de/spa-server).

Disk susceptibility of the strains was tested by using the Kirby-Bauer disk-diffusion method. Clinical Laboratory Standards Institute guidelines (M31-A3) were followed for inoculum standardization. After plates were incubated for 18 h, inhibition zones were measured in millimeters and interpreted according to Neo-Sensitabs manufacturer's instructions (http://rosco.dk). *S. aureus* ATCC 25923 was included for internal quality control.

MRSA was not isolated from any laying hen samples. This finding may indicate that MRSA is absent or present only in low numbers in laying hens, possibly because of the limited use of antimicrobial drugs in these animals. Use of certain antimicrobial drugs in human hospitals has been shown to be a risk factor for acquiring MRSA infection, especially when the chosen treatment is inappropriate or insufficient (*11*). Antimicrobial-drug use may also be a risk factor for MRSA colonization of animals. The antimicrobial drugs used in the flocks included in this study were tylosin, amoxicillin, trimethoprim-sulfamethoxazole, lincomycin, tetracycline, and colistin.

MRSA was isolated from 8 broiler chickens from 2 of the 14 farms sampled. Low prevalence in poultry has also been found by Kitai et al. (*6*) and Lee (*5*), although they sampled chicken carcasses from slaughterhouses and did not find any livestock-associated strains. Given our relatively small sample size, our data did not permit us to estimate the within- and between-flock prevalence.

In the MRSA-positive flocks, the number of positive samples varied between 1/5 (20%) and 5/5 (100%). From the 1 MRSA-positive farm that was sampled twice, MRSA was isolated on both occasions. This finding indicates that MRSA may persist on a farm and colonize future flocks. MRSA was found in nearly equal numbers from the nares samples and the cloaca samples. Of the 8 MRSA-positive animals (16 samples), MRSA was found in all samples except for 1 cloacal swab, for a total of 15 MRSA isolations.

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Table. Comparison of *spa* type methicillin-resistant *Staphylococcus aureus* isolated from pigs and poultry

Source	*spa* type	Composition*
Pig	t011	008 – 16 – 02 – 25 – – – – – 34 – 24 – 25
Pig	t034	008 – 16 – 02 – 25 – 02 – 25 – 34 – 24 – 25
Pig	t108	008 – 16 – 02 – 25 – – – – – – – 24 – 25
Pig	t567	008 – – – 02 – 25 – – – – – – – 24 – 25
Pig	t943	008 – 16 – 02 – 25 – – – 25 – – – 24 – 25
Pig	t1254	106 – 16 – 02 – 25 – – – – – 34 – 24 – 25
Pig	t1255	008 – 16 – – – – – – – – – 34 – 24 – 25
Poultry	t1456	008 – 16 – 02 – 25

*Variable number tandem repeat composition in the 3' end of the *spa* gene.

Susceptibility testing showed that all 15 isolated strains were resistant to erythromycin, kanamycin, tobramycin, lincomycin, tylosin, tetracycline, and trimethoprim. All strains were susceptible to chloramphenicol, ciprofloxacin, linezolid, mupirocin, quinopristin-dalfopristin, rifampin, and sulfonamides.

Molecular typing showed that the strains all belonged to *spa* type t1456 of the livestock-associated ST398, which is typically not typeable by pulsed-field gel electrophoresis. To our knowledge, this *spa* type has not been found in other animal species (*11*,*12*). Its relatedness to other *spa* types isolated from pigs is shown in the Table. A shortening of variable number tandem repeat composition seems to be present in *spa* type t1456. Whether t1456 is a clone typically associated with poultry, or specifically broiler chickens, and whether it is spreading internationally needs further investigation.

Conclusions

We confirmed the presence of MRSA in broiler chickens, but we were unable to find it in laying hens. All isolates belonged to 1 *spa* type, t1456, and thus differed from the other strains belonging to ST398 isolated from other animal species in Belgium and abroad. Whether this *spa* type is typically associated with poultry still needs to be confirmed. More detailed data are also needed to gain further insight in the true within- and between-flock prevalence of MRSA in poultry and its evolution over time.

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Human Bocavirus and KI/WU Polyomaviruses in Pediatric Intensive Care Patients

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We evaluated the prevalence of human bocavirus and KI and WU polyomaviruses in pediatric intensive care patients with and without lower respiratory tract infection (LRTI). The prevalence of these viruses was 5.1%, 0%, and 2.6%, respectively, in children with LRTI and 4.8%, 4.8%, and 2.4%, respectively, in those without LRTI.

Through use of molecular diagnostic tests such as real-time PCR in the clinical setting, our scope of etiologic viral agents of lower respiratory tract infection (LRTI) has increased. Respiratory viruses can now be detected in most pediatric intensive care patients with LRTI (*1*). Recently, 3 new viruses were described: human bocavirus (HBoV) and KI (KIPyV) and WU (WUPyV) polyomaviruses (*2*–*4*). These viruses were first identified in respiratory samples obtained from children with respiratory tract infections. An association between the viruses and respiratory tract symptoms was postulated, but, to date, evidence supporting that association is incomplete (*5*–*7*). This study evaluates the prevalences of HBoV, KIPyV, and WUPyV infections in pediatric intensive care patients with acute respiratory insufficiency caused by LRTI.

The Study

Patients <5 years of age who were admitted for LRTI to the pediatric intensive care unit (PICU) of Wilhelmina Children's Hospital, Utrecht, the Netherlands, were enrolled from October through May during 2005–2008. Patients were excluded if they had any of the following: asthma exacerbation, immunocompromised state, indication for antimicrobial drugs other than for LRTI, and repeated PICU admission for LRTI during the study period. Control group participants were children <18 years of age (median 2.2 years) who were admitted to the PICU from October 2005 through March 2006 for reasons other than LRTI.

Clinical data were obtained by using standardized forms to extract data from electronic charts. Underlying illnesses were defined as chronic pulmonary disease, congenital heart disease, immunodeficiency, malignancy, neurologic disease, or gastrointestinal disease (*8*). To assess the severity of illness, we used the lowest ratio during the first 24 hours of the partial pressure of oxygen in arterial blood ($PaO_{2)}$ to the inspired oxygen fraction (FiO_2). These ratios were acquired from the Pediatric Intensive Care Evaluation database, which contains validated clinical data for all Dutch PICU admissions.

Nasopharyngeal aspirates were collected from all patients in the LRTI group as part of the investigation of their illnesses. In the control group, nasopharyngeal aspirates were taken from intubated patients, and throat swabs were taken from extubated children as part of routine surveillance to identify transmission of respiratory syncytial virus (RSV). Because RSV surveillance was conducted as part of normal patient care, patient consent/ethical approval was not needed, according to the Medical Ethical Research Council of our institution.

Specimens from patients in the LRTI group were initially examined for RSV, influenza viruses, parainfluenza viruses, adenoviruses, rhinoviruses, coronaviruses, human metapneumovirus, and *Mycoplasma pneumoniae* by using real-time PCR as previously described (*1*,*9*,*10*). Specimens from patients in the control group were initially examined for RSV. All samples were retrospectively tested for HBoV, KIPyV, and WUPyV also by using real-time PCR as previously described (*11*,*12*). After nucleic acid extraction using the MagNA Pure LC 1.0 nucleic acid isolation system (Roche Diagnostics, Rotkreuz, Switzerland), amplification was carried out in a 25-μL reaction mixture on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Positive controls for the KIPyV and WUPyV PCR were provided by S. Bialasiewicz and T.P. Sloots, University of Queensland, Queensland, Australia, and the positive control for HBoV was provided by T. Allander, Karolinska Institute, Stockholm, Sweden. Internal control viruses were used to monitor efficient extraction and amplification. Real-time PCR results were expressed in cycle threshold (Ct) values. Ct values are inversely correlated with viral load; i.e., low Ct values indicate high viral loads.

Of 90 LRTI patients enrolled, 78 (86.7%) had sufficient material stored for HBoV, KIPyV, and WUPyV testing. Eighty-eight control patients were enrolled, of which 83 (94.3%) had sufficient material stored to be included. Table 1 provides patients' demographic and clinical characteristics. The main clinical conditions of control patients were cardiac disease requiring surgery (33.7%), trauma

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Table 1. Demographic and clinical characteristics for PICU patients with LTRIs and for controls, the Netherlands, 2005–2008*

Characteristics of patients	LRTI group, n = 78	Control group, n = 83
Sex, no. (%)		
M	43 (55.1)	44 (53.0)
F	35 (44.9)	39 (47.0)
Nasopharyngeal aspirates, no. (%)	78 (100.0)	57 (68.7)
Mechanical ventilation, no. (%)	76 (97.4)	79 (95.2)
Age, mo, median (IQR)	1.5 (4.2)	26.1 (134)
PaO_2/FiO_2, mm Hg, median (IQR)	130 (74.3)	250 (206)
Time on ventilator, d, median (IQR)	9 (5)	4 (9)
Time in hospital, d, median (IQR)	10 (6)	6 (11)

*PICU, Pediatric Intensive Care Unit, Wilhelmina Children's Hospital, Utrecht, the Netherlands; LRTI, lower respiratory tract infection; IQR, interquartile range; PaO_2/FiO_2, ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction.

(8.4%), sepsis (8.4%), and upper respiratory tract infection (8.4%). A total of 57 (68.7%) nasopharyngeal aspirates and 26 (31.3%) throat swabs from the 83 patients who had sufficient samples were tested.

In LRTI patients, HBoV was found in 4 (5.1%) and WUPyV in 2 (2.6%) of the 78 patients. No samples tested positive for KIPyV. Table 2 shows Ct values and clinical characteristics for LRTI patients whose samples were positive as well as for controls whose samples were positive for these viruses. Other respiratory viruses were found in 70 (89.7%) of the 78 children. RSV was found in 52 (66.7%), influenza viruses in 3 (3.8%), parainfluenza viruses in 2 (2.6%), adenoviruses in 4 (5.1%), rhinoviruses in 20 (25.6%), coronaviruses in 6 (7.7%), human metapneumovirus in 5 (6.4%), and *Mycoplasma pneumoniae* in 1 (1.3%) of the patients. Multiple respiratory viruses were found in 3 of the 4 LRTI patients with HBoV infection and in both patients with WuPyV infection (Table 2). One patient had a single infection with HBoV (i.e., no other virus was detected). This patient was born at 31 weeks of gestational age and had a history of a grade IV idiopathic respiratory distress syndrome. She was admitted to the PICU at

Table 2. Clinical characteristics of PICU patients with LRTIs and of PICU controls whose samples tested positive for HBoV, KIPyV, or WUPyV infections, the Netherlands, 2005–2008*

Patient no.	Diagnosis	Virus (Ct value)	Sample type	Sex	Age, mo	Immunocompromised	Other underlying disease	Length of stay, d	Other viruses
LRTI group									
1	LRTI	HBoV (39.4)	NPA	F	13	No	Pulmonary dysplasia (home mechanical ventilation)	10	Adeno, hMPV
2	LRTI	HBoV (15.0)	NPA	F	9	No	IRDS, PVL	10	Adeno
3	LRTI	HBoV (16.6)	NPA	M	13	No	Recurrent wheezing	5	RSV
4	LRTI	HBoV (15.0)	NPA	F	19	No	IRDS	7	None
5	LRTI	WUPyV (19.6)	NPA	M	1	No	None	11	RSV
6	LRTI	WUPyV (34.1)	NPA	F	41	No	Mitochondrial encephalopathy	18	Influenza
Control group									
7	Sepsis	HBoV (36.5)	NPA	F	27	No	None	7	NA
8	Infectious meningitis	HBoV (34.7)	NPA	F	100	No	22Q11 deletion (cardiac and neurologic disease)	3	NA
9	URTI	HBoV (37.9)	NPA	M	41	No	Spinal muscular atrophy	74	NA
10	Observation after brain biopsy	HBoV/ KIPyV (32/39)	NPA	F	127	Yes	AML, BMT, Brochiolitis obliterans	46	NA
11	VSD closure	WUPyV (33.8)	Throat swab	F	15	No	VSD	2	NA
12	Septic shock	WUPyV (34.4)	NPA	M	40	No	None	19	NA
13	Cardiac malformation	KIPyV (34.1)	NPA	F	4	No	Cardiac malformation	14	NA
14	Septic shock	KIPyV (23.1)	NPA	M	152	Yes	AML, aplasia	11	NA
15	ALTE	KIPyV (26.7)	NPA	M	2	No	None	8	NA

*PICU, Pediatric Intensive Care Unit, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; LRTI, lower respiratory tract infection; HBoV, human bocavirus; KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; Ct, cycle threshold; NPA, nasopharyngeal aspirate; Adeno, adenovirus; hMPV, human metapneumovirus; RSV, respiratory syncytial virus; IRDS, idiopathic respiratory distress syndrome; PVL, periventricular leukomalacia; NA, not assessed; URTI, upper respiratory tract infection; AML, acute myeloid leukemia; BMT, bone marrow transplant; VSD, ventricular septal defect; ALTE, apparent life-threatening event.

19 months of age with a severe LRTI. Bacterial throat and blood cultures remained negative.

In the control group, HBoV was found in samples from 4 (4.8%) patients, KIPyV was found in 4 (4.8%), and WUPyV was present in 2 (2.4%) samples from the 83 patients whose samples could be tested. One patient was found to have a co-infection with HBoV and KIPyV.

Median Ct values for HBoV, KIPyV, and WUPyV combined were 18.1 (interquartile range [IQR] 20.4) for the LRTI group and 34.4 (IQR 5.1) for the control group ($p = 0.09$; Figure). The Ct values indicate that, on average, the viral load in the LRTI group might be higher than in the control group.

Conclusions

In the present study, the prevalences of HBoV, KIPyV, and WUPyV in PICU patients with LRTI (n = 78) or without LRTI (n = 83) were similar (5.1%, 0%, 2.6%; and 4.8%, 4.8%, 2.4%, respectively). Most HBoV- and KIPyW-positive LRTI patients were co-infected with other viruses. One LRTI patient with a HBoV single infection was identified. In this patient, HBoV was present in a high quantity.

Two limitations of our study deserve further discussion. First, LRTI patients were younger than controls (LRTI, 100% <5 years; controls, 40% ≥5 years). However, the positivity rates for HBoV, KIPyV, and WUPyV in control children <5 years were similar to rates of control children ≥5 years of age (6/49 vs. 3/34, respectively). Hence, the influence of this limitation is likely minor. Studies have also shown that the highest incidence of KIPyV/WUPyV infection occurs in children ≈1 year of age, slightly older than the children in our LRTI group (*13–15*). The young age of the LRTI group may have resulted in a lower than expected positivity rate for this group. Second, all LRTI patients had nasopharyngeal aspirates taken; however, 68.7% of controls had provided nasopharyngeal aspirates. HBoV and KIPyV/WUPyV infections were more common in controls who had nasopharyngeal aspirate samples taken than in those who had throat swab samples taken (8/57 vs. 1/26). This difference in positivity for nasopharyngeal aspirates strengthens our conclusion that these viruses are not found more frequently in PICU children with LRTI.

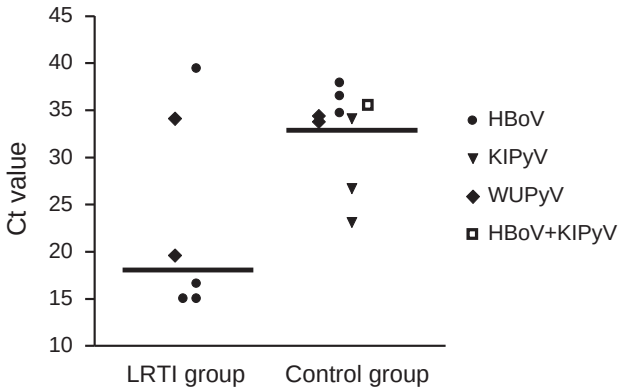

Figure. Cycle threshold (Ct) values of lower respiratory tract infection (LRTI) and control patients with human bocavirus (HBoV), KI polyomavirus (KIPyV), and WU polyomavirus (WUPyV) infections. LRTI patients are those admitted to the Pediatric Intensive Care Unit, Wilhelmina Children's Hospital, University Medical Center Utrecht, the Netherlands; control patients are patients admitted to the Pediatric Intensive Care Unit with other diagnoses. Horizontal bars represent group medians (difference 16.3 Ct, $p = 0.09$).

Sampling errors make precise quantification of viral loads difficult. Nevertheless, in the LRTI group, low Ct values, which indicate high viral loads, were found in nasopharyngeal samples taken from 3 patients infected with HBoV (Ct ≈ 15) and from 1 patient infected with WUPyV (Ct = 19). Ct values found in nasopharyngeal samples from patients in the control group were much higher, 32–39. A possible explanation for this difference is that high viral loads in the young LRTI population represent symptomatic primary infection, whereas the low viral load in the older controls might represent asymptomatic long-term shedding. Further studies are needed to show the clinical implications of infections with these viruses.

Prevalences of HBoV, KIPyV, and WUPyV infections in children in the PICU is low (≈<5% for LRTI patients and controls), and these agents are unlikely to be a major cause of LRTI at the PICU. However, HBoV might be pathogenic in some PICU patients because 1 person with a HBoV single infection in a high quantity was identified. Further studies using quantitative viral detection are needed to investigate the probability that HBoV, KIPyV, and WUPyV represent etiologic agents of LRTI.

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Rocky Mountain Spotted Fever in Dogs, Brazil

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Clinical illness caused by *Rickettsia rickettsii* in dogs has been reported solely in the United States. We report 2 natural clinical cases of Rocky Mountain spotted fever in dogs in Brazil. Each case was confirmed by seroconversion and molecular analysis and resolved after doxycycline therapy.

Rickettsia rickettsii, the etiologic agent of Rocky Mountain spotted fever (RMSF), is the most pathogenic of the rickettsiae for humans and some animals. RMSF has been reported in North, Central, and South America, where different tick species serve as vectors (*1*). Although serologic studies among healthy dogs in Brazil have indicated past infection by *R. rickettsii* (*2*,*3*), clinical illness caused by *R. rickettsii* in dogs has been reported solely in the United States (*4*,*5*).

In Brazil, the most common vector-borne disease of dogs is canine monocytic ehrlichiosis (CME), caused by *Ehrlichia canis* (*6*). Clinical signs (fever, depression, petechial hemorrhages, thrombocytopenia) in dogs with overt RMSF infection or CME are often similar (*5*). Doxycycline is the treatment of choice for *R. rickettsii* infection in dogs (*7*) and the most commonly prescribed treatment for CME in Brazil. Thus, clinical cases of RMSF among dogs in Brazil could be being misdiagnosed as CME. We describe 2 natural cases of RMSF in dogs in Brazil.

The Cases

On August 23, 2007, a 4-year-old, female, Dogue de Bordeaux (dog 1) was brought to a veterinary clinic in São Paulo because of a high load of ticks noticed 5 days after she had been to a farm in the Itu Municipality (23°15′S, 47°17′W), state of São Paulo. The dog was treated with fipronil and sent home. Tick taxonomic identification was not performed. The next day, the dog had diarrhea and hematochezia and was taken back to the clinic, where laboratory test results were within reference ranges, except for a slight leukocytosis (18,000 cells/mm^3) and elevated alkaline phosphatase level (278.6 U/L). Metronidazol was prescribed, and the dog was again sent home. Three days later, the dog was febrile (40.5°C), anorexic, and lethargic. Blood was sent to a private laboratory, where a battery of PCR tests failed to detect DNA of *Babesia* spp., *Borrelia* spp., *Mycoplasma* spp., or *Ehrlichia* spp. The dog was treated with subcutaneous imidocarb and oral doxycycline. The next day, the dog was still febrile (39.4°C) and anorexic, and neurologic signs (ataxia and vestibular syndrome with spontaneous nystagmus) had developed. The animal was hospitalized; doxycycline was switched to the subcutaneous route; and the next day oral prednisone was added. Blood values remained within reference range, except for a slight leukocytosis (17,600 cells/mm^3). On August 30, neurologic improvement was noted, and the dog had no fever (38.5°C) and started to eat. Despite slight nystagmus, the dog was discharged the next day. On September 3, (8 days after doxycycline therapy began), the dog showed no clinical abnormality, and a new blood sample was collected for serologic testing. Another blood sample collected on September 10 showed hematologic parameters within reference range except for leukopenia (6,900 cells/mm^3).

Serologic evaluation was performed by indirect immunofluorescence assay (IFA) by using antigens of 6 *Rickettsia* isolates from Brazil (*8*). Plasma from the sample collected on August 24 showed an IFA endpoint titer of 128 for *R. rickettsii* and no reactivity for the remaining rickettsial antigens at a 1:64 dilution. Plasma from the sample collected on September 3 showed the following endpoint titers for rickettsial antigens: *R. rickettsii* 2,048, *R. parkeri* 512, *R. amblyommii* 512, *R. felis* 512, *R. rhipicephali,* and 512; *R. bellii* 256.

DNA was extracted from the blood samples collected on August 24 and September 3 (before and after antimicrobial drug therapy) by using the DNeasy Tissue Kit (QIAGEN, Chatsworth, CA, USA). Samples were tested by 2 PCR protocols: one targeting a 147-bp fragment of the rickettsial *gltA* gene (*9*), and the other, a heminested PCR, targeting a fragment of the rickettsial *ompA* gene (*10*). Extracted DNA from the first blood sample yielded expected products by both PCR protocols. No product was obtained from the second blood sample. Sequencing of the *ompA* product resulted in a 452-bp fragment 100% identical to the corresponding sequence of the Bitterroot strain of *R. rickettsii* from the United States (GenBank accession no. U43804). *Ehrlichia* spp. were not detected by PCR (*6*) in either sample.

The second case was noted on August 28, 2007, when a 10-month-old, female, miniature Schnauzer (dog 2) was examined at the same veterinary clinic for anorexia, lethargy, fever (40.2°C), vomiting, and tick infestation. This dog had visited the same farm at the same time as dog 1. No neurologic signs were observed. Dog 2 was treated with

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fipronil and sent home with atropine, imizol, ranitidine, dipirone, and doxycycline. Blood collected on August 28 had values within reference ranges, except for thrombocytopenia (thrombocytes 150,000/mm^3). IFA for rickettsial antigens showed no reactivity at the 1:64 dilution for the 6 rickettsial antigens, but serum from a second sample collected on September 3 (when the dog was showing no clinical signs) showed the following endpoint titers: *R. rickettsii* 4,096 *R. parkeri* 512, *R. amblyommii* 512, *R. felis* 256, *R. rhipicephali* 256, and *R. bellii* 256.

DNA was extracted from the samples collected on August 24 and September 3 (before and after antimicrobial drug therapy) and processed by the PCR protocols cited above. Extracted DNA from the first sample yielded expected product for the *gltA*-PCR, which was not sequenced. No other PCR product was obtained.

Conclusions

Definitive diagnoses of naturally acquired *R. rickettsii* infection in 2 dogs in Brazil are supported by 1) paired serum samples with >8-fold rise in antibody titer to *R. rickettsii* antigen; 2) titers to *R. rickettsii* ≥4-fold higher than titers to other rickettsial antigens known to occur in Brazil; 3) detection of rickettsial DNA in canine blood, confirmed to be *R. rickettsii* in at least 1 of the dogs; 4) compatible clinical signs and laboratory abnormalities (i.e., thrombocytopenia in at least 1 dog); 5) response to doxycycline; and 6) compatible epidemiologic history (i.e., prior contact with ticks in an RMSF-endemic area). This sixth statement is supported by the fact that Itu municipality is an area where RMSF laboratory-confirmed cases in humans have been reported since 2003 (www.cve.saude.sp.gov.br). Owners of the 2 dogs reported here noted various capybaras (*Hydrochoerus hydrochaeris*) in the area where their dogs had become infested with ticks (data not shown). Capybaras are one of the main hosts of *Amblyomma cajennense* ticks, the most common important vector of *R. rickettsii* in Brazil (*9*,*11*).

In a recent study of experimental infection, dogs exposed to a Brazil isolate of *R. rickettsii* had fever, lethargy, anorexia, anemia, and thrombocytopenia; 1 also had ocular lesions (*12*). These clinical signs have been reported in the United States for dogs with active *R. rickettsii* infection (*4*,*5*) and were also noted in the present study under natural conditions, except for anemia and ocular lesions. Studies in the United States have shown that neurologic dysfunction occurs in as many as 43% of dogs with RMSF; vestibular dysfunction is possibly the most frequent neurologic abnormality (*13*). These results suggest that clinical illness caused by *R. rickettsii* in dogs has similar patterns in Brazil and the United States.

Veterinarians in Brazil should include *R. rickettsii* infection in their differential diagnoses of CME and other acute nonspecific febrile illnesses of dogs, especially because *R. rickettsii* is highly pathogenic for humans. In the United States, several cases of human infection have been preceded by RMSF in dogs (*14*,*15*). Accurate diagnosis of RMSF in dogs should lead to dog owners understanding risk for infection from ticks in their location (*14*) and provide valuable information for the surveillance of RMSF in humans.

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Crimean-Congo Hemorrhagic Fever Virus in High-Risk Population, Turkey

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In the Tokat and Sivas provinces of Turkey, the overall Crimean-Congo hemorrhagic fever virus (CCHFV) seroprevalence was 12.8% among 782 members of a high-risk population. CCHFV seroprevalence was associated with history of tick bite or tick removal from animals, employment in animal husbandry or farming, and being >40 years of age.

Crimean-Congo hemorrhagic fever virus (CCHFV) infection was first defined in Turkey in 2003 from persons who became sick during a 2002 CCHFV outbreak (*1*,*2*). During 2002–2007, CCHFV was confirmed serologically, virologically, or by both types of testing, in ≈1,800 persons, mainly in the Tokat and Sivas provinces of Turkey (Figure 1) (*3*). This region was then considered an epicenter for CCHFV epidemics (*4*). This study determined the seroprevalence of CCHFV in a high-risk population living in that region after 4 epidemic seasons and assessed transmission routes of CCHFV infection.

The Study

In June and September 2006, persons living in 56 villages of the 14 districts of Tokat and Sivas provinces (Figure 1) who had a risk for CCHFV infection other than occupational risk (i.e., healthcare, slaughterhouse work, and veterinary care) were randomly selected for the study. Villages and districts were selected based on residences of patients who were diagnosed with CCHFV infection and treated at Cumhuriyet University Hospital, Sivas, Turkey, during the 2005 CCHFV outbreak. Men and women were included in the study, but children <7 years of age were excluded because of difficulties in drawing blood samples and obtaining parental consent.

Using EPI Info version 6 software (Centers for Disease Control and Prevention, Atlanta, GA, USA) and assuming a CCHFV seroprevalence of 10% in the study population with 99% confidence levels, we calculated error limits of ± 3% and a design effect of 1. The estimated sample size required was 664, but the target sample size of high-risk persons was increased to 782. Another 100 persons who were not at high risk for CCHFV infection, but who lived in urban areas in the high-risk region and agreed to provide blood samples, were also included in the study. The study protocol was approved by the Cumhuriyet University Hospital Human Ethics Committee.

The CCHFV Seroprevalence Study Team in Turkey included a physician and a nurse who went to the selected villages and approached the heads of the village and selected families. They explained the objectives of the study and asked for written informed consent from participants or parents of participating minors and then administered an interview-based questionnaire and collected a blood sample. The questionnaire considered the following variables: age; sex; history of tick bite, tick removal from animals, animal abortion, and animal slaughtering activity; close contact with a CCHFV patient or an animal; and occupation. Blood samples (10 mL each) were collected and later tested for antibodies to CCHFV by using immunoglobulin G (IgG) ELISA kits (Vector-Best; Kolsovo, Novosibirsk, Russia). SPSS version 10.0 (SPSS, Chicago, IL, USA) for Windows software was used for statistical analysis. Chi-square and Fisher exact tests were used to compare categorical variables. Statistical significance was defined as a 2-tailed p value ≤0.05. Univariate analysis was used to identify the risk factors for seropositivity of CCHFV in the 782 participants.

Of the 782 high-risk persons, 100 were positive for IgG against CCHFV (seroprevalence 12.8%). The sex ratio was ≈1:1 (390 females, 392 males). Forty-seven (12.1%) of 390 female participants and 53 (13.5%) of 392 male

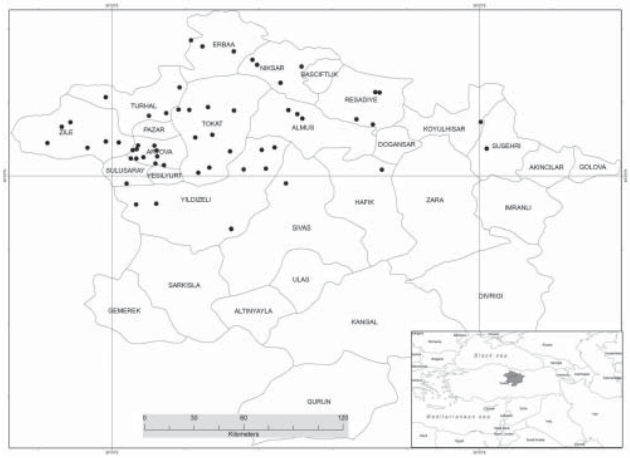

Figure 1. Districts of Tokat and Sivas provinces, Turkey, from which 782 persons at high risk for Crimean-Congo hemorrhagic fever virus infection were sampled, 2006. Sample sites are indicated by black dots. (Map provided by Zati Vatansever and reproduced with permission.)

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participants were seropositive for CCHFV (p>0.05). Mean age was 41.5 years. Of the 100 serum samples collected in the urban population, only 2 (males 44 and 56 years of age) were seropositive. The CCHFV seroprevalence in the 782 persons at high risk increased significantly with age (p<0.001). The highest proportion (23.5%) of seropositivity was found in persons 61–70 years of age (p<0.001) (Table 1). Figure 2 shows distribution of the CCHFV seroprevalence in high-risk persons by age groups. The only variables significantly associated with presence of antibody against CCHFV were history of tick bite (p = 0.002) or of tick removal from the animals (p = 0.03), employment in animal husbandry (p = 0.01) or farming (p = 0.02), and age >40 years (p<0.001) (Table 2).

Conclusions

Serologic evidence of CCHFV in Turkey was reported in the 1970s (*4*). In 2003, the CCHFV seroprevalence among 40 veterinarians in the Tokat region was 2.5% (*5*). Another seroprevalence study conducted in 2003 among healthcare workers providing care to CCHFV patients in Turkey detected no seropositive persons (*6*). The present survey indicates that the seroprevalence of CCHFV is higher in persons living in rural areas than in urban areas of the CCHFV epicenter in Turkey (12.8% vs 2.0%). However, because special markets for animal trading are located on the outskirts of large cities in Iran, CCHFV seroprevalence was found to be higher among persons living in urban areas than in persons living in rural areas of this country (*7*). Living in a rural area is a risk factor for exposure to the tick vector and for acquiring CCHFV infection (*8*,*9*). Expected seroprevalence of CCHFV among high-risk persons during epidemics has been found to be 10% (*3*); however, seroprevalence has been reported to be as low as 0.5% in nonepidemic situations (*10*). Other studies conducted in rural parts of Iran and Senegal during epidemics showed that the CCHFV seroprevalence was 13%, comparable to our findings (*9*,*11*).

In the present study, history of tick bite and history of tick removal from animals were found to be significantly associated with CCHFV seropositivity. The overall tick-bite frequency was 62% (483/782) among persons at high risk and has been reported among 40%–60% of CCHFV patients in Turkey (*4*). We also determined that the occupations of animal husbandry and farming were significantly associated with CCHFV seropositivity. Vector ticks are generally present on the ground and on animals, which explains the risk for CCHFV infection in persons who work in farming and animal husbandry. Personal protective measures such as regular examination of clothing and skin for ticks, tick removal, and use of repellents are important to prevent CCHFV infection (*12*).

We did not identify any association between seroprevalence and gender but found that CCHFV seropositivity increased with age. In these regions of Turkey, women contribute to farming and animal husbandry tasks and are exposed to ticks and livestock as often as men are. However, age >40 years was significantly associated with CCHFV seropositivity and reflects the age of workers in Turkish agricultural areas (*4*,*8*,*13*). Increased CCHFV seroprevalence with age may result from increased opportunities of contact with vector ticks (*14*).

Table 1. Demographics and seroprevalence of CCHFV in persons living in rural and urban areas of Tokat and Sivas provinces, Turkey, 2006*

Characteristic	Persons living in rural area (n = 782)	Persons living in urban area (n = 100)
Age, y		
Mean ± SD	41.5 ± 18.6	41.9 ± 18.4
Range	7–83	7–80
Gender, no. (%)		
Female	390 (49.8)	53 (53)
Male	392 (50.2)	47 (47)
Total seroprevalence, no. positive (%)	100 (12.8)	2 (2)
Seroprevalence by gender, no. positive/no. tested (%)†		
Female	47/390 (12.1)	0/53 (0)
Male	53/392 (13.5)	2/47 (4.3)
Seroprevalence by age, y, no. positive/no. tested (%)‡		
7–20	4/138 (2.9)	0/14 (0)
21–30	9/100 (9)	0/18 (0)
31–40	14/134 (10.5)	0/15 (0)
41–50	20/126 (15.90	1/18 (5.6)
51–60	23/157 (14.6)	1/17(5.9)
61–70	20/85 (23.5)	0/13 (0)
71–83	10/45 (22.2)	0/5 (0)

*CCHFV, Crimean-Congo hemorrhagic fever virus.
†p value = 0.59 for persons living in rural area; for persons living in urban area, data are insufficient for statistical analysis.
‡p value <0.001 for persons living in rural area; for persons living in urban area, data are insufficient for statistical analysis.

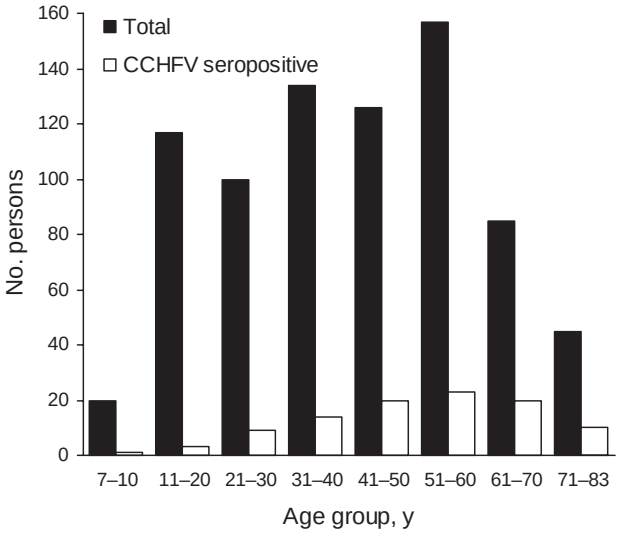

Figure 2. Distribution of seroprevalence of immunoglobulin G against Crimean-Congo hemorrhagic fever virus (CCHFV) by age groups for 782 high-risk persons living in rural areas of Tokat and Sivas provinces, Turkey, 2006.

Exposure to blood and tissues of viremic animals during slaughter is a source of infection (*12*,*14*). However, we did not identify any association between CCHFV seropositivity and contact with animals. This finding may result from a low number of viremic animals in our study region. It is known that domestic animals generally have low levels of viremia, which lasts a short time (*15*). However, in our study region, 79% of animals have been found to be seropositive against CCHFV (*4*).

In the study population, 89 (11.4%) persons had a history of close contact with a CCHFV-infected patient. Among these 89 persons, 14 (15.7%) were seropositive, but this transmission route for CCHFV was not statistically significant for our study population. However, protection against this potential transmission route is especially important for healthcare workers in hospitals that provide care to CCHFV case-patients (*12*).

This study indicated that tick exposure is the most statistically significant transmission route for CCHFV in a high-risk population in Turkey. Effective tick prevention aids such as tick repellents may help reduce the risk. On the other hand, the absence of CCHFV seropositivity in 87.2% of the population after 4 CCHFV outbreaks in Turkey may suggest that this population remains at risk for infection in the future. This knowledge may help public health authorities determine appropriate CCHFV intervention and prevention methods.

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Table 2. Demographic features and risk factors associated with CCHFV seroprevalence (univariate analysis) for persons living in rural areas of Tokat and Sivas provinces, Turkey, 2006*

Risk factor category	No. seropositive persons/total population (%)	p value
Age >40 y	73/410 (17.8)	<0.001
History of tick bite	78/483 (11.5)	0.002
Tick removal from the animals	69/450 (15.3)	0.03
Animal abortion	19/135 (14.1)	0.67
Slaughtering activity	25/151 (16.6)	0.18
Contact with CCHFV patient	14/89 (15.7)	0.44
Contact with an animal	97/734 (16.6)	0.26
Job		
Farmer	93/656 (14.2)	0.02
Animal husbandry	94/664 (14.2)	0.01
Milking	35/263 (13.3)	0.79
Student	1/38 (2.6)	0.11
Total no. seropositive persons	100/782 (12.8)	–

*CCHFV, Crimean-Congo hemorrhagic fever virus.

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Sudden Increases in Listeriosis Rates in England and Wales, 2001 and 2003

Benjamin J. Cairns and Robert J.H. Payne

The monthly incidence of listeriosis infections in England and Wales had 2 sudden increases during April 2001 (41%) and March 2003 (48%). Although no causative association is demonstrated, these increases correspond to key dates relating to the onset and aftermath of the 2001 foot and mouth disease outbreak in the United Kingdom.

Prevention of listeriosis (infection caused by *Listeria monocytogenes*) is a serious food safety issue, particularly for pregnant women, the elderly, and those who are immunocompromised. Death occurs in 20%–30% of cases, making listeriosis a leading cause of food poisoning deaths in Europe and the United States (*1*). An increasing rate of listeriosis has been reported in several European countries (*2*). Our study focused on the large increase in the number of reported listeriosis cases in England and Wales during 2 months in separate years (April 2001 and March 2003). These increases were permanent and cumulative; after each increase, monthly incidence of listeriosis did not return to previous levels. These increases primarily reflect a higher rate of bacteremic listeriosis in those ≥60 years of age and are not otherwise correlated with geography, gender, ethnicity, socioeconomic factors, or infectious serotypes (*3*).

The Study

We compared monthly listeriosis data from England and Wales with temperature records from 1989 through 2007 to determine the influence of various potential predictors on the number of listeriosis cases. UK Health Protection Agency (HPA) data listing total monthly cases of human listeriosis in England and Wales during 1990–2007 (*4*,*5*) are aggregate. All age categories and regions were included and were collated by the HPA Centre for Infections from voluntary reporting by microbiology laboratories and from referrals of cultures. These publicly available data were also validated by the HPA, and in our analysis we used revised figures based on that validation. Our analysis covered the period from 1990, when active surveillance of listeriosis began, through 2007. Pregnancy-associated cases (mother and neonate) were counted as 1 case. Undated cases that could not be assigned to a particular month were excluded from analysis. We used the UK Met Office mean monthly area temperature time series for 1989–2007 and 30-year means averaged for 1961–1990 (*6*).

Exploratory linear regression analyses suggested a positive correlation between the number of listeriosis cases and the monthly mean UK ambient temperature, as well as suggesting a change in this relationship after 2000 ($p = 0.001$; Figure 1, panel A). However, residual variability was not constant, and the monthly counts are likely to be overdispersed due to clustering of cases (*3*). The data were fitted again by using a negative binomial generalized linear model with a logarithmic link function, a common model for time series of foodborne illness cases (*7*,*8*). To separate seasonality of listeriosis rates from dependence on temperature, we considered the 30-year mean monthly temperatures from 1961–1990, as well as monthly temperature anomalies (observed mean temperature minus 30-year mean). To determine whether temperatures could have a delayed effect on listeriosis incidence, we also included mean and anomaly temperature variables lagged by 1 or more months. To allow for 2 break points at which the incidence of listeriosis may have suddenly changed, dummy variables were used to represent periods before, between, and after the months in which these increases might have occurred. A best-fit model was selected according to the corrected Akaike Information Criterion (AIC) (*9*) by using stepwise regression at all combinations of 2 break point months from January 1996 through December 2007. To examine changing effects on incidence, interactions between break point indicators and other variables were considered, even if the main effects were not yet in the model. Main effects were subsequently added to the final model if any interaction terms were included.

The best-fit model included 2 break points (Tables 1, 2; Figure 1, panel B; Figure 2). The overall rate of cases increased by 40.83% in April 2001 (adjusted $p = 0.001$, 95% confidence interval [CI] 17.29%–68.93%); a further increase of 47.76% occurred in March 2003 (adjusted $p<0.001$, 95% CI 27.92%–71.13%). According to the corrected AIC, there is statistical evidence of changes in the incidence of listeriosis in a range of months around these best-fit values, and weak support for a change in early 2005, apparently due to low numbers of cases in the first months of that year and the following winter (Figure 1, panel B). Most of the seasonality in the number of cases was accounted for by the 30-year mean monthly temperatures. Each extra degree Celsius of mean monthly temperature corresponded to a 2.42% increase in cases in the current month (adjusted $p = 0.044$, 95% CI, 0.55%–4.33%) and a 4.09% increase in the following month (adjusted $p<0.001$,

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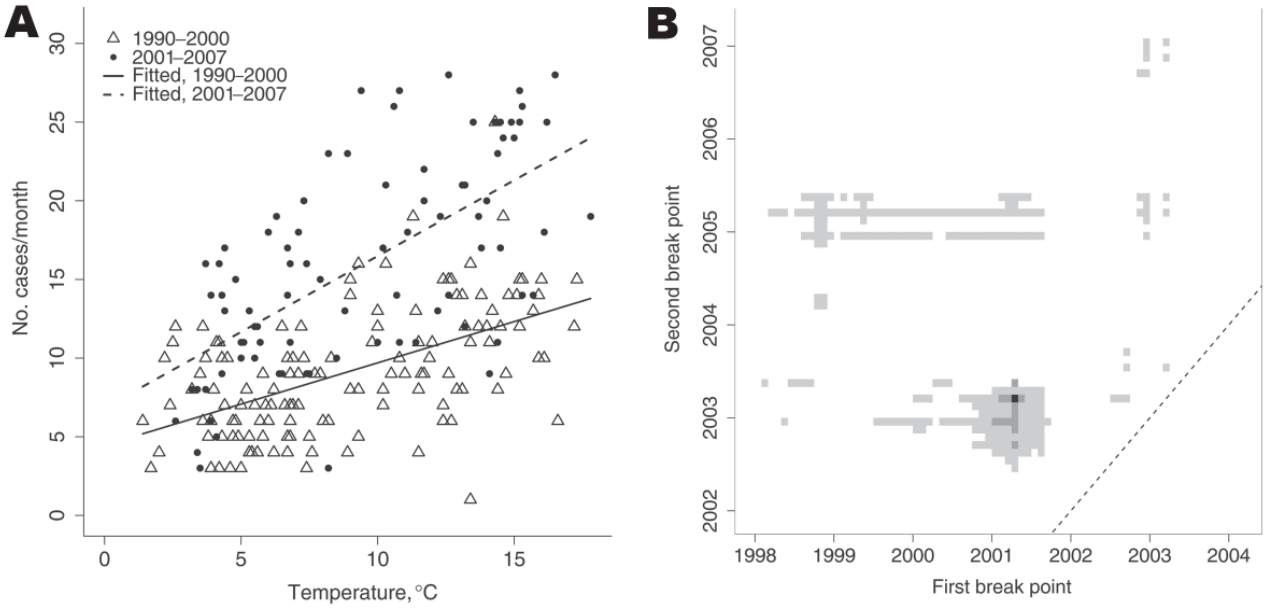

Figure 1. Exploratory analyses of changing rates of listeriosis, England and Wales. A) Listeriosis cases compared with mean observed monthly UK temperatures, 1990–2000 (triangles) and 2001–2007 (circles). Shown are an increased overall incidence in 2001–2007 (dashed line) versus 1990–2000 (solid line) and a significant change in the linear dependence of incidence on temperature (p = 0.001). B) Best-fit pair of break points and other pairs of break points with support, according to the corrected Akaike Information Criterion (AIC). The 2 break-point months are varied to find the lowest value (black square). Pairs of break points with good support relative to the best model (corrected AIC within 4 of the best fit; dark gray squares) or moderate to weak support (corrected AIC within 10 of the best fit; light gray squares) are also shown. Pairs of break points with little or no support (corrected AIC >10 greater than the best fit; white area) include those models for which only 1 break point exists (squares along the dashed line).

95% CI 2.19%–6.03%). The 1-month lag reflects delays between food production and consumption plus the known long incubation of listeria infections. Similar lags have been observed for other enteric pathogens (*6*,*11*). A relationship also appears to exist between the rate of listeriosis and the 1-month lagged temperature anomaly starting in April 2001, which corresponded to an additional 5.71% of listeriosis cases per degree Celsius (95% CI –2.10%–14.15%) when compared with the main effect of temperature anomaly in 1990–2007. This increase was not significant (adjusted p = 0.312), although the power of this test is only ≈15% at an adjusted significance level of 0.05 (post hoc power analysis performed by computer simulation of the fitted model in the R statistical computing environment). The model also includes a slight negative trend in cases over time (–0.13% per month, 95% CI –0.26%–0.01%), but evidence for this trend is very weak (adjusted p = 0.188).

The changes in the incidence of listeriosis appear to have been quite sudden (Figure 2). Causative explanations based on gradual demographic or behavioral changes have previously been ruled out (*3*), and those based on dynamic processes seem unlikely because no evidence exists for epidemiologic feedback between the source of infections and clinical cases. One possibility is that the increases are due to contamination of a small number of food products, as has been suggested as an explanation for an upsurge in listeriosis rates in the late 1980s. However, a restricted range of strains was responsible for most of the additional

Table 1. Coefficient names and descriptions for the best-fit negative binomial generalized linear model of listeriosis incidence

Variable	Coefficient description
(Intercept)	Log of overall monthly no. cases in March 2001
GEAPR01	Change in log cases (April 2001)
GEMAR03	Change in log cases (March 2003)
MEANTEMP	Log cases/°C 30-year mean temperature of the current month
MEANTEMP1	Log cases/°C 30-year mean temperature of the previous month
MONTHNUM	Overall linear trend of log cases/month
ANOMALY1	Overall log cases/°C previous month's temperature anomaly
ANOMALY1×GEAPR01	Change in log cases/°C previous month's temperature anomaly from April 2001
θ (theta)	Negative binomial response distribution size parameter

Table 2. Estimated coefficients for terms in the best fit model for the monthly incidence of listeriosis*†

Variable	Coefficient	95% Confidence interval	Unadjusted p value	Adjusted p value
(Intercept)	1.5433	1.3961–1.6879	<0.001	<0.001
GEAPR01	0.3424	0.1594–0.5243	<0.001	0.001
GEMAR03	0.3904	0.2462– 0.5373	<0.001	<0.001
MEANTEMP	0.0239	0.0055–0.0424	0.011	0.044
MEANTEMP1	0.0401	0.0217–0.0586	<0.001	<0.001
MONTHNUM	–0.0013	–0.0027–0.0001	0.063	0.188
ANOMALY1	0.0246	–0.0246–0.0739	0.327	0.327
ANOMALY1×GEAPR01	0.0556	–0.0212–0.1323	0.156	0.312
θ (theta)	600.41	NA	NA	NA

*NA, not applicable.
†p values are for 2-sided tests of significance of the difference of each coefficient from zero; adjusted p values are according to Holm's method (*10*). Percentage changes in the main text can be derived from these coefficients by taking exponentials, subtracting 1, and multiplying by 100.

cases at that time (*12*), and no evidence exists of such a pattern since 2001 (*3*). An alternative possibility is that the phenomenon is a consequence of changes in government policy or business practices that have had more widespread effects on food processing, distribution, or preparation.

We found notable coincidences between the dates of the increases in listeriosis infection rates and the dates of events associated with the 2001 foot and mouth disease (FMD) outbreak in the United Kingdom. The April 2001 increase in listeriosis rates occurred shortly after the outbreak of FMD in February 2001, allowing for a delay similar to the incubation period of listeria infections. The March 2003 increase in listeriosis rates occurred in the same month as the relaxation of movement restrictions on livestock instituted after the 2001 FMD outbreak (*13*). The diverse and widespread consequences of the 2001 FMD outbreak are well-documented (*14*,*15*), and it seems plausible that such major disturbances to agricultural production could be the ultimate cause of the large increases in listeriosis rates that have been observed. The coincidence of these events raises the possibility that the change in listeriosis rates would be an unrecognized outcome of the 2001 UK FMD crisis, although we caution that our analysis of the of these cases does not demonstrate whether a causative link exists.

Conclusions

Listeriosis incidence in England and Wales has increased notably since the beginning of 2001, with 2 separate, sudden increases recorded in April 2001 and March 2003. Gillespie et al. argue that blame for these increases cannot be ascribed to any of a variety of specific factors (*3*). Instead, more widespread changes affecting food production, processing, or consumption could be the root of the problem. Incidence of this serious disease has risen sub-

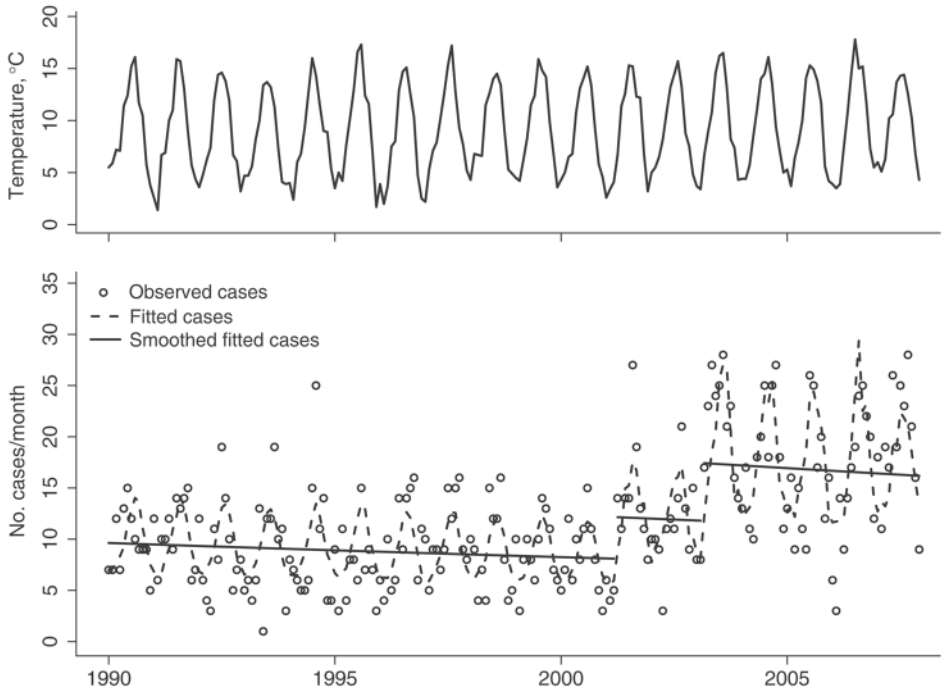

Figure 2. Monthly listeriosis cases and temperature observations, England and Wales, 1990–2007. The monthly number of listeriosis cases (circles, lower plot) is strongly seasonal, with a close relationship to the monthly mean temperature (solid line, upper plot). Overall listeriosis incidence per month underwent 2 sudden increases, at break points around April 2001 and March 2003. Our fitted statistical model (dashed line, lower plot) provides a close fit to the observed data; the seasonally-adjusted fitted model (solid line, lower plot) shows the large magnitudes of the jumps in the rate of cases at the 2 break points.

stantially in England and Wales, and an understanding of why will be important for management of listeriosis as a public health issue.

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Coordinated Implementation of Chikungunya Virus Reverse Transcription–PCR

Marcus Panning, Remi N. Charrel, Oliver D. Mantke, Olfert Landt, Matthias Niedrig, and Christian Drosten

A preformulated chikungunya virus real-time reverse transcription–PCR, quality-confirmed oligonucleotides, and noninfectious virus controls were distributed by the European Network for the Diagnosis of Imported Viral Diseases. An international proficiency study with 31 participants demonstrated that ad hoc implementation of molecular diagnostics was feasible and successful.

Chikungunya fever, caused by chikungunya virus (CHIKV), is an acute febrile illness that causes severe and long-lasting arthralgia (*1*). A recent and ongoing epidemic in the Indian Ocean area extended far beyond this region and caused hundreds of imported cases worldwide (*2–4*). Chikungunya fever is difficult to clinically distinguish from co-endemic diseases such as malaria or dengue fever. Laboratory testing is required for appropriate case management and public health response (*5*). Pilot studies have shown that reverse transcription–PCR (RT-PCR) reliably detects acute infections in humans (*3*,*6*), but many laboratories were not ready to conduct such tests when this epidemic occurred.

During 2006 and 2007, the European Network for the Diagnosis of Imported Viral Diseases (ENIVD) received requests by many laboratories for assistance with CHIKV diagnostics. On the basis of experiences during the outbreak of severe acute respiratory syndrome (SARS) in 2003 (*7*), an ENIVD member laboratory distributed a then-unpublished real-time RT-PCR protocol that had been evaluated with a large number of clinical samples from imported cases to laboratories asking for assistance (*3*). To determine efficacy of RT-PCR testing for CHIKV, we distributed testing materials to 31 participating laboratories in an external quality assurance study. Laboratories sent their results to ENIVD for analysis of efficacy.

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The Study

Information distributed to laboratories asking for assistance with CHIKV RT-PCR included reaction chemistry setup, cycling profile, and primer and probe sequences. A quantified CHIKV in vitro RNA transcript containing 9×10^{10} subgenomic RNA copies/μL was used as a noninfectious positive control. Additional measures were taken to provide proper primers and probes because these components are most vulnerable to variation when assays are adapted from protocols, e.g., because of synthesis errors or poor purification. Primers and probes were synthesized in large reference lots and stored centrally at an oligonucleotide factory. Samples of these lots were validated by the reference laboratory and confirmed to provide full sensitivity as achieved with the original primers used in developing the prototype assay (*3*). Recipients of protocols were invited to order and use aliquots of primers directly from the validated reference lot.

To receive feedback on performance of this method and other methods of CHIKV detection, a proficiency study was organized among ENIVD members. All participants were informed about the option of obtaining the preformulated assay. Laboratories in Europe (22), Asia (6), South America (2), and Africa (1) participated.

Inactivated and stable testing material was generated from cell culture supernatants of 4 CHIKV strains from the epidemic in the Indian Ocean area (1 each from Seychelles, Mauritius, Réunion Island, and India) and 1 East/Central Africa strain (S27). Virus solutions were inactivated by heating at 56°C for 1 h and gamma irradiation with 30 kGy. Residual infectivity was excluded by 3 blind passages of a sample of each solution on Vero cells. Solutions were diluted in human fresh-frozen plasma, aliquoted (100 μL), and lyophilized. Test aliquots were reconstituted in 100 μL of water, and CHIKV RNA was quantified by RT-PCR (*3*). Lyophilized samples were shipped at ambient temperature to participating laboratories. Each shipment contained a coded panel of 9 CHIKV RNA positive– and 3 CHIKV RNA–negative lyophilized samples with virus concentrations shown in Table 1. Participants were asked to test the material with any molecular assay routinely used for detecting CHIKV in human plasma or with the preformulated test. We requested test results and assay details (PCR formulations and extraction methods). A total of 36 sets of results were received by the study coordinator, including 3 double sets from 3 laboratories that used 2 methods each. One laboratory provided triple sets of results from 3 tests.

We used 2 criteria to define successful participation in the external quality assessment study. First, those samples

Table 1. Positive samples in external quality assessment panel for detection of CHIKV by reverse transcription–PCR*

Sample code	Origin of strain	Virus RNA concentration, copies/mL	Laboratories with positive detection, %
CHIK #2	Réunion Island	10,487,171	100
CHIK #9	Réunion Island	745,257	77.4
CHIK #4	Réunion Island	86,197	83.9
CHIK #12	Réunion Island	7,040	48.4
CHIK #5	Réunion Island	1,076	22.6
CHIK #6	India	918,259	96.8
CHIK #10	Seychelles	526,268	87.1
CHIK #1	Mauritius	564,192	83.9
CHIK #11	East Africa	1,131,422	87.1

*CHIKV, chikungunya virus.

containing ≥7,040 RNA copies/mL should be correctly identified. Analogous to previous external quality assessments (*8*–*11*), we chose this threshold because it is ≈5–10× above the limit of detection of current CHIKV RT-PCR protocols (*3*,*12*). Second, no false-positive results were allowed in virus-free samples.

Samples containing 10,487,171 RNA copies/mL were correctly detected by all participating laboratories (Table 1). Fifteen (48%) of the laboratories were able to detect samples containing ≥7,040 RNA copies/mL. Only 22.6% correctly detected the sample with 1,076 copies/mL. Of 31 laboratories, 14 (45.2%) met all proficiency criteria. Seventeen laboratories missed the proficiency criteria because of a lack of sensitivity. Two of these laboratories reported ≥1 false-positive result. Both laboratories had used a nested RT-PCR, which likely indicated cross-contamination during RT-PCR procedures. No other laboratories reported false-positive results.

To project performance of a hypothetical average laboratory, cumulative fractions of positive results reported for each test sample were correlated against RNA concentrations in samples and subjected to probit analysis. This procedure used a dose-response model, which predicted for the average laboratory that a 50% certainty of detection was achieved for CHIKV plasma concentrations ≥10,000 RNA copies/mL (95% confidence interval [CI] 3,162–19,952 copies/mL) (Figure). A 95% certainty of detection was achieved for CHIKV plasma concentrations ≥7,943,282 copies/mL (95% CI 2,511,886–39,810,717 copies/mL).

To evaluate critical criteria in laboratory practice, we determined whether particular components of laboratory procedures had any systematic influence on laboratory performance. Selection of criteria was based on experiences from earlier external quality assessment studies (*8*,*9*,*11*). We evaluated automated versus manual RNA extraction methods, 1 widely distributed procedure for RNA extraction (viral RNA mini kit; QIAGEN, Hilden, Germany), any real-time RT-PCR, any nested RT-PCR, or the preformulated RT-PCR distributed with this study. Cumulative fractional positive results of all low- and medium-concentration samples (≤86,197 copies/mL) were subjected to multifactor analysis of variance, which eliminated influence of other defined factors in each analysis. The only technical factor that increased sensitivity was the preformulated RT-PCR (Table 2). Thirteen (42%) of 31 participants used this assay. Another factor with nonsignificant benefit (p = 0.08) was use of automated RNA extraction.

Conclusions

Because of little disease activity before the epidemic, laboratories inside and outside epidemic regions were not prepared to detect CHIKV when the epidemic occurred. In a similar situation during the SARS epidemic in 2003, we demonstrated that rapid provision of a commercial test kit

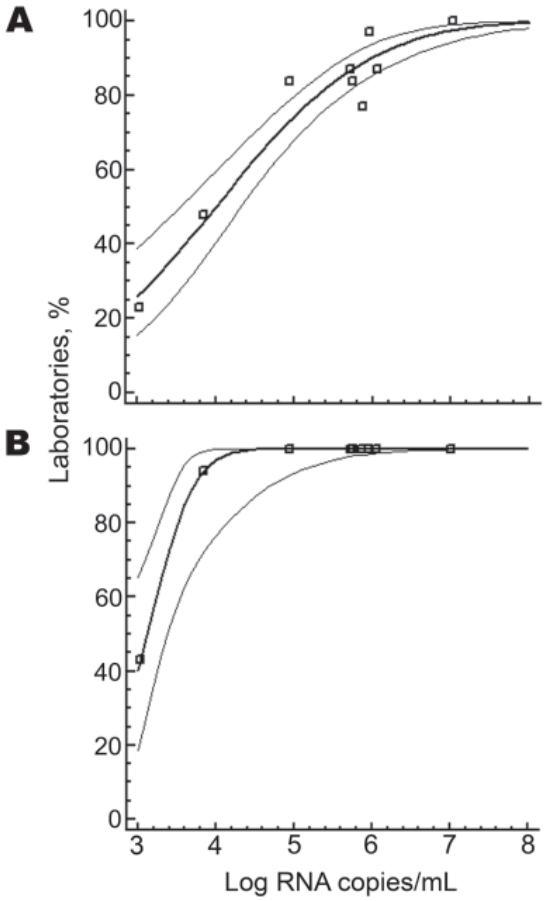

Figure. Probit analysis of laboratories with a positive result (y axes) for chikungunya virus in relation to viral RNA concentration in positive samples (x axes). A) Laboratories using in-house reverse transcription–PCRs (RT-PCRs) (n = 18) had a 50% certainty of having a positive result at 10,000 RNA copies/mL (95% confidence interval [CI] 3,162–19,952). B) Laboratories using a preformulated RT-PCR (n = 13) had a 50% certainty of having a positive result at 1,288 RNA copies/mL (95% CI 416–2,344). Data points represent individual samples in the test panel. Thick line is the regression line calculated on the basis of a probit model (dose-response curve), and thin lines are 95% CIs. Data fit into the model with p<0.00001.

Table 2. Possible technical factors influencing performance of laboratories in detection of CHIKV*

Factor	No. laboratories	p value for positive influence on sensitivity
QIAGEN† viral RNA extraction kit	23	0.2
Any automated RNA extraction procedure	8	0.08
Preformulated CHIKV real-time RT-PCR protocol	13	0.03
Any real-time CHIKV RT-PCR	27	0.3
Any nested CHIKV RT-PCR	6	0.37

*CHIKV, chikungunya virus; RT-PCR, reverse transcription–PCR.
†Hilden, Germany.

could greatly assist laboratories worldwide, enabling them to perform state-of-the art molecular diagnostics during the epidemic (*7*,*9*). However, for chikungunya fever, commercial firms did not rapidly prioritize development of CHIKV test kits. ENIVD attempted to assist implementation of molecular diagnostics on an ad hoc basis by distributing a validated CHIKV RT-PCR and all required reagents.

Our proficiency study showed surprisingly good overall performance of participating laboratories than most of our previous external quality assessments (*8*,*10*). Analysis of factors identified that this success was primarily due to the preformulated assay. In our earlier external quality assessments on detection of emerging viruses, many participants used diagnostic methods reported in the literature, which did not provide technical features such as real-time PCR (*8*,*9*,*11*). The assay distributed in this study was technically advanced, and its efficient adaptation was supported by providing quality-controlled oligonucleotides and controls. This in-house assay was readily implemented by a large number of laboratories. It improved diagnostic proficiency similar to the commercial assay distributed during the SARS epidemic (*9*). We showed that novel PCR diagnostics for emerging diseases can be implemented on an international scale. However, enhanced support by reference laboratories through efficient collaborative networks of laboratories is indispensable. Public health organizations should be encouraged by these data to strengthen and extend networking between diagnostic laboratory facilities.

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Extended-Spectrum β-Lactamase–Producing *Enterobacteriaceae* in Malian Orphanage

Didier Tandé, Nelle Jallot, Flabou Bougoudogo, Tracey Montagnon, Stéphanie Gouriou, and Jacques Sizun

We show high rates of extended-spectrum β-lactamase–producing *Enterobacteriaceae* carriage among the staff and children at an orphanage in Bamako, Mali. *Enterobacteriaceae* colonized in 100% and 63%, respectively, of the 38 children and 30 adults studied. Use of antimicrobial drugs appeared excessive and inappropriate; decontamination and hygiene protocols were also questioned.

Extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* pose a major health problem because the incidence rate of infection is particularly high (*1*–*3*), and delays in the prescription of appropriate antimicrobial drug therapy for these infections are a risk factor for poor prognosis and death (*4*). In the 36 months preceding our study, children adopted by French families, and who were followed up at the Brest University Hospital, were shown to be carriers of ESBL-producing *Enterobacteriaceae*. All of these children had resided at the state orphanage in Bamako, Mali.

The dominant, and consequently widespread, manifestation of ESBL-producing *Enterobacteriaceae* in these children was the catalyst for our prospective investigation. We studied the carriage rate of these bacteria for the children and personnel of the orphanage and also the living conditions within the orphanage.

The Study

This prospective cross-sectional study was conducted during September 10–19, 2003. The study population included 44 staff members and 39 children who were residing in the orphanage on the day the bacteriologic samples were taken. Adults were included on a voluntary basis and gave informed consent. Informed consent for the children was obtained from their legal representative, the director of the orphanage. Anonymity of participants was guaranteed.

We examined clinical data from charts as well as lifestyle habits, including personal and environmental hygiene. Environmental samples and children's stool samples were obtained. Adults obtained their own stool samples. Environmental samples comprised potable tap water (1 sample) and surface samples (2 silicone bottle nipples, 1 child toilet, 2 faucets, and 1 sink that served as both a dishwashing and child-bathing station).

Samples were stored at 4°C and were sent by air within 18 hours to the microbiology laboratory of Brest University Hospital for analysis. Immediately upon arrival, the samples were injected into a Drigalski medium (bioMérieux, Marcy l'Etoile, France) supplemented with ceftazidime at 2 mg/L. This medium allows for selective isolation of resistant gram-negative bacteria. Colonies that developed on this medium showed different shapes and were systematically identified by using the API20E system (bioMérieux). Antimicrobial-drug susceptibility patterns were determined by the disk-diffusion method, as defined by the Committee for Antimicrobial Susceptibility Testing of the French Society for Microbiology (*5*). *Enterobacteriaceae* were considered ESBL producers if synergy between third-generation cephalosporins and amoxicillin associated with clavulanic acid was detected (*5*,*6*).

Of the 30 adults sampled, 19 (63%) were found to be colonized with 1–3 ESBL-producing *Enterobacteriaceae* of the same or different species (9 samples showed 1 strain; 7 showed 2 strains, and 3 showed 3 strains). More carriers were found in the caregiver adult group, who had direct contact with the children, than in those with less interactive responsibilities (e.g., administration and housekeeping staff), 90% (16/18) versus 25% (3/12), respectively ($p<0.0016$).

We included 38 of the 39 children in the analysis; no stool sample was available for the remaining child. All children sampled carried 1–3 ESBL-producing *Enterobacteriaceae* (21 with 2 strains and 9 with 3 strains). Bacteria samples from children were colonized more often than that of adults with >1 ESBL-producing *Enterobacteriaceae*, 78% (30/38) and 52% (10/19), respectively ($p = 0.04$). Water samples collected contained no ESBL-producing *Enterobacteriaceae*. The other 6 environmental samples testing positive showed 1 (4 samples), 2 (1 sample), or 3 (1 sample) strains of ESBL-producing *Enterobacteriaceae*.

We isolated 118 strains of ESBL-producing *Enterobacteriaceae*: *Escherichia coli* (56%), *Klebsiella pneumoniae* (36%), *K. oxytoca* (4%), and *Citrobacter freundii* (4%). Depending on the species, 36%–79% of strains were resistant to aminoglycosides, 20%–50% were resistant to fluoroquinolones, and >94% were resistant to cotrimoxazole.

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Clonal relatedness of the 52 *E. coli* ESBL-producing strains was assessed by pulsed-field gel electrophoresis (Figure). Among these, 12 unique subtypes and 15 clusters of 2–7 clonal strains were identified. Dissemination might follow an allodemic pattern, corresponding to the spread of multiple specific clones or genetic elements.

The high colonization rate for caregiver adults within our study (90%) could logically and predominantly be attributed to direct contamination from the children. Furthermore, the excessive use of antimicrobial drugs also contributes to the emergence and spread of multidrug-resistant bacteria, as previously stated by Harbarth and van de Sande-Bruinsma (*7,8*). The health records of the 39 children showed that 88 courses of antimicrobial drugs had been prescribed to 27 children during their stay at the orphanage during the 1,921 weeks that the children lived in the orphanage. Many of the antimicrobial drugs prescribed at the orphanage were used for infections of unlikely bacterial etiology. Many authors have emphasized that the inappropriate use of antimicrobial drugs, particularly among the children of various nonindustrialized nations, leads to antimicrobial resistance (*9,10*).

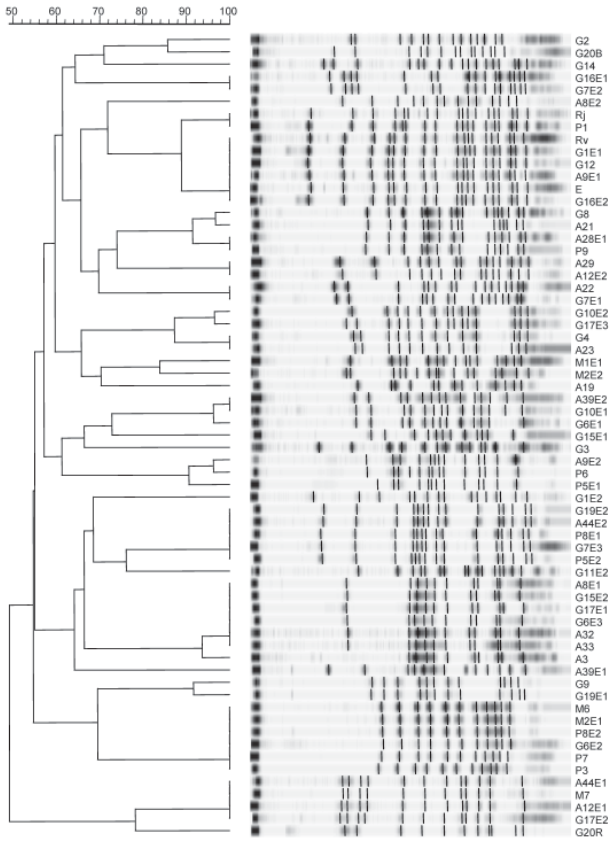

Figure. Representative *Xba*I pulsed-field gel electrophoresis profiles of extended-spectrum β-lactamase–producing *Escherichia coli*. Isolates denoted A originate from adults, P from children <4 months of age, M from children 4–12 months of age, G from children >12 months of age, and E and R from environmental samples.

However, this phenomenon also exists in developing nations (*11*). The antimicrobial drugs most frequently prescribed were third-generation cephalosporins and penicillins, which according to Colodner, favor the carriage of ESBL-producing *Enterobacteriaceae* (*12*). The dosages prescribed were not always adapted to the precise weight of the children, thereby favoring the development of resistance. In addition, the duration of antimicrobial drug therapy, which also favors the development of resistance, was often limited for economic reasons, (*13,14*).

We also suggest that dissemination of ESBL-producing *Enterobacteriaceae* within the orphanage might be explained by close contact between persons and massive contamination of the environment. With the exception of the water supply, all environmental samples tested positive despite compliance with posted hygiene rules and the best efforts and intentions of personnel. Feeding bottles were prepared on a bench top next to the sink, which was also used for bathing infants, and this sink was not regularly decontaminated during the day. Water was served in 1 shared drinking cup. Moreover, traditions such as eating from a shared plate and washing only 1 hand, along with a lack of material and financial means, appeared to clearly favor ESBL-producing *Enterobacteriaceae* colonization in the children. Childcare personnel washed their hands with soap and a diluted bleach solution before and after each caregiving interaction. Taps were operated manually without any particular contamination precautions. These environmental hygiene conditions contributed to the dissemination of the multidrug-resistant strains of *Enterobacteriaceae*.

Although this study gives a precise idea of the situation at the orphanage, our study has limitations. 1) The kinetics of colonization within the orphanage remain unknown; 2) the lack of a control group without ESBLs does not allow a comparison of the antimicrobial drug use; and 3) the rate of ESBL-producing *Enterobacteriaceae* carriage overall in Malian children of similar age and sex has not been scientifically determined.

Conclusions

This study provides evidence that the carriage of ESBL-producing *Enterobacteriaceae* is prevalent at the orphanage in Bamako; dissemination is widespread among children and staff and within the environment. We identified 2 key factors responsible for this type of epidemic: 1) inadequate hygiene conditions favoring the spread of ESBL-producing *Enterobacteriaceae* in the environment and 2) the qualitative and quantitative inappropriate use of antimicrobial drugs. We therefore strongly recommend implementation of appropriate hygiene practices to limit colonization. Of equal importance is the coupling of these measures with a targeted and reasonable approach to the use of antimicrobial drugs.

Our results raise questions about the importance of quickly and accurately identifying persons who carry ESBL-producing *Enterobacteriaceae* because initiation of appropriate antimicrobial drug therapy may be delayed for infected patients and infection could spread rapidly. Testing for ESBL-producing *Enterobacteriaceae* in stools appears to be justified as part of the initial examination of all adopted children who have lived in an institution where these microorganisms are endemic.

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Dr Tandé is a clinical microbiologist who works closely with the infectious disease specialists at Brest University Hospital, Brest, France. He is particularly involved in the study of changes in microbial ecology caused by antimicrobial drug therapy.

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Highly Pathogenic Avian Influenza Virus (H5N1) Outbreak in Captive Wild Birds and Cats, Cambodia

Stéphanie Desvaux, Nick Marx, Sivuth Ong, Nicolas Gaidet, Matt Hunt, Jean-Claude Manuguerra, San Sorn, Malik Peiris, Sylvie Van der Werf, and Jean-Marc Reynes

From December 2003 through January 2004, the Phnom Tamao Wildlife Rescue Centre, Cambodia, was affected by the highly pathogenic influenza virus (H5N1). Birds from 26 species died. Influenza virus subtype H5N1 was detected in 6 of 7 species tested. Cats from 5 of 7 species were probably infected; none died.

On January 24, 2004, the first confirmed outbreak of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 in Cambodia was reported to the Office International des Epizooties (*1*). During the previous month, an unusually high mortality rate had been noted among captive wild birds at the Phnom Tamao Wildlife Rescue Centre (PTWRC) in Takeo Province, 45 km South from Phnom Penh. We report the results of a retrospective investigation of this outbreak.

The Study

During the outbreak period, PTWRC housed 600–1,000 wild animals (70 species of mammals, birds, and reptiles). The center is divided into 3 main sections that cover 37 ha. Birds were kept in sections S1–1, S1–2, and S2, and the cats were in all sections (Figure). The information on bird deaths at PTWRC was systematically recorded by WildAid staff members who were at the Centre at the time of the outbreak. In June 2004, a complete investigation was conducted at PTWRC, and semistructured interviews of key informants were used to identify deaths of domestic poultry in the surrounding villages. Every bird death between December 15, 2003, through January 15, 2004, was defined as a suspected case of HPAIV (H5N1). For S1, the cumulative mortality rate could not be estimated because the exact bird population was not known and the birds were difficult to observe in that section (the semicaptive waterfowl population is able to mix with the wild population and disperse to breed). For S2, information was complete (Table 1).

The first case, in a crested serpent eagle (*Spilornis cheela*), was reported on December 15, 2003, in S2 (Figure). On December 19, the outbreak had reached every section and continued until January 12; a total of 86 birds, representing 8 taxonomic orders and 12 families, died (Table 1). Of 7 cat species, cats from 5 species were reported sick (16/39 total cats) (Table 2). In S2, 80% of the reported bird deaths were observed from December 15 to 21. Of the 29 wild bird species kept in S2 at the beginning of the outbreak, no birds from 12 species showed signs of disease (Table 1). Mortality rates varied among the orders, 0–100%

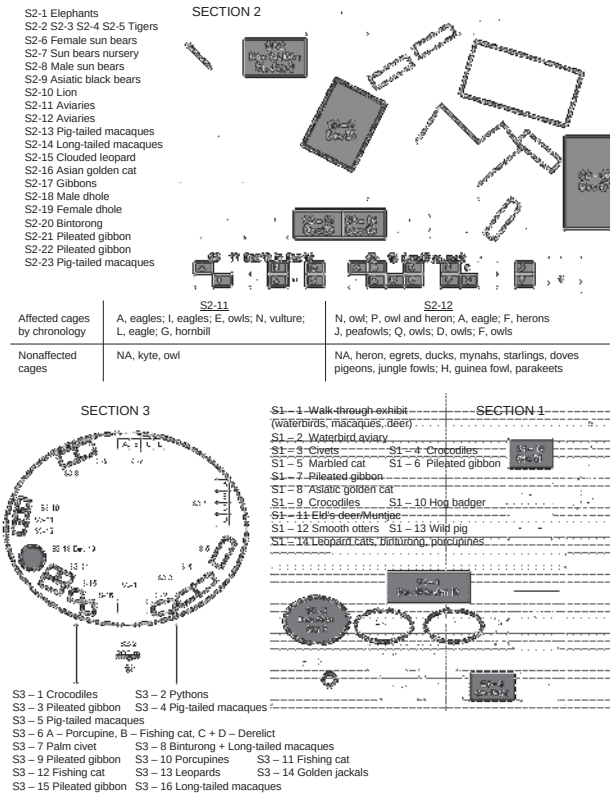

Figure. Map of the 3 main sections of the Phnom Tamao Wildlife Rescue Centre, Cambodia, during outbreak of highly pathogenic avian influenza virus (H5N1), December 15, 2003–January 13, 2004. Shaded areas indicate contaminated cages (labeled with date of outbreak). NA, exact cage not available.

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(Table 1). The only mammals present in the aviaries in S2, slow lorises (*Nycticebus* sp.), did not become ill. None of the 27 animal keepers, who were 20–50 years of age, were reported to have gotten sick.

Most of the birds died within a few hours without showing any clinical signs of infection. A few birds died 1–2 days after onset of clinical signs (anorexia, extreme lethargy, occasional dark green diarrhea, respiratory dis-

Table 1. Cumulative deaths during an outbreak of highly pathogenic avian influenza virus (H5N1), Phnom Tamao Wildlife Rescue Centre, Cambodia, December 15, 2003–January 13, 2004*

Order	Family	Species (common name), no. sampled	No. dead birds in S1	No. birds not dead in S1	Cumulative deaths in S2, % (dead/total at risk)	
					Per species	Per order
Anseriformes	Anatidae	*Anas poecilorhyncha* (Indian spot-billed duck)	NP	NP	0 (0/4)	0 (0/4)
Ciconiiformes	Ardeidae	*Ardea cinerea* (grey heron), n = 2	4	2	NP	47 (9/19)
		Ardeola speciosa (Javan-pond heron), n = 1	7	0	100 (7/7)	
		Butorides striatus (little heron)	NP	NP	0 (0/1)	
		Egretta garzetta (little egret)	NP	NP	18 (2/11)	
	Ciconidae	*Ephippiorhynchus asiaticus* (black-neck stork)	1	3	NP	
		Leptoptilos dubius (greater adjutant stork)	2	1	NP	
		Leptoptilos javanicus (lesser adjutant stork)	3	21	NP	
		Mycteria leucocephala (painted stork)	6	20	NP	
		Ciconia episcopus (wooly necked stork)	0	3	NP	
		Anastomus oscitans (Asian openbill stork)	0	5	NP	
Colombiformes	Colombidae	*Treron curvirostra* (thick-billed green pigeon)	NP	NP	0 (0/7	0 (0/17)
		Streptopelia chinensis (spotted dove)	NP	NP	0 (0/10)	
Coraciiformes	Buceritidae	*Buceros bicornis* (great hornbill)	NP	NP	100 (1/1)	100 (1/1)
Falconiformes	Accipitridae	*Gyps bengalensis* (white-rumped vulture)	NP	NP	100 (1/1)	93 (13/14)
		Haliastur indus (Brahminy kite)	NP	NP	0 (0/1)	
		Ichthyophaga ichtyaetus (grey-headed fish eagle)	3	0	100 (4/4)	
		Ictinaetus malayensis (black eagle)	NP	NP	100 (1/1)	
		Milvus migrans (black kite)	1	0	NP	
		Spilornis cheela (crested serpent eagle), n = 1	1	0	100 (5/5)	
		Spizaetus cirrhatus (changeable hawk eagle), n = 1	0	0	100 (2/2)	
Galliformes	Numididae	*Agelastes sp* (guineafowl)	NP	NP	33.3 (1/3)	36 (5/14)
	Phasianidae	*Pavo muticus* (green peafowl)	0	3	100 (3/3)	
		Gallus gallus (red jungle fowl)	NP	NP	12.5 (1/8)	
Gruiformes	Gruidae	*Grus antigone* (Sarus crane)	3	0	NP	
Passeriformes	Corvidae	*Corvus macrorynchos* (large-billed crow), n = 1	2	0	100 (3/3)	25 (3/12)
	Sturnidae	*Gracula religiosa* (hill mynah)	NP	NP	0 (0/3)	
		Acridotheres tristis (common mynah)	NP	NP	0 (0/4)	
		Acridotheres javanicus (white-vented mynah)	NP	NP	0 (0/1)	
		Sturnus contra (Asian pied starling)	NP	NP	0 (0/1)	
Pelecaniformes	Anhigindae	*Anhinga melanogaster* (oriental darter)	0	1	NP	
	Pelecanidae	*Pelecanus philippensis* (spot-billed pelican)	3	2	NP	
Psittaciformes	Psittacidae	*Psittacula eupatria* (Alexandrine parakeet), n = 1†	1	0	50 (1/2)	0 (1/146)
		Psittacula roseate (blossom-headed parakeet)	NP	NP	0 (0/20)	
		Psittacula alexandri (red-breasted parakeet)	NP	NP	0 (0/20)	
		Psittacula finschii (grey-headed parakeet)	NP	NP	0 (0/104)	
Strigiformes	Strigidae	*Bubo nipalensis* (spot-bellied eagle owl), n = 1	0	0	100 (1/1)	92 (12/13)
		Ketupa ketupu (buffy fish owl)	NP	NP	100 (3/3)	
		Ketupa zeylonensis (brown fish owl)	NP	NP	86 (6/7)	
		Strix seloputo (spotted wood owl)	NP	NP	100 (2/2)	
	Tytonidae	*Tyto alba* (barn owl)	5	0	NP	
Total		8 sampled	42	61	18.3% (44/240)	

*S1, aviary section in which cumulative mortality rate could not be estimated because exact bird population was not known and birds were difficult to observe; S2, aviary section in which captive bird population was exactly known and number of dead birds was precisely recorded; NP, species not present in S1 or S2.
†Only sample that was negative for highly pathogenic avian influenza virus (H5N1); all other birds sampled were positive.

Table 2. Morbidity rates for wild cats during outbreak of highly pathogenic avian influenza virus (H5N1), Phnom Tamao Wildlife Rescue Centre, Cambodia, December 15, 2003–January 13, 2004

Order	Family	Species (common name)	Cumulative morbidity rate, % (sick/at risk), no. sampled
Carnivora	*Felidae*	*Panthera leo* (lion)	100 (2/2)
		Panthera tigris (tiger)	80 (8/10), n= 1*
		Catopuma temminckii (Asiatic golden cat)	100 (2/2), n = 1*
		Panthera pardus (leopard)	100 (3/3), n = 1*
		Neofelis nebulosa (clouded leopard)	100 (1/1), n =1*
		Prionailurus bengalensis (leopard cat)	0 (0/16)
		Prionailurus viverrinus (fishing cat)	0 (0/5)
Total			41 (16/39)

*All serum samples were positive (date of collection: March 4, 2004).

tress, and neurologic abnormalities). The cats were sick for 5–7 days and exhibited anorexia and lethargy but no respiratory illness.

Laboratory investigations of the organs from 8 birds sampled in December 2003 were performed (Table 1). For those birds, West Nile virus infection was ruled out by reverse transcription–PCR (RT-PCR), according to the procedure described by Lanciotti et al. (*2*). All birds sampled, except a parakeet, were positive for influenza subtype H5N1 by RT-PCR (*3*) (Table 1). Molecular characterizations of hemagglutinin (H)5 and neuraminidase (N)1 were performed from the influenza virus (H5N1) strains from PTWRC as previously described (*4*). H5 amino acid sequences were identical in the coding region to the sequence of isolates obtained from poultry cases in Cambodia (ill poultry from a flock with high mortality rates) and similar (≥96.5%) to HPAIV (H5N1) strain H5 sequences from Vietnam and Thailand in 2004 (data not shown). All belonged to the H5 clade 1 (*4*). Amino acid sequences from N1 from Cambodia were very close to each other (≥97.12% identity) and to 2004 Vietnamese and Thai N1 sequences (>96%) (data not shown). The HA and NA sequences of the isolates were deposited in GenBank (accession nos. ISDN186319–ISN186324, ISDN186329, ISDN186330–ISDN186665, and ISDN242365).

Retrospective investigation of the villages surrounding the PTWRC and Phnom Penh showed that chickens from 2 flocks in which deaths had been reported in mid-December had been provided to the PTWRC, either for the restaurants or for the captive animal feeding. Furthermore, at the time of the outbreak, many wild crows were found dead in the forest surrounding the PTWRC.

The 4 cat serum samples, each from a different species, were positive for HPAIV (H5N1) with serum neutralization test (*5*); titers ranged from 10 to 40 (Table 2). None of the affected cats died.

Conclusions

The sources of introduction of HPAIV (H5N1) within the PTWRC were probably multiple: virus-infected chicken bought to feed the carnivorous species, infected live chickens brought to restaurants near S2 (i.e., the first place where deaths were detected), and contact between infected wild and captive birds. The introduction through infected chickens is supported by the absence of an outbreak at the PTWRC after the feeding of chickens to carnivorous species was discontinued; however, deaths in domestic poultry continued in the area. In addition, almost all carnivorous bird species in S2 died (93% of Falconiformes and 92% of Strigiformes) as did most species usually fed chicken meat in captivity (herons, storks, crows, great hornbill, pelican). Diet was also the origin of the outbreak among tigers and leopards in Thailand (*6*,*7*). The dispersion of the disease between PTWRD sections was probably due to poor biosecurity measures.

The clinical outcome of wild birds with suspected HPAIV (H5N1) infection at PTWRC ranged from severe illness and death to complete absence of clinical signs, as described (*8*). Several species from the orders Ciconiiformes, Galliformes, Passeriformes, Gruiformes, Coraciiformes, and Pelecaniformes were affected during the outbreak. This observation is consistent with data published earlier, except for Coraciiformes represented by 1 bird in our study (*9*). Only the carnivorous species (*Corvus macrorynchos*) among the 5 species of Passeriformes in the aviaries showed clinical signs and later was confirmed by RT-PCR to be positive for HPAIV (H5N1). This outbreak confirms that Falconiformes and Strigiformes are sensitive to HPAIV (H5N1) infection and disease (*10–12*) and shows that numerous species of these orders can be affected by HPAIV (H5N1) (Table 1). Psittaciformes and Columbiformes were not visibly affected by the outbreak although they were kept in large numbers in S2, where large numbers of deaths occurred. As non–water-bird species, they do not belong to groups in which avian influenza is commonly reported (*13*). Anseriformes, represented in PTWRC by only 4 birds (*Anas poecilorhyncha*), did not show any clinical signs. Heterogeneity in the susceptibility of wild ducks to HPAIV (H5N1), including asymptomatic infection, has been demonstrated (*14*); this species also belongs to the group of wild ducks found asymptomatically infected with HPAIV (H5N1) in the People's Republic of

China during the winter of 2005 (*15*).

The serologic evidence of influenza virus (H5N1) infection in 4 species of wild cats is in agreement with previous infection in Thailand (*6*,*7*). The report of illness in the Asiatic golden cat (*Catopuma temminckii*) and the clouded leopard (*Neofelis nebulosa*) broadens the host range of the virus among mammals.

This report confirms the great variability of wild bird and mammal responses to HPAIV (H5N1) infection. It also confirms the broadening range of susceptible species that may be specific to this clade 1 virus.

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Hepatitis E Virus Antibodies in Patients with Chronic Liver Disease

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In the United States, the seroprevalence rate for hepatitis E virus (HEV) is ≈20%. This study examined HEV seroprevalence in persons with and without chronic liver disease. Our data indicate that HEV seropositivity is high in patients with chronic liver disease and that HEV seroprevalence increases significantly with age.

The hepatitis E virus (HEV) is an enterically transmitted, hepatotropic, single-stranded RNA virus. It causes a self-limiting acute infection, but the infection does not develop into chronic disease in immunocompetent persons. Although symptoms are generally mild, they may be severe in certain cases, especially in pregnant women (*1*). HEV is endemic in regions where the water supply may be contaminated with animal waste, such as in Central Asia, the Middle East, and parts of South America and Africa. Large waterborne epidemics have occurred in refugee camps in Somalia (*2*) and Sudan (*3*), causing sickness and death. In regions where HEV is endemic, seroprevalence and illness rates for hepatitis E infection vary from 40%–90% (*4*,*5*). In the United States, seroprevalence studies detected antibodies against HEV (anti-HEV) in ≥21% of blood donors (*6*). Despite this indication of its presence in the United States, HEV infection is rarely diagnosed in this country except in travelers to HEV-endemic areas (*7*).

Recent reports have demonstrated that zoonotic (swine) reservoirs may exist in non–disease-endemic regions (*8*) and that unrecognized HEV may be the source of cryptogenic hepatitis in certain cases (*9*). Data concerning immunocompromised patients are scarce; however, higher seroprevalence of HEV has been reported in HIV-positive persons than in the general population. Additionally, in patients with compromised immune systems, symptoms of HEV infection and viremia may be prolonged (*10*,*11*). We conducted a study to determine the seroprevalence of anti-HEV in persons with and without chronic liver disease to uncover potential associations between clinical markers of liver disease and HEV seroprevalence and to identify risk factors associated with HEV positivity.

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The Study

We performed an observational cross-sectional analysis of de-identified serum samples and data from patients with chronic liver disease who were monitored at the University of Cincinnati, Cincinnati, Ohio. Data and samples were collected during 1995–2006. Healthy donor samples were obtained from controls (generally healthcare and laboratory personnel) who had no evidence of liver disease. Limited demographic, clinical, and laboratory data were collected from medical records and existing databases for patients with chronic liver disease and for controls. Tests for anti-HEV immunoglobulin (Ig) G and IgM were performed by ELISA (MP Biomedicals Asia Pacific Pte Ltd, Singapore; formerly Genelabs Diagnostic Pte Ltd) according to the manufacturer's instructions. Assay validity was evaluated according to the manufacturer's specifications. Demographic, clinical, and laboratory values were reported as mean ± SD for normally distributed variables and as median (range) for nonnormal continuous variables. Between-group comparisons were assessed by analysis of variance with the Sidak adjustment for multiple comparisons, the Kruskal-Wallis test, the χ^2 test, or Fisher exact test, as appropriate to the data distribution. Logistic regression was used to determine predictors of IgG positivity. A 2-tailed p value ≤0.01 was considered significant in all cases.

A total of 167 persons with a mean age of 39.6 years (±10.9 years) were evaluated. Of these, 129 had chronic hepatitis (46 [35.7%] of whom were co-infected with HIV), and 38 were healthy controls. Demographic and laboratory characteristics are displayed in Table 1. The most

Table 1. Characteristics of 167 persons evaluated in study of association between HEV and chronic liver disease*

Characteristic	No. (%) persons
Gender	
M	100 (59.9)
F	67 (40.1)
History of chronic liver disease	
Yes	129 (77.2)
No	38 (22.8)
HIV co-infection	
Yes	46 (27.6)
No	121 (72.4)
HEV IgG	
Positive	49 (29.3)
Negative	118 (70.7)

*HEV, hepatitis E virus; IgG, immunoglobulin G.

common causes of chronic liver disease were hepatitis C virus (HCV) infection, present in 59 (45.7%) of the 129 persons, and HCV/HIV co-infection, present in another 39 (30.2%). Other causes of liver disease included hepatitis B virus (HBV) infection (7.8%), HBV/HIV co-infection (6.2%), autoimmune hepatitis (3.1%), and idiopathic hepatitis (6.9%). Of those with liver disease, 89.9% had viral infections and 30.2% had alanine aminotransferase (ALT) levels >2× the upper limit of the reference range. Eighty-four patients had risk factors assessed, and 68 of those had a known risk factor for viral hepatitis. These risk factors included being an injection drug user or man who has sex with men, using cocaine nasally, having had a blood transfusion, and having hemophilia. Forty-nine (29.3%) of the 167 persons evaluated tested positive for HEV IgG antibody, and most of these persons (45/49, 91.8%) had chronic liver disease. None were positive for anti-HEV IgM, a marker for acute HEV infection. Optical densities (ODs) for anti-HEV IgG differed significantly ($p<0.001$); the group with liver disease had greater values than the controls (mean 0.63 ± 0.96 and 0.13 ± 0.38, respectively; median 0.12 and 0.03, respectively), as shown in Figure 1. No relationship was shown between anti-HEV IgG ODs and the cause of liver disease. Because the odds of HEV seropositivity increase with age, correlation between age and ODs was examined; ODs increased significantly with age ($p<0.001$, Spearman rank correlation $r = 0.305$) (Figure 2).

To determine characteristics associated with testing positive for HEV, univariable logistic regression was conducted. Predictors of significance included age ($p = 0.002$), male gender ($p = 0.009$), liver disease ($p = 0.001$), and HIV co-infection ($p = 0.04$). A multivariable regression model was then developed with age and liver disease and age by liver disease interaction as predictors. The interaction was not significant ($p = 0.97$) and, therefore, was removed from the model. The final model showed that the chance of being HEV positive increases as age increases (adjusted odds ratio [AOR] 1.05, $p = 0.002$). Also, probability of HEV positivity is significantly greater if other liver disease is present (AOR 7.78, $p<0.001$) (Table 2). Male gender was significantly associated with HEV positivity (AOR 2.07, $p = 0.009$), but no associations were found between HEV positivity and race/ethnicity, viral versus nonviral liver disease, ALT level, or HIV seropositivity.

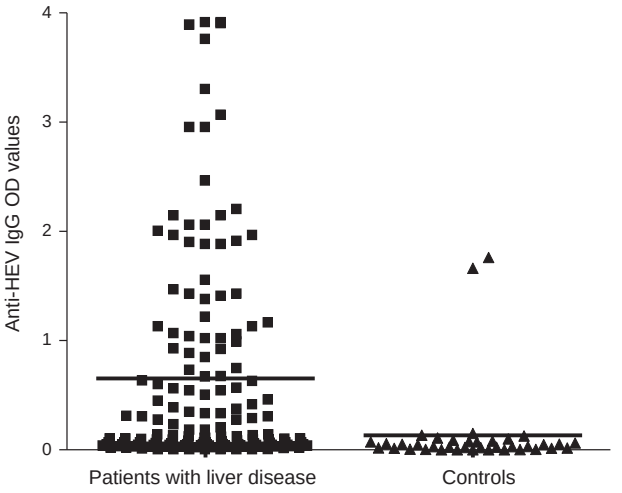

Figure 1. Relationship between anti-hepatitis E virus (HEV) immunoglobulin (Ig) G and chronic liver disease. Scattered plot of the optical density (OD) of anti-HEV IgG for both liver disease patients and control groups is shown; each point represents a subject. Means (0.63 and 0.13 for patients with chronic liver disease and for control group, respectively) are plotted as horizontal lines.

Conclusions

HEV in the United States and other industrialized nations is not thought to present a substantial risk of illness and death; however, recent data suggest that in these regions, HEV may be a cause of cryptogenic hepatitis. In Japan, HEV RNA has been detected in blood donors with elevated liver enzymes (*12*). A recent report from France found that acute HEV in persons who had not traveled to HEV-endemic regions may cause fulminant hepatitis, especially in those with active alcohol use and other chronic liver disease (*13*). Despite these associations and evidence of decompensation in patients with cirrhosis from preexisting liver disease in developing countries (*14*,*15*), whether HEV causes fulminant hepatitis among those with chronic liver disease who live in the United States is unknown.

Our study has limitations that should be noted. First, the intent of this analysis was to determine whether an association between HEV and chronic liver disease was detectable. We therefore used an observational study design.

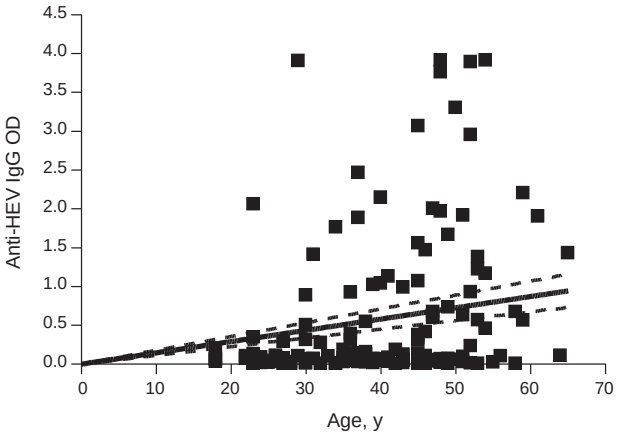

Figure 2. Correlation between the optical density (OD) of anti-hepatitis E virus (HEV) immunoglobulin (Ig) G and the age of persons. OD values of the anti-HEV IgG were plotted against the age of the enrolled persons. Correlation between age and OD value is significant ($p <0.0001$, $r = 0.305$).

Table 2. Hepatitis E virus immunoglobulin G multivariable logistic regression results

Risk factor	Adjusted odds ratio	SEM	p value	95% Confidence interval
Age	1.05	0.02	0.002	1.02–1.09
Male gender	2.07	0.07	0.009	1.17–3.66
History of chronic liver disease	7.78	3.25	0.001	3.43–17.64

The results of the study should be carefully interpreted to avoid assumptions of a cause-effect relationship, as this study was a cross-sectional survey. Second, 2 discrete populations were included. Differences in sample collection dates, geographic location, and other variables could affect results, although no evidence of any confounding effect was detected. Third, as is the case in many retrospective analyses, we were unable to collect all risk factors and characteristics of persons in the study. Underreporting of injection drug users, for example, would undermine power for detection of an association between drug use and HEV seropositivity. However, collecting data on behavior risk factors for viral hepatitis poses a challenge even in prospective studies, and our results are consistent with expectations.

Whether HEV superinfection mediates other liver disease in persons living in the United States can be determined only by prospective cohort studies. Such follow-up is warranted to determine the role of HEV superinfection as a participating factor in liver diseases.

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Detection of Novel SARS-like and Other Coronaviruses in Bats from Kenya

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Diverse coronaviruses have been identified in bats from several continents but not from Africa. We identified group 1 and 2 coronaviruses in bats in Kenya, including SARS-related coronaviruses. The sequence diversity suggests that bats are well-established reservoirs for and likely sources of coronaviruses for many species, including humans.

The 2003 outbreak of severe acute respiratory syndrome (SARS) generated renewed interest in coronaviruses (CoV) and the source for the SARS CoV that caused the outbreak in humans (*1*). Serologic studies demonstrated that the virus had not previously circulated in human populations to any large extent and suggested a source of zoonotic origin (*2–4*). A likely natural viral reservoir for the virus was not identified until horseshoe bats (*Rhinolophus* spp.) in several regions in the People's Republic of China were demonstrated to harbor SARS-like CoVs (*5*). Subsequently, a number of other SARS-like CoVs, as well as CoVs from antigenic groups I and II, were identified from bats in Asia, Europe, and North America, and coronavirus antibodies were detected in African bat species (*6–11*). It is not surprising that a growing number of CoVs have been detected in bats. To date, >60 viral species have been detected in bats because their biodiversity (second only to rodents), high population densities, wide distribution, and ability to fly over long distances allow them to harbor and easily spread multiple infectious agents. Bats have long been known as natural hosts for lyssaviruses and more recently have been recognized as potential reservoirs for emerging human pathogens, including Ebola, Marburg, Nipah, and Hendra viruses in addition to SARS-CoV (*12*,*13*).

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The Study

Given the association of bats with emerging infectious diseases, field surveys were performed during July–August 2006 in the southern portion of Kenya (Figure 1). The selection of sites was based on preliminary data regarding bat roost locations and observations of bats in the field during the survey. Attempts were made to collect specimens from 10–20 animals of each species present in each location. Bats were captured manually and by using mist nets and hand nets; adults and subadults of both sexes were captured. Each bat was measured, sexed, and identified to the genus or species level when possible. Blood samples and oral and fecal swabs were collected; the animals were then euthanized in compliance with field protocol. Blood, fecal swabs, and selected tissue samples were transported on dry ice from the field and stored at –80°C.

Fecal swabs (n = 221; Table) were screened for the presence of CoV RNA using 2 semi-nested reverse transcription-PCR (RT-PCR) assays. For the pan-coronavirus RT-PCR, conserved primers were designed from highly conserved regions of the RNA-dependent RNA polymerase (RdRp) gene 1b based on available CoV sequences (1st and 2nd round forward 5′-ATGGGITGGGAY TATCCWAARTGTG-3′; 1st round reverse 5′-AATTAT ARCAIACAACISYRTCRTCA-3′; 2nd round reverse 5′-CTAGTICCACCIGGYTTWANRTA-3′). For the pan–bat

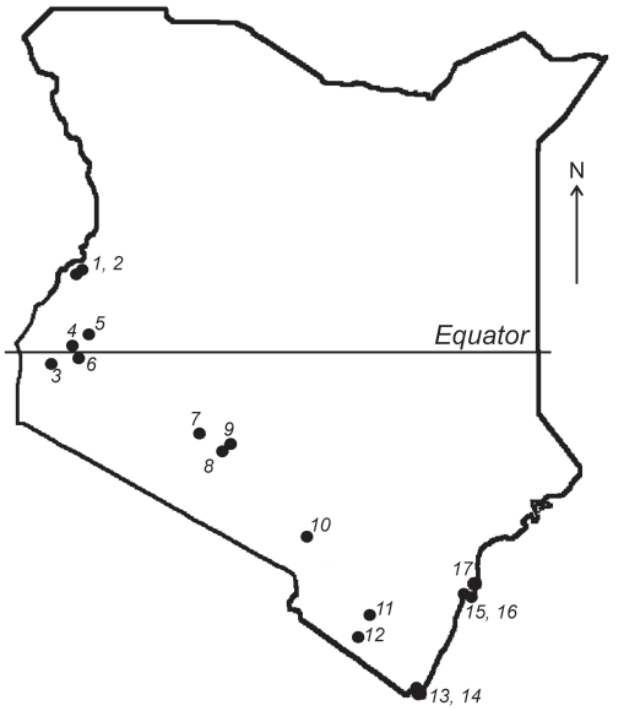

Figure 1. Map of Kenya showing the locations of 17 bat collection sites.

Table. Results of detection of CoV RNA in fecal swabs of bats from Kenya*

Bat species	Geographic location	PCR results, no. positive/no. tested	Clusters
Cardioderma cor	15	0/10	
	12	1/3	BtCoVA970-like
Chaerophon sp.	6	1/14	BtHKU7-like
	17	6/19	BtHKU7-like, BtKY18-like, SARSCoV-like
	3	0/5	
Chaerophon pumilus	3	2/3	HCoV229E-like
	11	0/4	
Coleura afra	11	0/1	
	14	0/1	
Eidolon helvum	4	6/10	BtKY18-like
Epomophorus wahlbergi	9	0/3	
Hipposideros commersoni	14	1/10	BtHKU9-like
Hipposideros ruber	2	0/4	
	5	0/2	
Lissonycteris angolensis	5	0/10	
Miniopterus africanus	10	1/8	BtCoV1A-like
Miniopterus inflatus	5	7/12	BtCoV1A-like, BtHKU8-like
Miniopterus minor	13	1/16	BtCoV1A-like
Miniopterus natalensis	1	1/7	BtCoV1A-like
Neoromicia tenuipinnis	6	0/4	
Otomops martinsseni	7	2/19	BtHKU7-like
Pipistrellus sp.	8	0/1	
Rhinolophus hildebrandtii	10	0/4	
Rhinolophus sp.	14	0/1	
	13	0/1	
	8	0/5	
Rousettus aegyptiacus	1	2/10	BtKY18-like
	2	2/9	BtCoVA970-like, BtHKU9-like
	16	6/9	BtCoVA970-like, BtHKU9-like
	13	2/11	BtHKU9-like
Taphozous hildegardeae	14	0/3	
Taphozous sp.	11	0/2	
Total		41/221 (19%)	

*CoV, coronavirus; SARS, severe acute respiratory syndrome.

coronavirus RT-PCR, conserved primers were designed from the same highly conserved regions based on available bat CoV sequences and presumed to be more specific to bat coronaviruses (1st and 2nd round forward 5′-ATGGGITGGGAYTATCCWAARTGTG-3′; 1st round reverse 5′-TATTATARCAIACIACRCCATCRTC-3′; 2nd round reverse 5′-CTGGTICCACCI GGYTTNACRTA-3′). Total nucleic acids were extracted from 200 μL of a phosphate buffered saline suspension of each swab by using the QIAamp Mini Viral Elute kit (QIAGEN, Santa Clarita, CA, USA), according to the manufacturer's instructions. The seminested RT-PCR was performed by using the SuperScript III One-Step RT-PCR kit and Platinum Tag Kit (Invitrogen, San Diego, CA, USA). The positive PCR products were purified by gel extraction by using the QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's instructions; they were then sequenced on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Of 221 bat fecal swabs examined, 41 (19%) were positive by at least 1 of the 2 seminested RT-PCR assays (Table). One specimen had 2 distinct CoV sequences, each amplified by 1 of the 2 PCR assays, giving a total of 42 distinct CoV sequences. To characterize the overall diversity of CoV sequences, in this study a phylogenetic tree (Figure 2) of the 121-bp fragment of RdRp was generated from 39 coronaviruses from bats in Kenya and 47 selected human and animal coronaviruses from the National Center for Biotechnology Information database based on the Bayesian Monte Carlo Markov Chain method (*14*). Three of the 42 sequences were not of sufficiently high quality to include in this tree. Some nodes had low Bayesian posterior probabilities (Figure 2). Longer sequences from these viruses are needed to refine their phylogenetic relationships.

Among the 39 sequences in the tree, 23 belonged to previously defined group 1 and were mapped into 5 different sequence clusters. The 121-bp sequences in these 5 clusters had an average nucleic acid (NA) sequence

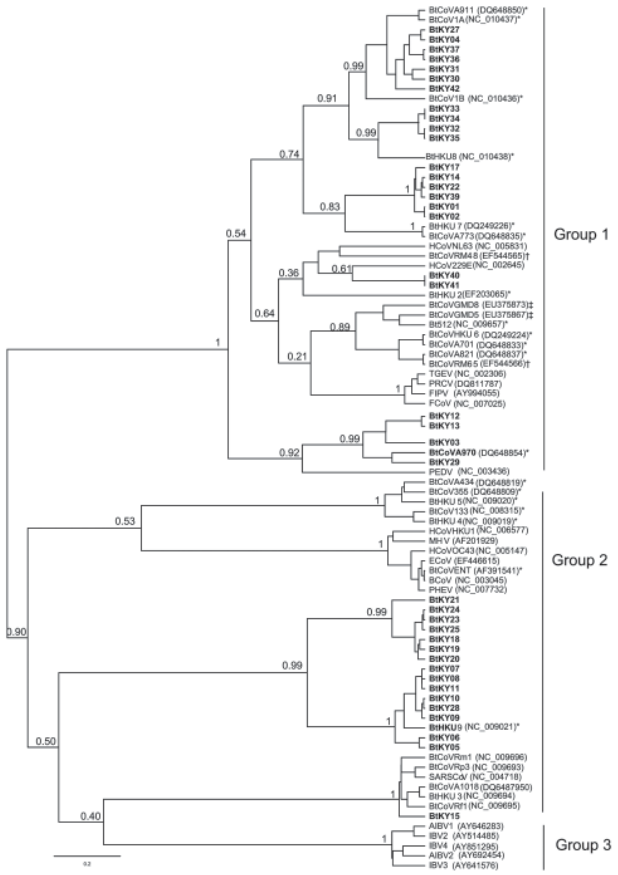

Figure 2. Phylogenetic tree generated using Bayesian Markov Chain Monte Carlo analysis implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST; http://beast.bio.ed.ac.uk) by using a 121-nt fragment of the RdRp gene 1b from 39 coronaviruses (CoVs) in bats from Kenya. CoVs from this study are shown in **boldface**; an additional 47 selected human and animal coronaviruses from the National Center for Biotechnology Information database are included. The Bayesian posterior probabilities were given for deeper nodes. CoV groups (1 to 3) based on International Committee on Taxonomy of Viruses recommendation are indicated. Bat coronaviruses from the People's Republic of China (*), northern Germany (†), and North America (‡) are labeled. Scale bar indicates number of nucleotide substitutions per site.

identity of 88%, 85%, 81%, 77%, and 80% when compared with the next closest previously characterized CoVs (i.e., BtCoV1A, BtHKU8, BtHKU7, HCoV229E, and BtCoVA970, respectively). The remaining 16 sequences would likely be placed into group 2. Two sequences from *Chaerophon* spp. bats (location 17) were closely related to a SARS-like CoV cluster, including 1 sequence shown in Figure 2 (BtKY15) and another (BtKY16) that was 1 of the 3 low-quality sequences excluded from the tree. These 2 NA sequences show ≈89% identity with the nearest previously characterized bat: SARS-like CoV, BtCoVRF1, shows ≈80% NA sequence identity to SARS CoV (Urbani strain) and ≈63% NA sequence identity to the human group 2 CoV HCoVOC43. The 15 remaining NA sequences were grouped into 2 clusters. One cluster contains the recently described BtHKU9 with >95% NA sequence identity, and the other cluster (BtKY18-like cluster) contains no other previously known CoVs, with <75% NA sequence identity to BtHKU9.

The pattern of CoV detections by bat species and location demonstrates several features concerning coronaviruses in bats. A given bat species in the same location can harbor several distinct CoVs as noted for *Chaerophon* spp. (location 17), *Miniopterus inflatus* (location 5), and *Rousettus aegyptiacus* (location 2 and 16); similar CoVs can also been seen in the same type of bat in different locations, as noted for BtCoV1A-like cluster CoVs being detected in *Miniopterus* spp. bats of 4 species from different locations. One *M. inflatus* bat from location 5 harbored 2 different, but closely related, CoVs, 1 (BtCoV 36) from the BtCoV1A-like cluster and 1 (BtCoV 35) from the BtHKU8-like cluster (Figure 2). CoVs of these 2 closely related clusters were detected in *Miniopterus* spp. bats, but not detected in other bat genera, including those that shared roosts with *Miniopterus* spp. bats. This finding is consistent with studies from China in which BtCoV1A-like and BtHKU8-like CoVs were frequently identified but only in *Miniopterus* spp. bats (*15*). This may suggest that viruses of the BtCoV1A-like cluster and the BtHKU8-like cluster are specifically adapted to *Miniopterus* spp. bats and not easily transmitted to other bat species.

In contrast, other genetically similar CoVs were detected in several different bat species. For example, CoVs from th BtHKU7-like cluster were detected in both *Chaerophon* spp. and *Otomops martinsseni* bats; CoVs from the BtCoVA970-like cluster were detected in *Cardioderma cor* and *Rousettus aegyptiacus* bats; CoVs from the BtKY18-like cluster were detected in *Chaerophon* spp., *Eidolon helvum*, and *R. aegyptiacus* bats; and CoVs from the BtHKU9-like cluster were detected in *Hipposidereos commersoni* and *R. aegyptiacus* bats.

Conclusions

These data demonstrate that the CoV diversity in bats previously detected in Asia, Europe, and North America is also present, possibly to a greater extent, in Africa. The extent of this diversity among CoVs may be shown more clearly through additional studies in bats, and increased demonstration of CoV diversity in bats may require a reconsideration of how they should be grouped. The frequency and diversity of CoV detections in bats, now in multiple continents, demonstrate that bats are likely an important source for introduction into other species globally. Understanding the extent and diversity of CoV infection in bats

provides a foundation for detecting new disease introductions that may, like SARS, present a public health threat.

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Rickettsia spp. in Ticks, Poland

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Ticks are recognized as the main vectors and reservoirs of spotted fever group rickettsiae. We searched for the most prevalent *Rickettsia* spp. in Poland and found *R. slovaca* and *R. helvetica* bacteria in ticks in southern and central Poland; *R. raoulti* was found in ticks in all parts of Poland.

Ticks are ectoparasites infesting many mammals, including humans and their pets. In Poland, *Ixodes ricinus* ticks are widely distributed throughout the country whereas *Dermacentor reticulatus* ticks are limited to the northeast region. Both *I. ricinus* and *D. reticulatus* ticks are known to be the main vectors and reservoirs of the spotted fever group rickettsiae (SFG) worldwide. Detection of the SFG pathogens requires a 2-step procedure: PCR and sequencing of selected genes (16S rRNA, citrate synthase [*gltA*], outer membrane protein A [*ompA*], *ompB,* and 17-kDa protein) (*1*–*6*). The aim of the present study was to identify and characterize rickettsial species occurring in ticks in Poland.

The Study

We collected 214 ticks in 3 regions of Poland: the Warsaw region (central Poland) (107 *I. ricinus* ticks), the Radomsko region (southern Poland) (47 *I. ricinus* ticks), and the Bialowieza Primeval Forest National Park (northeastern Poland) (60 *D. reticulatus* ticks). The specimens were collected from April 2005 through August 2007 and identified on the basis of morphologic characteristics. All *I. ricinus* ticks were obtained from dogs and cats, and *D. reticulatus* ticks were collected from vegetation.

DNA was extracted from *I. ricinus* specimens by using the QIAamp DNA Tissue Kit (QIAGEN, Hilden, Germany). *D. reticulatus* specimens were crushed in Eppendorf (Hamburg, Germany) tubes, after which DNA extraction was performed by boiling the specimens in 200 μL of 0.7 M NH_4OH for 30 min.

Bacterial DNA was examined for the *Rickettsia* spp. *gltA* gene by using *Rp*CS.409d and *Rp*CS.1258n primers. Each positive specimen was amplified with paired primers *Rr*190-70 and *Rr*190-701, which were specific for the SFG rickettsiae *ompA* gene. In the absence of amplifiable fragments of the *ompA* gene, molecular identification was conducted by using PCR with paired primers *Rr*17.61p and *Rr*17.492n, which are able to anneal the specific 17-kDa outer membrane protein gene (Table 1).

The QIAquick PCR Purification Kit (QIAGEN) was used to purify PCR products for sequencing. All amplicons were sequenced with the ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's recommendations. All sequences were edited by using the AutoAssembler software (Applied Biosystems), identified with the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and compared with sequences available in GenBank.

The DNA of *Rickettsia* spp. was found in 70 (32.7%) *I. ricinus* and *D. reticulatus* ticks by using PCR and primers specific for the *gltA* gene. All 70 tick samples that tested positive for the genus *Rickettsia* were subjected to amplification with primers specific for the *ompA* gene. Twenty-eight *I. ricinus* and 34 *D. reticulatus* ticks were positive in PCR for the specific *ompA* fragment. In DNA samples extracted from 8 *I. ricinus* specimens (7 negative for the *ompA* gene and 1 having a faint reaction to the *gltA* gene), gene fragments characteristic of the 17-kDa protein were found.

Sequences of the *gltA* gene fragment (688 nt) from 62 samples were identical to the *R. raoultii* Marne and *R. raoultii* Khabarovsk strain sequences (GenBank accession nos. DQ365803.1 and DQ 365804.1). Results were confirmed by sequencing the *ompA* fragment; all 62 showed 99%–100% nucleotide similarity with the *ompA* gene (610 nt) of the *R. raoultii* Marne and the *R. raoultii* Khabarovsk strains (GenBank accession nos. DQ365799.1 and DQ365801.1).

Seven sequences of the *gltA* gene fragment amplicons were identical with the *R. helvetica gltA* gene (GenBank accession nos. U59723.1 and DQ131912.1). These samples also had 100% identical sequences of the 17-kDa protein gene characteristic of *R. helvetica* (GenBank accession nos. EF392726.1 and AJ427881.1).

The sequence of 1 amplicon with primers specific for the *ompA* gene showed 99% similarity with the *R. slovaca ompA* gene (GenBank accession no. U43808.1). Sequencing of PCR products with primers specific for the *gltA* and 17-kDa protein gene were not conducted due to the small amount of DNA in the sample and also due to poor amplification.

R. raoultii DNA was detected in 34 (56.7%) of the 60 *D. reticulatus* specimens from Bialowieza and in 28 (18.2%) of the 154 *I. ricinus* specimens tested, including 25 (23.4%) of 107 from the Warsaw region in central Poland and 3 (6.4%) of 47 *I. ricinus* specimens from the Radomsko region in the south. *R. helvetica* and *R. slovaca* were noted

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Table 1. Oligonucleotide primers used for PCR amplification and sequencing of rickettsial species in ticks, Poland, 2005–2007

No.	Primers	Fragment gene (size, bp)	Nucleotide sequences (5′ → 3′)	Reference
1	*Rp*CS.409d	Citrate synthase	CCTATGGCTATTATGCTTGC	(*5*)
	*Rp*CS.1258n	(850)	ATTGCAAAAAGTACAGTGAACA	
2	*Rr*190-70	Outer membrane protein A	ATGGCGAATATTTCTCCAAAA	(*3*)
	*Rr*190-701	(632)	GTTCCGTTAATGGCAGCATCT	
3	*Rr*17.61p	17-kDa genus-common antigen	GCTCTTGCA ACT TCT ATG TT	(*7*)
	*Rr*17.492n	(434)	CATTGTTCGTCAGGTTGGCG	

exclusively in DNA extracted from the *I. ricinus* ticks. In 3 (2.8%) of the 107 ticks from the Warsaw area, DNA of *R. helvetica* was found. In the Radomsko area, 4 (8.5%) of 47 specimens had *R. helvetica* DNA, and 1 (2.1%) had *R. slovaca* DNA (Table 2).

Conclusions

The DNA of *R. raoultii* was found in *Ixodes* spp. ticks in southern Poland and in *Dermacentor* spp. ticks in northeastern Poland. Until recently, *R. raoultii* had been reported only in *D. nutallii*, *Rhipicephalus* sp., and *Dermacentor* spp. ticks in Europe and Asia (i.e., Siberia and the Astrakhan area) (*8*–*11*).

SFG rickettsiae in Poland have been previously noted solely in *D. reticulatus* ticks (*12*). The *gltA* gene fragment sequence demonstrated similarities with *R. honei* and other unidentified SFG rickettsiae. The nucleotide sequences of amplified fragments of the *ompA* gene were 98% homologous to RpA4 *Rickettsia* sp.

Our findings show that *R. raoultii* occur in all regions of Poland. These bacteria were noted in 18.2% of *I. ricinus* and 56.7% of *D. reticulatus* specimens. Their occurrence in various species of ticks may suggest that they are capable of being distributed all over Europe.

Although the pathogenic role of these genotypes has not yet been established, their ability to cause infection cannot be ruled out. In fact, the RpA4 strain has been isolated from a patient with symptoms resembling those of *R. slovaca* infection (D. Raoult, unpub. data) (*10*). *R. slovaca*, a member of the SFG rickettsiae, was isolated from *D. marginatus* ticks in Slovakia in 1968 (*13*). Research has established that this bacterium is widely distributed and has been isolated from *D. marginatus*, *I. ricinus*, *Haemaphisalis* spp., and Argas ticks in many countries (*13*). From a medical standpoint, *R. slovaca* infection seems to be the most important SFG rickettsiosis in central Europe because of the severe and characteristic symptoms that occur after being bitten by these ectoparasites.

R. helvetica, which was detected in Switzerland in 1979, is also widespread in Europe. This species has recently been found in *I. ricinus* ticks in the northern part of Poland (*14*). Our study also confirms the occurrence of *R. helvetica* in central and southern Poland. These reports suggest that *R. helvetica* can infect *I. ricinus* ticks and may be extensively distributed in several European countries, including Poland. The pathogenicity of *R. helvetica* as a self-limiting illness associated with headache, myalgias, rash, or eschar has been confirmed. Also, several patients with perimyocarditis associated with *R. helvetica* have been observed in Sweden (*15*).

Our findings indicate that SFG rickettsiae transmitted by ticks could penetrate biotopes in various parts of Europe. Though the pathogenicity of the newly recognized species of the genus *Rickettsia* has not yet been proven definitively, it is prudent for clinicians in Poland and other European countries to be alert to possible appearances of infections caused by these pathogens.

Dr Chmielewski is a research scientist in the Laboratory of Rickettsiae, Chlamydiae and Spirochetes at the National Institute of Public Health–National Institute of Hygiene in Warsaw, Poland. His research interests include humoral immune response to *Borrelia burgdorferi*, *Coxiella burnetii*, and *Rickettsia* spp. and molecular biology diagnostic methods for rickettsial and borrelial infections.

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Table 2. *Rickettsia* spp. detected in ticks (N = 214) from 3 regions of Poland, 2005–2007

Rickettsia sp.	Total no. (%) ticks positive	Białowieża,* *Dermacentor reticulatus* ticks (n = 60), no. (%) positive	Radomsko,† *Ixodes ricinus* ticks (n = 47), no. (%) positive	Warszawa,‡ *I. ricinus* ticks (n = 107), no. (%) positive
R. helvetica	7 (3.3)	0	4 (8.5)	3 (2.8)
R. slovaca	1 (0.5)	0	1 (2.1)	0
R. raoultii	62 (29.0)	34 (56.7)	3 (6.4)	25 (23.4)

*Eastern Poland.
†Southern Poland.
‡Central Poland.

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Merkel Cell Polyomavirus in Respiratory Tract Secretions

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Merkel cell polyomavirus (MCPyV), associated with Merkel cell carcinoma, was detected in 27 of 635 nasopharyngeal aspirate samples by real-time PCR. MCPyV was more commonly found in adults than in children. Presence in the upper respiratory tract may be a general property of human PyVs.

Polyomaviruses (PyVs) are highly prevalent, small DNA viruses, capable of persistence in the host. To date, 5 human PyVs have been described: JC (JCPyV), BK (BKPyV), KI (KIPyV), WU (WUPyV), and Merkel cell (MCPyV). The discovery of KIPyV and WUPyV in respiratory tract samples has led to many studies of the role of these viruses in respiratory tract disease (*1*–*4*). Moreover, JCPyV and BKPyV viral DNA has been detected in tonsils (*5*,*6*), and BKPyV has been found in respiratory tract secretions (*7*). MCPyV was reported in 2008 and was identified in Merkel cell tumors, a rare form of skin cancer (*8*). We hypothesized that presence in the upper respiratory tract is a trait shared by all human PyVs and investigated whether MCPyV could also be found in respiratory secretions.

The Study

We used 635 of 637 NPA extracts that had been collected and stored as part of a previous study. Two extracts were insufficient for analysis. A total of 340 samples were from children (median age 5 months, range 10 days–3 years), and 295 samples were from adults (median age 59 years, range 16–93 years). The samples had been sent to Karolinska University Hospital for diagnosis of respiratory tract infections in 2004–2005. Patient identifiers were removed, and the only available clinical information was the patient's age and sex, month of sampling, and name of referring clinic.

An initial screening by nested PCR with the published MCPyV primer sets LT3, LT1, and VP1 (*8*) identified a strongly positive sample, NPA370, that was used as a positive control for subsequent experiments. Two hydrolysis probe–based, real-time PCRs (rtPCRs) were designed to target the large T antigen (LT) gene and the capsid VP1 gene of MCPyV. Primers and probe targeting the LT gene were (LT.1F) 5′-CCACAGCCAGAGCTCTTCCT-3′, (LT.1R) 5′-TGGTGGTCTCCTCTCTGCTACTG-3′, and (LT probe) 5′-FAM-TCCTTCTCAGCGTCCCAGGCTTCA-TAMRA-3′. The resulting amplicon was 146 bp. Primers and probe targeting the VP1 gene were (VP1.1F) 5′-TGCCTCCCACATCTGCAAT-3′, (VP1.1R) 5′-GTGTCTCTGCCAATGCTAAATGA-3′, and (VP1 probe) 5′-6FAM-TGTCACAGGTAATATC-MGBNFQ-3′. The resulting amplicon was 59 bp. Reactions were performed in 20 μL of 1×TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nmol/L of LT.1 primers or 450 nmol/L of VP1.1 primers, 250 nmol/L of LT probe or 500 nmol/L of VP1 probe, and 5 μL of template. Cycling conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 5 s, and 60°C (LT assay) or 58°C (VP1 assay) for 1 min in a Roche Lightcycler 480 (Roche, Basel, Switzerland).

Due to a limited amount of the positive sample NPA370, control plasmids were constructed for both assays by cloning amplicons of NPA370 into pCR4-TOPO (Invitrogen, Carlsbad, CA, USA): pMCPyVLT.1 containing a 258-bp LT gene amplicon (FJ472933) and pMCPyVVP1.1 containing a 179-bp VP1 gene amplicon (FJ472932). Serial dilutions of the plasmids were used to determine assay sensitivity, and pMCPyVLT.1 was also used to determine a genome copy number correlate for the LT assay. In both assays, plasmid control with 2 copies/reaction was reproducibly positive, corresponding to 400 copies/mL of sample. Specificity of both assays was assessed by a range of templates: a plasmid containing the complete KIPyV genome; a WUPyV-positive sample NPA213; 4 urine samples positive for either BKPyV or JCPyV; and a panel of samples containing respiratory syncytial virus, influenza A and B viruses, adenovirus, bocavirus, parainfluenza virus, metapneumovirus, herpes simplex virus type 1 and 2, varicella-zoster virus, human herpesvirus 6, parvovirus B19, cytomegalovirus, echovirus 30, *Mycoplasma pneumonie*, *Chlamydophila pneumoniae*, and *Legionella pneumophila*. All the above samples were negative by LT and VP1 assays. To check for contamination, we included at least 4 water controls per run of 10–86 samples; no amplification was observed.

Of the 635 NPA samples, 44 (6.9%) were positive for MCPyV DNA by the LT assay, 84 (13.2%) were positive by the VP1 assay, and 27 (4.3%) were positive by both assays. With a few exceptions, viral DNA copy numbers were low, as determined by cycle threshold values (mean LT/VP1 = 38.6/39.0) and plasmid equivalent counts of the LT assay (Table). To further validate these findings, 10 double-positive samples and all LT-positive (+)/VP1-negative

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Table. Consensus results of 2 real-time PCRs for MCPyV in adults and children*

pMCPyVLT.1 equivalents			No. children	No. adults			
Per reaction	Per mL of sample	No. samples	(<15 y)	(≥15 y)	No. male	No. female	Coinfection†
<2 (negative)	<400 (negative)	608	338	269	308	299	229‡
2–100	400–20,000	22	2	20	15	8	7§
101–617	>20,200–123,000	5¶	0	5	4	1	1#

*MCPyV, Merkel cell polyomavirus.
†Viruses detected by immunofluorescence, virus culture, and real-time PCR.
‡Adenovirus, enterovirus, herpes simplex virus type 1, influenza A virus, influenza B virus, KI polyomavirus, metapneumovirus, parainfluenza virus type 1 and 3, respiratory syncytial virus, WU polyomavirus.
§Influenza A and B viruses.
¶Includes NPA370.
#Influenza A virus.

(–) samples were amplified by conventional PCR using the LT primers. All double-positive samples gave the expected 146-bp product, which was confirmed to have MCPyV sequence (FJ472034–43), but the LT+/VP1– samples did not generate specific products. Whether this was due to lower sensitivity of the conventional PCR or occasional unspecific amplification in the LT rtPCR could not be determined. The VP1 PCR product was too short to enable direct sequencing. Therefore, only samples positive by both assays were considered positive for MCPyV.

By these criteria, 25 of 295 samples from adults and 2 of 340 samples from children were positive. Therefore, significantly more adults than children were MCPyV positive ($p<0.001$, χ^2 test). MCPyV was particularly prevalent among the elderly. The median age for MCPyV carriers was 74 years. A skew in gender in MCPyV carriers was not statistically significant. None of the MCPyV-positive samples were positive for KIPyV or WUPyV.

Conclusions

MCPyV DNA was detected in 4.3% of NPA samples from patients with respiratory disease as determined by combined results of 2 rtPCRs. Viral DNA was detected in low copy numbers, which makes reproducibility of positive results challenging (*9*). Therefore, a prevalence rate of 4.3% is probably an underestimation of samples containing MCPyV DNA at concentrations below the reproducible detection limit. A similar result of discordant LT and VP1 positive Merkel cell tumor samples has been reported (*8*). Contamination by the plasmid template is unlikely because water controls were consistently negative and LT and VP1 positivity were significantly associated ($p<0.001$, χ^2 test). Notably, both assays were easily reproducible for the 5 samples that had relatively high copy numbers (Table).

The prevalence of MCPyV in this collection of NPA samples is higher than that of KIPyV and WUPyV (*10*). Also, significantly more adults (particularly the elderly) were MCPyV positive than were children. This result is in contrast to results with KIPyV and WUPyV, which have mainly been found in children (*1*,*2*). Possible reasons for this age-related prevalence include incidence increases with exposure time, activation of latent virus increases with age, and possible previous exposure to transmission of MCPyV that is no longer in effect (*11*). Alternatively, a larger number of adult patients may be immunosuppressed than are the children. However, most MCPyV-positive samples (22/27) were sent for influenza diagnostics from the infectious diseases unit, not a hematology or oncology unit; hence, patients were probably not severely immunosuppressed.

The presence of MCPyV in respiratory secretions indicates that it is shed into the respiratory tract or present in cells of the respiratory tract, similar to KIPyV, WUPyV, BKPyV, and possibly JCPyV. Its presence also suggests that the respiratory tract may be a route of transmission. The use of stored nucleic acid extracts did not allow us to determine whether detected MCPyV DNA was intracellular or virion-associated. Thus, the newly discovered human PyVs can all be found in the respiratory tract. Conclusions about their primary target organs and pathogenicity cannot be drawn without epidemiologic support and further investigation on different sample types.

Dr Goh is a postdoctoral fellow at Karolinska Institute. Her research interests focus on novel human viruses and their prevalence in clinical samples.

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Merkel Cell Polyomavirus DNA in Respiratory Specimens from Children and Adults

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Merkel cell polyomavirus (MCPyV) DNA was detected in 7 (1.3%) of 526 respiratory tract samples from patients in Australia with upper or lower respiratory tract symptoms. Partial T antigen and major capsid protein sequences of MCPyV identified in respiratory secretions showed high homology (99%–100%) to those found in Merkel cell carcinoma.

In the past 2 years, several previously unknown human polyomaviruses (PyVs) have been identified: KI virus (KIPyV) (*1*) and WU virus (WUPyV) (*2*) from respiratory samples, and more recently, Merkel cell polyomavirus (MCPyV), most commonly from Merkel cell carcinoma tissue (*3*). MCPyV has been found in integrated and episomal states (*3*); however, a mode of transmission for MCPyV has not yet been proposed. In this study, we sought to identify MCPyV in respiratory specimens by using real-time PCR.

The Study

This study was conducted exclusively at the Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital and Health Service, Brisbane, Queensland, Australia. The necessary ethical approval for this study was obtained from the Ethics Committee of the Royal Children's Hospital.

Specimens tested in this study (n = 526) were collected from January through December 2003 from 418 pediatric patients (birth to 14 years of age) (n = 450; age range 3 days–13.5 years; mean 1.7 years; median 0.8 years) and 71 adult patients (n = 76; age range 14.3 years–80.1 years; mean 47.1 years; median 52.5 years) who were hospitalized or sought treatment at hospitals in Queensland, Australia, for upper or lower respiratory tract symptoms. Most (95.6%) samples were nasopharyngeal aspirates (NPAs); the remainder were bronchoalveolar lavage specimens, bronchial washing specimens, and endotracheal aspirates. These samples were collected as part of a previous study that tested for influenza viruses A and B, adenoviruses, human metapneumovirus, respiratory syncytial virus, and parainfluenza viruses 1, 2, and 3, in addition to KIPyV and WUPyV (*4*).

Samples were screened by using an MCPyV real-time PCR that was specific for the VP2/3 region. Briefly, 10 pmol of each primer MCPyV-2.0–4367F (5′-GGCAGCATCCCGGCTTA-3′) and MCPyV-2.0–4399R (5′-CCAAAAAGAAAAGCATCATCCA-3′) and 4 pmol of dual-labeled probe MCPyV-2.0–4371-Prb (5′-FAM-ATACATTGCCTTTTGGGTGTTTT-BHQ1-3′) in a 25-μL reaction using QIAGEN Quantitect Probe master mix (QIAGEN, Doncaster, Victoria, Australia) with 2 μL of template were run on a LightCycler 480 (Roche Diagnostics, Castle Hill, New South Wales, Australia) under the following conditions: incubation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Quantitative real-time PCR was not performed because of limited applicability due to inherent variability in respiratory sample collection. The presence of MCPyV in samples positive by real-time PCR was then confirmed by using partial large T antigen (LT3) and major capsid protein (VP1) conventional MCPyV detection PCR assays of Feng et al. (*3*). All PCRs were performed in a unidirectional workflow through dedicated suites for reagent preparation, PCR setup, and amplification. Ten random real-time PCR–negative samples, 10 water control samples, and template-negative control samples were used to exclude amplicon or sample cross-contamination. A clinical sample that was positive for MCPvV by all 3 assays and confirmed by sequencing was used as a positive control for all PCRs.

Thirty-one (5.9%) samples produced positive results in the real-time PCR screening. Of these, 8 (1.5%) could be confirmed by only 1 conventional PCR, and 7 (1.3%) yielded positive results in all 3 MCPyV PCRs. All positive detections were in NPA samples. This variation in detection rates could have been due to the unforeseen nonspecificity of the real-time PCR, or to the inherent lower sensitivity of conventional PCRs, because most real-time PCR–positive samples produced late signals at cycle threshold values ≈40. We chose a conservative algorithm, in which a sample must have been positive in all 3 assays to be considered a true positive. This rule may have led to an underestimation of MCPyV prevalence in this sample population.

All MCPyV-positive samples were collected during the spring and early summer months (October–December

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2003). Five (1.1%) of these samples originated from pediatric patients, which suggests early acquisition of virus. In fact, all 4 MCPyV-positive patients who were not immunosuppressed were <2 years of age (Table 1). This age range coincides with ages of those who experience primary infections of many other human viruses (*5*,*6*). Of the 3 immunosupressed MCPyV-positive patients, 2 were adults (2.6%), and 1 was a 6.6-year-old child. Further information on each MCPyV-positive patient is listed in Table 1.

We could not discern whether the higher frequency of detections in adults was representative of the general adult population or whether the detections were overrepresented due to a small sample size or the immune status of the adult cohort. The integration status of the detected MCPyV DNA was not able to be ascertained; however, for 1 patient for whom multiple samples were tested, MCPyV was neither detected 2 months before nor 3 months after MCPyV-B4 was detected, which suggests that, at least in this particular patient, MCPyV may have had transitory presence or activity. Multiple samples were not available for any of the other MCPyV-positive patients.

Three MCPyV (MCV-B1/B2/B3)–positive samples were further investigated through bidirectional sequencing of their LT3 (FJ009185, FJ009186, and FJ009187, respectively) and VP1 (FJ009188, FJ009189, and FJ009190, respectively) assay amplification products. The sequences of the 3 samples showed high homology to those of the 2 previously described MCPyV strain sequences, MCC350 (EU375803) and MCC339 (EU375804) (*3*); similarities in the LT3 and VP1 target regions ranged from 99.6%–100.0% and 99.0%–99.6%, respectively (Table 2).

Clinical notes were available for 5 of the 7 MCPyV-positive patients and indicated that these patients exhibited a variety of upper and lower respiratory tract symptoms. MCPyV was the sole viral agent detected in 6 of the 7 samples (Table 1). However, due to limited remaining volumes, study samples were not screened for other known respiratory pathogens, including coronaviruses and rhinoviruses, which may have led to a higher codetection rate, similar to that for KIPyV (*1*,*4*) and WUPyV (*2*,*4*).

Conclusions

To date, MCPyV has been most commonly studied and detected in the context of Merkel cell carcinoma (*3*,*7*), but it has also been identified in Kaposi sarcoma tissue (*3*), primary squamous cell carcinoma tissue (*7*), keratoacanthoma tissue from an organ recipient (*8*), in normal sun-exposed skin (*7*), and in a small number of control tissues (normal skin, small bowel, hemorrhoids, appendix, and gall bladder) (*3*). In this study, MCPyV was detected in 1.3% of respiratory samples collected from symptomatic persons. This description of the presence of MCPyV in the respiratory tract raises questions about the transmission and respiratory pathogenicity of this newly described PyV.

Analogous to MCPyV, both KIPyV (*1*,*4*) and WUPyV (*2*,*4*) have been found globally in respiratory specimens with a high prevalence of codetected viruses, but clear evidence for a causal association with respiratory illness has yet to be identified. These PyVs may be merely transmitted through the respiratory route or detected during periods of reactivation (*9*). Similarly, JC and BK PyVs are suspected of being transmitted by inhalation and are occasionally detected in respiratory samples, yet patients generally remain asymptomatic or exhibit nonspecific upper respiratory tract symptoms (*10*,*11*). Like these other PyVs, MCPyV may potentially be transmitted through the respiratory route and become latent in other sites, such as epidermal tissue, by systemic spread, in a similar fashion to murine PyV (*12*). Clearly, further studies using larger sample populations, expanded respiratory pathogen detection methods, and control groups are needed to elucidate what role, if any, MCPyV plays in upper and lower respiratory tract illness.

On the basis of the ages and immune status of the MCPyV-positive patients, we can hypothesize that MCPyV may have an infectious cycle similar to that of other human PyVs, in which the virus is acquired early in life, under-

Table 1. Detection of MCPyV in nasopharyngeal aspirates in persons with respiratory signs and symptoms, Queensland, Australia, 2003*†

			Patient characteristics				
Sample	Ct	Codetections	Age	Sex	Immune status	Clinical history	Signs and symptoms
MCV-B1 (LT3: FJ009185, VP1: FJ009188)	29.0	–	52.0 y	M	Suppressed	Acute myeloid leukemia	Flu-like
MCV-B2 (LT3: FJ009186, VP1: FJ009189)	33.0	–	6.6 y	F	Suppressed	Heart transplant, T-cell lymphoma	Coryza, fever, sore throat, ear pain
MCV-B3 (LT3: FJ009187, VP1: FJ009190)	31.5	–	47.7 y	M	Suppressed	Heart transplant	–
MCV-B4	36.9	–	8 mo, 8 d	M	Competent	–	Bronchiolitis
MCV-B5	37.5	–	1 mo, 26 d	M	Competent	–	Rhinorrhea, cough
MCV-B6	37.4	–	9 mo, 27 d	F	Competent	–	
MCV-B7	38.9	Adenovirus, WUPyV	1.6 y	F	Competent	–	Fever, convulsions

*MCPyV, Merkel cell polyomavirus; Ct, cycle threshold; WUPyV, WU polyomavirus.
†Clinical histories and symptoms were noted when available. Associated GenBank accession numbers are shown in parentheses.

Table 2. Nucleotide variation in LT3 and VP1 sequences from 3 MCPyV strains detected in respiratory secretions from patients, Queensland, Australia, 2993*†

	Nucleotide position and change				
	LT3	VP1			
Isolate name	843	3825	3875	3909	4022
MCC350‡ (EU375803)	**G**	**T**	**C**	**C**	**T**
MCC339‡ (EU375804)	C	C	G	T	A
MCV-B1 (LT3: FJ009185, VP1: FJ009188)	C	C	–	–	–
MCV-B2 (LT3: FJ009186, VP1: FJ009189)	C	C	G	–	A
MCV-B3 (LT3: FJ009187, VP1: FJ009190)	C	C	–	–	–

*LT3, partial large T antigen; VP1, major capsid protein; MCPyV, Merkel cell polyomavirus.
†Nucleotide changes are in relation to the prototype strain (**boldface**). Dashes indicate homology with MCC350. Nucleotide positions are given in a 5′ → 3′ early to late orientation, respective to alignment with MCC350. GenBank accession numbers are shown in parentheses.
‡Original MCPyV strain sequences described by Feng et al. (*3*).

goes a period of latency, and then becomes reactivated in the event of immunosuppression (*10*). Sequence data from positive specimens indicate that MCPyV found in respiratory secretions is similar to the viruses identified within Merkel cell carcinomas. Whether and how the virus is translated from the respiratory tract to skin cells, if it is able to be transported systemically, and its role in the malignant transformation of Merkel cells are all questions worthy of further investigation.

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Guillain-Barré Syndrome after Chikungunya Infection

To the Editor: *Chikungunya virus* is an RNA alphavirus (group A arbovirus) in the family *Togaviridae*. The known vectors are *Aedes aegypti* and *Ae. albopictus* mosqitoes. Chikungunya infection, after an incubation period of 2–10 days, has the main clinical manifestations of fever, polyarthralgia, and rash. Treatment consists of rest and medication for pain. Outcome is marked by incapacitating arthralgia, which can persist for several weeks or months (*1*). Complications are rare and consist of mild hemorrhage, myocarditis, and hepatitis (*2*). Neurologic manifestations are less well known (*3*). Infection is confirmed by the identification of genomic products in acute-phase blood specimens, (reverse transcription–PCR [RT-PCR]) or, more recently, by serum immunoglobulin (Ig) M or a 4-fold increase in other antibodies. In 2006, chikungunya virus was found on Réunion Island; seroprevalence on the island was estimated to be 38.2% among 785,000 inhabitants (95% confidence interval 35.9%–40.6%) (*4*).

Guillain-Barré syndrome (GBS) is an acute inflammatory demyelinating polyneuropathy; incidence worldwide is 0.6–4/100,000 persons/year. In two thirds of patients, neuropathic GBS occurs after an infection (*5*,*6*).

Cases of GBS have been described in association with the arboviruses dengue and West Nile but not with chikungunya virus. We report 2 cases of acute and severe GBS related to infection with chikungunya virus.

The first patient was a 51-year-old woman who in 2006 was admitted to an intensive care unit in Réunion Island's Centre Hospitalier Departemental for treatment of polyradiculoneuropathy. Her medical history consisted of poorly treated type 2 diabetes and hypertension. Three weeks before hospital admission, she had had fever, arthralgia, rash, and diarrhea. One week later, rapidly progressing motor weakness and sensory disturbances developed, e.g., tingling in all limbs. She had facial diplegia, and her tendon reflexes were absent. Cerebrospinal fluid (CSF) contained increased protein (1.44 g/L) but not increased leukocytes (1/mm^3). Electromyography displayed typical signs of demyelinating sensorimotor neuropathy with increased distal motor latency and reduced motor conduction velocity. Sensory nerve action potential was absent. Antichikungunya IgM was found in serum at 15 days after onset of signs and symptoms. This seroconversion confirms an acute infection by an alphavirus. Serum genomic product (RT-PCR, TaqMan method) (*7*) was negative for chikungunya virus. Antichikungunya IgM and IgG were also found in CSF.

The patient's respiration rapidly deteriorated, and she required tracheal intubation and mechanical ventilation for 12 days. She was given intravenous immunoglobulin for 5 days (TEGELINE; LBF Biomedicaments, Courtaboeuf, France). She recovered and was extubated on day 12. Two months after onset of symptoms, the patient reported a satisfactory recovery; she was able to walk, and her sensory disturbances had rapidly disappeared.

The second patient was a 48-year-old woman who in 2006 was admitted to the intensive care unit in Réunion Island's Centre Hospitalier Departemental unit for a rapidly developing polyradiculoneuropathy. She had no relevant past medical history. Two weeks before her admission, she had been febrile and had had arthralgia and a rash. Later, weakness with facial diplegia and sensory disturbances developed, e.g., tingling in all limbs. Tendon reflexes were absent. CSF contained increased protein but not increased leukocytes. Electromyography displayed signs of a peripheral neuropathy and evidence of a conduction block. At the time of hospital admission, antichikungunya IgM and IgG were detected in 2 serum samples. RT-PCR for chikungunya virus in serum and CSF was negative.

The patient's respiration rapidly deteriorated, and she required tracheal intubation and mechanical ventilation for 9 days. After receiving intravenous immunoglobulin for 5 days, she recovered quickly. Return of a productive cough and satisfactory muscle tone enabled her to be removed from mechanical ventilation on day 9.

For the 2 patients reported here, GBS diagnosis was based on a typical clinical acute motor and sensory polyradiculoneuropathy, which evolved in 3 characteristic stages: rapid deterioration, plateau, and slow recovery (*6*). Also typical of GBS are normal CSF counts, increased CSF proteins, and electromyography data (peripheral neuropathy, conduction block). The widespread screening for organisms known to be associated with GBS produced negative results. However, antichikungunya IgM was found in serum and CSF, although genomic products in serum and CSF were negative, which was not surprising, given the brief period (4–5 days) of viremia (*8*). These findings strongly supported a disseminated acute chikungunya infection and enabled us to conclude that chikungunya virus was probably responsible for the GBS.

Epidemiologic data also support a causal relationship between chikungunya infection and GBS. The incidence rate of GBS increased ≈22% in 2006 (26/787,000 [3.3/100,000] persons) over the rate in 2005 (21/775,000 [2.7/10,000] persons) and then declined to a rate closer to baseline in 2007 (23/800,000 [2.87/100,000] persons).

These 2 cases of GBS on Réunion Island were related to an acute and documented chikungunya infection. In the absence of an effective treatment, patients with these suspected infections should receive supportive care for classic GBS.

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Cockroaches (*Ectobius vittiventris*) in an Intensive Care Unit, Switzerland[1]

To the Editor: *Ectobius vittiventris* (Costa) is a field-dwelling cockroach and 1 of 4,000 cockroach species worldwide (*1*). We describe a cockroach infestation of an intensive care unit (ICU). Successful management required knowledge of the ecology of cockroaches and highlighted the need for species-level identification to tailor control strategies.

The University of Geneva Hospitals are a 2,200-bed tertiary healthcare center. The 18-bed medical ICU is located on the ground floor next to an outdoor recreational area and admits ≈1,400 patients/year. Smoking inside hospital buildings by patients and healthcare workers (HCWs) is strictly prohibited. On August 25, 2006, ≈30 cockroaches were observed in the ICU hiding inside oxygen masks, moving around on the light panels below the ceilings, or dropping onto intubated patients during the night.

An outbreak investigation was initiated. All work areas, including sinks and material stock areas, were thoroughly searched for cockroaches. External pest control experts identified only 1 species, *E. vittiventris*, which had presumably entered the ICU through windows facing the outdoor recreational area. The investigation showed that despite verbal recommendations and being repeatedly forbidden to do so, HCWs had opened the windows secretly with screwdrivers so that they could smoke during night shifts. The infestation was halted within 3 days after information regarding the infestation was provided to HCWs and all windows were bolted shut. In contrast to measures required to deal with a reported infestation in a neonatal ICU (*2*), no other measures such as use of insecticides, review of the air circulation system, or changes in architectural structures were necessary to stop the infestation reported here.

Cockroaches can cause 2 potentially serious health problems. First, they may provoke allergic reactions (*3*). Second, they have been suggested as possible vectors of multidrug-resistant pathogens. In particular, cockroaches that live and breed in hospitals have higher bacterial loads than cockroaches in the community (*4–6*). Up to 98% of these "nosocomial" cockroaches may carry medically important microorganisms on their external surfaces or in their alimentary tracts (*4–9*) and may disseminate these microorganisms by fecal–oral transmission.

Cockroaches are capable of harboring *Escherichia coli* (*6*,*7*), *Enterobacter* spp. (*6*,*8*,*9*), *Klebsiella* spp. (*6*,*7*,*9*), *Pseudomonas aeruginosa* (*6*,*9*), *Acinetobacter baumannii* (*2*), other nonfermentative bacteria (*7*,*9*), *Serratia marcescens* (*7*,*9*), *Shigella* spp. (*6*), *Staphylococcus aureus* (*6*,*7*), group A streptococci (*6*,*7*,*9*), *Enterococcus* spp. (*6*,*7*), *Bacillus* spp. (*7*), various fungi (*6–8*), and parasites and their cysts (*6*). An outbreak of extended-spectrum β-lactamase–producing *Klebsiella pneumoniae* in a neonatal unit was attributed to cockroaches (*2*). Pulsed-field gel electrophoresis did not distinguish organisms from the insects from those colonizing infants or causing clinical disease (*2*). Unlike other investigators, we did not cultivate the cockroaches (*6*,*9*).

E. vittiventris cockroaches are easily confused with *Blattella germanica* (Linnaeus) (the German or croton cockroach), which is probably the most important cockroach pest worldwide (*1*,*9*). In contrast to *B. germanica* (*6*,*9*) and other species (online Technical Appendix, available from www.cdc.gov/EID/content/15/3/496-

[1]Parts of this research have been presented as a poster at the Annual Meeting of the Swiss Society for Infectious Diseases, Zurich, Switzerland, June 14, 2007.

Techapp.pdf), *E. vittiventris* cockroaches are considered to be harmless and have not been associated with human disease or transmission of pathogens. We did not observe any allergic reactions or an increase in colonization or infection rates of multidrug-resistant organisms. *B. germanica* cockroaches are nocturnal, cannot fly, are always encountered within human habitations, and require specialized measures for eradication (*10*).

E. vittiventris cockroaches live in outdoor areas, do not avoid light, and are active during daytime. Buildings are not a natural habitat. In summer, adult insects can fly inside at night, but because these cockroaches are unable to reproduce inside buildings (*1*), stopping entry from outside halts the infestation. Entry can be stopped by closing windows or using mosquito nets. There is no existing insecticide for eradication of *E. vittiventris* cockroaches (*10*), and even if there were, it would not be effective because insects from untreated areas outside would enter continuously (*1*).

E. vittiventris cockroaches have been recently discovered in Geneva (*10*) and have become the most frequently encountered cockroaches in urban areas of Switzerland for several years (*1*). The reason for this finding remains unknown. The summer of 2003 was remarkably hot and dry in central Europe, thus representing a subtropical climate that usually favors the growth and development of cockroach populations (*1*,*7*). If this warming trend persists, populations of *E. vittiventris* cockroaches may continue to expand, and similar infestations may occur.

In conclusion, effective control strategies for cockroach infestations depend on identification of cockroach species. In this report, permanent closure of all windows was sufficient to stop the infestation. However, to ensure compliance, it was critical to discuss the purposes of the intervention with HCWs.

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Cutaneous Anthrax, West Bengal, India, 2007

To the Editor: In most of India, anthrax is not common, probably because a large proportion of the population is Hindu and does not eat beef. However, sporadic cases and outbreaks have been reported (*1*–*6*).

On June 8, 2007, a healthcare facility reported 12 cases of cutaneous anthrax in the Muslim village of Sarkarpara (population 361). On August 4, 2007, another facility 50 km away reported 8 cases from the Muslim village of Charbinpara (population 835). These 2 outbreaks, both in Murshidabad district, West Bengal, were associated with the slaughtering of 4 cows. We investigated each outbreak to confirm diagnosis, estimate magnitude (incidence and severity), and identify risk factors. We conducted house-to-house searches to identify case-patients and collected smears from skin lesions.

From Sarkarpara, we identified 45 cases of cutaneous anthrax and 2

deaths (attack rate 12%, case-fatality rate 4%); from Charbinpara, we identified 44 cases and no deaths (attack rate 5%). In Sarkarpara, villagers had slaughtered a cow on June 2, 2007. The outbreak started on June 3, peaked on June 6 (1 cluster), and ended on June 10. In Charbinpara, villagers had slaughtered 3 cattle, 1 each day, on July 16, July 23, and August 1. The first case occurred on July 17 and was followed by 3 peaks (3 clusters) (Figure). In each village, attack rates were highest among persons 15–44 years of age. Microscopic examination at the district public health laboratory showed gram-positive, spore-bearing bacilli that were characteristic of *Bacillus anthracis* on 7 of 20 smears (5/10 from Sarkarpara and 2/10 from Charbinpara).

To test the hypothesis that exposure to meat of a slaughtered cow was associated with illness, we conducted a retrospective cohort study among families who had handled or eaten beef from cows slaughtered during the week before the outbreak. Through interviews, we collected information about possible exposures, including slaughtering, handling meat or skin, and eating beef.

In Sarkarpara, we enrolled 296 persons from 59 families in the cohort study. Persons who had slaughtered cows and handled meat and skins had a significantly higher risk for illness than those who had not. In Sarkarpara, risk associated with slaughtering cattle was 9.1 (95% confidence interval [CI] 6.0–13.7) and with handling meat 2.6 (95% CI 1.5–4.4) (online Appendix Table, available from www.cdc.gov/EID/content/15/3/497-appT.htm). Slaughtering cows or handling meat accounted for the largest proportion of cases; 8% and 33% of the population was engaged in these practices, respectively (population-attributable fraction [PAF] 39% [95% CI 37.0–41.2] and 34% [95% CI 18.5–42.9], respectively). PAF associated with handling skins was 2% (95% CI 1.8–2.0).

In Charbinpara, we included 687 persons from 118 families in the cohort study. Slaughtering cattle and distributing beef were strongly associated with illness (online Appendix Table). Slaughtering cows and handling meat were common practices and accounted for the largest proportion of cases (PAF 47% [95% CI 46.0–48.0] and 19% [95% CI 17.5–19.4], respectively). In Charbinpara, risk associated with slaughtering was 19.0 (95% CI 11.0–30.0) and with distributing was 11.0 (95% CI 6.8–19.0) (online Appendix Table). Of the persons who ate beef, anthrax developed in 17% in Sarkarpara and 7% in Charbinpara. However, when we restricted the analysis to those who did not handle meat or skin, eating beef was not associated with illness. No person whose sole exposure was eating beef became ill. Persons who slaughtered cattle were not in the butchering profession; they did not wear gloves or other protective equipment. Their helpers distributed the beef in the village without any protection. Persons involved in skin trading carried the skins to nearby villages to sell. Women in the villages boiled the beef for 30 minutes before serving.

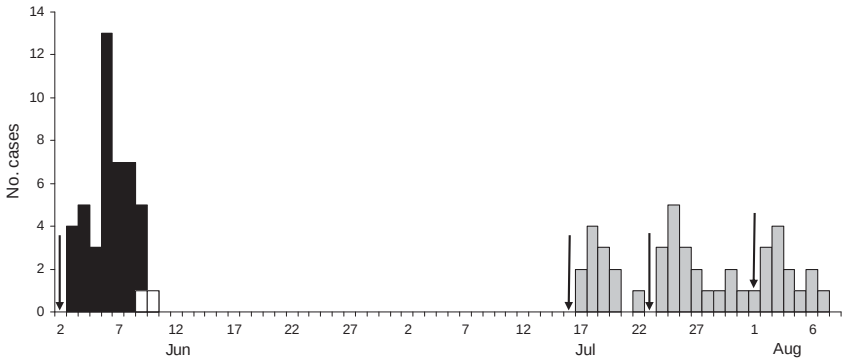

Figure. Cases of cutaneous anthrax, Mushidabad district, West Bengal, India, 2007. Dates indicate onset of skin lesion. Arrows indicate dates cattle were slaughtered. Black bars, cases in Sarkarpara village; gray bars, cases in Charbinpara village; white bars, deaths.

In Sarkarpara, healthcare workers knew the symptoms suggestive of anthrax and that this disease needed to be reported. As a result, this outbreak was reported early. In Charbinpara, healthcare workers knew nothing about the disease and did not report it. As a result, reporting was delayed until the third cluster. Late reporting prevented effective public health action. Because the source of infection in the 2 villages differed (different cattle), we were unable to formally establish a causal link between these 4 clusters.

Because the anthrax outbreak in Murshidabad was associated with slaughtering of ill cows and handling raw meat without taking any protective measures, we propose several recommendations. First, healthcare workers in anthrax-endemic areas need to be educated about promptly recognizing and reporting the disease. Second, persons in the community must be educated about using personal protective equipment during slaughtering of animals and handling of meat and skins. Community education should focus on those at risk, including Muslim communities who eat beef. Because anthrax occurs in only a few districts, India does not have a nationally organized control program (*7*). However, a focal prevention plan based on these recommendations would ultimately help reduce illness and death in anthrax-endemic districts.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Cat-to-Human Orthopoxvirus Transmission, Northeastern Italy

To the Editor: Kurth et al (*1*) recently described a cowpoxvirus chain of transmission from rat to human through an elephant in Germany. Zoonotic cowpoxvirus infections are well known in Europe (*2*,*3*). This virus can infect many animal species; serologic evidence of infection may approach 10% in cats in western Europe (*4*,*5*). Zoonotic orthopoxvirus (OPV) infection has been reported in several European countries, but it is rare south of the Alps, and no extensive description of cases is available for Italy. We describe 2 cases of zoonotic OPV in Friuli, northeastern Italy, in veterinary personnel scratched by cats.

In December 2005, a male veterinary student (patient A) who had been scratched by a cat with multiple cutaneous ulcerated lesions sought care at a local hospital; he had a lesion on his right hand, moderate fever, and malaise. Histopathologic findings from the cat indicated feline poxvirus infection. In July 2007, a female veterinarian (patient B) who lived in a different area of the same region and also had been scratched by a cat, sought care at the same hospital; she had a lesion close to the right sternoclavicular joint.

On the basis of patients' history of exposure and clinical presentation of the animals' disease, zoonotic transmission of OPV infection was suspected. Vesicle fluid and, subsequently, crusts from the patients' lesions, were sent to the virology laboratory of the reference center for poxvirus infections at National Institute for Infectious Diseases in Rome, where OPV diagnosis was based on electron microscopy, virus isolation, and detection of viral nucleic acid. In addition, serial blood samples were sent to the same laboratory for analysis of specific antibodies and cellular immune response.

The viruses were almost identical, according to the partial sequence of the *crmB* gene (EF612710 and FJ445748) and on the complete sequence of the hemagglutinin gene (EF612709 and FJ445747). The hemagglutinin (Figure) and the partial *crmB* (not shown) sequences from each isolate formed a distinct cluster within the OPV genus; similar results were obtained from concatenated analysis of both genes (not shown). The identity/similarity scores of the complete nucleotide sequences of *crmB* and hemagglutinin genes from patient A, in relation to other OPVs, were, respectively, Ectromelia (AF012825), 0.598 and 0.841; cowpox (X94355), 0.933 and 0.927; vaccinia (AY678276), 0.844 and 0.934; camelpox (AY009086), 0.941 and 0.940; monkeypox (DQ011153), 0.909 and 0.914; and variola (DQ437588), 0.922 and 0.906. On the basis of these results, species assignment of the isolated OPVs was not possible. Preliminary sequence data on additional genes (ATI, A27L, and CBP) from patient A's isolate supported the segregation of these OPVs in Italy from the other known OPV species. However, the available information is still not enough to infer whether the isolates from Italy belong to a novel or known OPV species. More extensive biologic and molecular characterization is in progress.

The 2 cases reported here, occurring >1 year apart, indicate that OPV is circulating in domestic, and possibly wild, local fauna; they underscore the need for physicians and veterinarians to become aware of the risk for OPV zoonoses. A surveillance program has been launched among local veterinary clinics to identify nonvaccinated veterinarians who have been exposed to OPV. A surveillance plan for cats at these clinics has also been started.

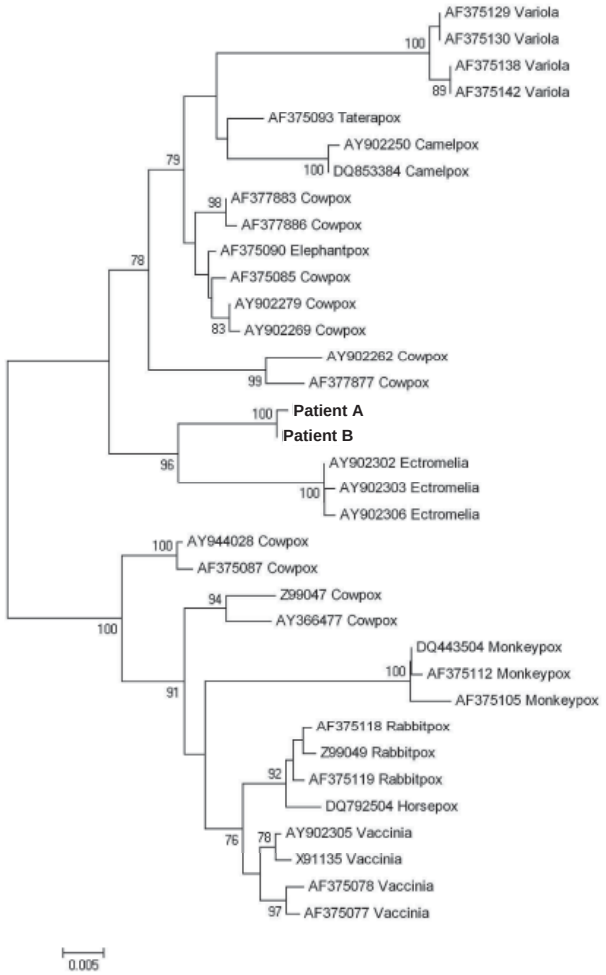

Figure. Phylogenetic tree of nucleotide sequences of the complete hemagglutinin open reading frame (930 bp) of orthopoxviruses (OPVs) isolated from the 2 patients described in this report and additional poxviruses available in GenBank (cowpox: AY902279, AY902269, AF375085, AF377883, AF377886, AY902262, AY944028, Z99047, AY366477, AF377877, AF375087; taterapox: AF375093; camelpox: AY902250, DQ853384; horsepox: DQ792504; elephantpox: AF375090; vaccinia: AY902305, X91135, AF375078, AF375077; rabbitpox: AF375119, AF375118, Z99049; variola: AF375129, AF375130, AF375138, AF375142; ectromelia: AY902302, AY902303, AY902306; monkeypox: DQ443504, AF375105, AF375112). Multiple alignment was generated with ClustalW 1.7 software in BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and the phylogenetic tree was constructed by using maximum-likelihood and neighbor-joining algorithms implemented in Mega 4.0 software (www.megasoftware.net). Bootstrap values >75 are shown at nodes. Location of sequences from the patients form a distinct cluster separated from other OPV sequences. Scale bar indicates genetic distance.

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Pertussis in Afghanistan, 2007–2008

To the Editor: Recent reports have raised concerns about transmission of pertussis among troops stationed in Afghanistan and indicated lack of data about pertussis in this country (*1*,*2*). To fill the knowledge gap about pertussis in Afghanistan, we analyzed data collected by the new Disease Early Warning System (DEWS) during Afghan Year 1386, which corresponds to March 21, 2007–March 20, 2008 (*3*) (Figure).

DEWS collects weekly data on 15 epidemic-prone diseases from 129 sentinel sites, mostly provincial and district hospitals, based on surveillance case definitions of the World Health Organization. The case definition for suspected pertussis is "a person with a cough lasting at least two weeks and having paroxysms of coughing; or inspiratory 'whoop'; or post-tussive vomiting and without other apparent cause." During the study period, 718 cases meeting the surveillance definition for suspected pertussis were recorded in weekly reports from patient visits at DEWS sentinel sites.

When a geographic cluster of at least 5 cases of pertussis is suspected in any area, the DEWS team travels to the area and either confirms that cases meet the clinical definition or rejects the outbreak alert. During outbreak response, DEWS personnel record all cases on a linelist (a rough database), collect throat swabs for laboratory culture, treat case-patients and susceptible contacts (infants and pregnant women) with erythromycin, and schedule follow-up vaccination services for the area. During the study period, the team responded to 56 outbreaks of pertussis in the field that involved 2,233 suspected cases and 32 deaths.

Despite difficult geographic features and lack of infrastructure, DEWS collected and transported 203 specimens from patients with suspected pertussis to the Central Public Health Laboratory in Kabul where *Bordetella pertussis* was successfully isolated in bacteriologic culture from 7 (≈3%) of the specimens. This finding compares with that of a recent study from Spain in which 7% of specimens were microbiologically confirmed (*4*).

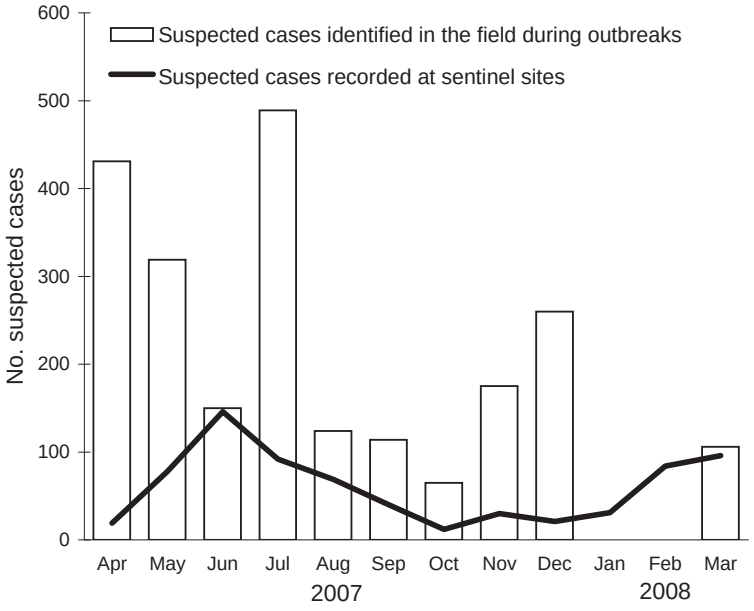

Figure. Suspected pertussis cases recorded at sentinel sites and from outbreaks, Afghanistan, April 2007–March 2008.

In 75% of the outbreak areas, vaccination coverage was <50%, and median age of patients with suspected cases was 4 years. Thus, Afghanistan needs to continue its focus on raising immunization coverage to >90% by administering the primary series of diphtheria-pertussis-tetanus vaccine to infants. This effort will help reduce transmission among infants and young children for whom pertussis is most lethal (*5*).

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Salmonella bongori 48:z_{35}:– in Migratory Birds, Italy

To the Editor: Serovars of *Salmonella*, other than subspecies *enterica*, are primarily associated with cold-blooded animals and infrequently colonize the intestines of warm-blooded animals. Strains of *S. bongori*, in particular, are rarely isolated from human patients (*1*,*2*). However, during 1984–2004, 27 strains of *S. bongori* with the antigenic formula 48:z_{35}:– were identified from 2 epidemic clusters in Italy (Messina, October 1984–May 1985, and Palermo, July–October 1998), and from sporadic cases of acute enteritis that occurred in several cities in Sicily. Almost all cases involved children 1 month to 3 years of age (*3*). By 2006, 8 additional isolates of the same serovar had been collected in southern Italy from the following sources: 1) a healthy human carrier; 2) 3 warm-blooded animals (2 apparently healthy pigeons and a dog with diarrhea); 3) 2 food products (soft cheese and the shell of a hen's egg); and 4) urban wastewater (*3*).

No cases of human infections caused by *S. bongori* 48:z_{35}:– have been reported in countries other than Italy. Except for the original strain isolated from a lizard in Chad in 1966 (*4*), the only recorded isolates of this serovar are 4 isolates from foodstuffs recovered in England in 1985 (M.Y. Popoff, pers. comm.).

Pulsed-field gel electrophoresis (PFGE) analysis of *Xba*I-digested DNA of the 35 isolates obtained from Sicily showed identical or similar profiles that differed from each other by 2–5 bands (>79% similarity), except for the profile obtained from the soft cheese isolate (<40% similarity) (*3*). Thus, all but one of the isolates could be considered closely or at least likely related to the oldest profile characterizing the putative ancestor clone.

Until now, the rare *S. bongori* 48:z_{35}:–, an apparent epidemiologic peculiarity of Sicily, could not have been traced to a well-defined source. Indeed, in past years, many infrequently isolated and new serovars of *Salmonella* have been identified in Sicily from wild reptiles, but *S. bongori* 48:z_{35}:– has never been isolated from these animals (*5*). Moreover, whether pigeons that live in urban areas have epidemiologic importance as sources of infection is questionable, because of the apparently exclusive infection of infants in the first months of life. The role of migratory birds has not previously been assessed.

To determine the prevalence of birdborne pathogens in the migratory bird fauna of the Mediterranean basin, we conducted a study in October 2006, during ringing (banding) activity at the station of the University of Palermo on Ustica, a small island in the Tyrrhenian Sea (38°42′N, 13°11′E) near the northern coast of Sicily. Apparently healthy birds trapped by mist nets during active migration were sampled. Fresh fecal samples or cloacal swabs from 239 birds belonging to the orders Passeriformes, Gruiformes, and Caprimulgiformes were analyzed to determine whether birds were colonized with *Salmonella*. Routine procedures for isolation of *Salmonella* spp. were used. Colonies with morphologic characteristics of *Salmonella* spp. were fully identified by standard biochemical and serologic testing.

Two isolates, from 2 blackcaps (*Sylvia atricapilla*), were identified as *Salmonella* spp. Morphologic features of the external flight apparatus and weight clearly indicated that the 2 individuals belonged to the migratory subspecies of blackcap (*6*).

The *Salmonella* isolates were characterized by serotyping at the Centre for Enteric Pathogens of Southern Italy, University of Palermo, as *S. bongori* 48:z35:–. Molecular typing by PFGE after digestion of DNA by *Xba*I showed a banding pattern similar to that of all previously identified *S. bongori* 48:z35:–. In particular, the DNA restriction pattern proved to be indistinguishable from patterns of the human isolates belonging to the epidemic clusters and of the pigeon isolates (*3*).

Previous studies have documented that carriage of *Salmonella* spp. by apparently healthy migrating birds is infrequent, although some isolates have been recovered (*7*,*8*). Prevalence has been shown to be higher during breeding season and during wintering in some urban-associated bird species, especially in those that feed on refuse, such as corvids and gulls (*9*). However, to our knowledge, *S. bongori* in migratory birds has not previously been reported.

Our findings suggest that passerine migratory birds may play a role in the introduction or persistence of *S. bongori* 48:z35 in southern Italy. The bacteria also have the potential for gaining access to the food chain, as confirmed by their presence in the shell of hen eggs. Moreover, a bird-environment-food network could perpetuate a reservoir of *S. bongori* 48:z35:–. Most small passerine migratory birds, including blackcaps, do not share a niche with humans and are most likely to be found in rural habitats (*10*). However, some species are developing an increasing ability to live in urban and suburban environments, especially where winter feeding by humans attracts birds, forcing changes in the species balance. Because of their ability to fly through long distances during annual migrations, wild birds could also play a role in the epidemiology of zoonoses. Thus, risk assessment of *Salmonella* carriage in wild birds warrants further investigation.

Acknowledgments

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Comet Sign (and Other) in *Pyemotes* Dermatitis

To the Editor: Recently, Pascal Del Giudice et al. published an interesting article (*1*) about dermatitis in France caused by *Pyemotes ventricosus* in which they highlight the presence of the comet sign in a number of their patients. It is, they assert, a sign that because of its peculiarity could be useful for diagnosing this type of dermatitis in outbreaks and sporadic cases.

Some years ago, we studied 3 outbreaks (with >100 cases) of dermatitis caused by *P. ventricosus* parasitic mites in Castellón, Spain, produced by different infected materials (*2*). When we published the results, we concentrated on the epidemiologic characteristics and the discovery of the mite; perhaps we paid too little attention to the appearance of the lesions, of which we did not provide images. Nevertheless, we also observed the descriptions by Del Giudice et al., which we now show in the Figure. In 2 patients (Figure, panels A and B), the comet sign can be clearly assessed; the patients were 2 women who had had direct contact with the infected material against their legs. The other patient (Figure, panel C) displayed 56 macules with 1 pruritic central vesicle. We did not observe facial lesions on any of the case-

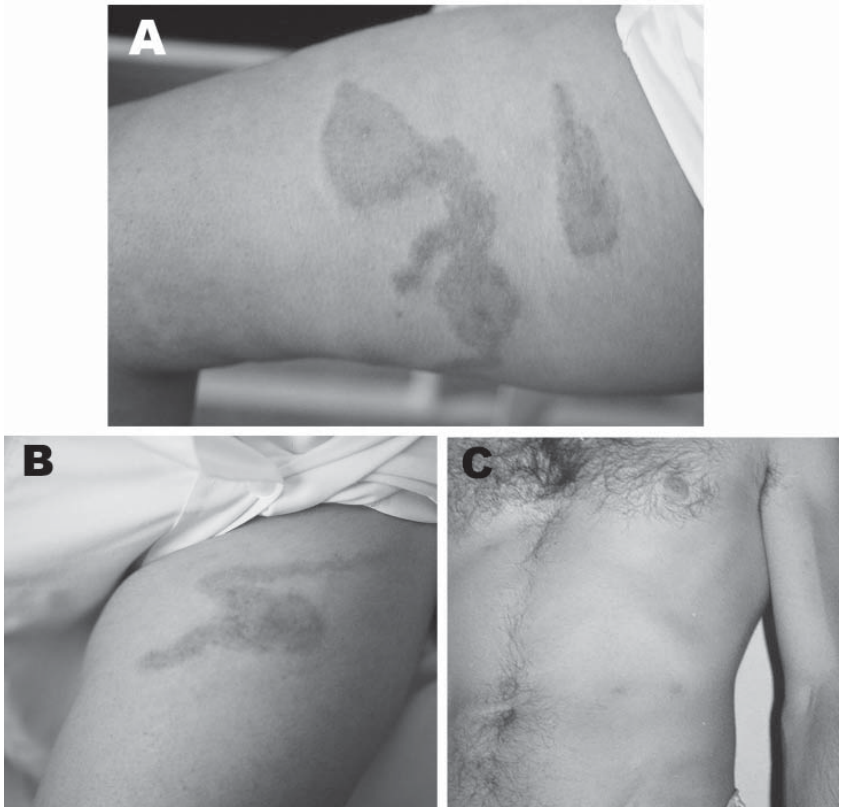

Figure. Photographs of 3 persons with skin lesions of *Pyemotes* dermatitis during the same outbreak in Castellón, Spain, showing the comet sign in 2 affected women (panels A, B), and macular form of the lesions in 1 of the affected investigators (panel C).

patients (but we did observe lesions on the necks of some patients).

Our data coincided with those of the French study and reinforce the specificity of this dermatologic sign. However, this was not the only coincidence; cases also occurred among the investigators after contact with the infected material in each of the outbreaks. Perhaps both signs may characteristic this dermatitis: the comet sign and "the sign of the infected investigators" of the outbreaks.

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Helicobacter pylori Infection in Patients Undergoing Upper Endoscopy, Republic of Georgia

To the Editor: *Helicobacter pylori* infection is the principal cause of chronic active gastritis and peptic ulcer disease and a major contributor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (*1*). Approximately 50% of the world's population is infected (*2*), but only 10%–20% of infected persons become symptomatic (*3*). The annual incidence rate of *H. pylori* infection is ≈4%–15% in developing countries, compared with ≈0.5% in industrialized countries (*4*). Studies in the Republic of Georgia (ROG), a developing country with an economy in transition, suggested that >70% of adults are infected with *H. pylori* (*5*,*6*) and that the prevalence rate of gastric cancer is 18 cases per 100,000 population, ≈6- to 9-fold higher than in the United States (National Center for Disease Control, Tbilisi, ROG, unpub. data, 2003). We investigated the prevalence of infection in patients in ROG in whom gastritis, peptic ulcer, and gastric cancer had been diagnosed.

We performed a cross-sectional study of patients referred for upper endoscopy from all regions of ROG to 23 tertiary-care medical centers in the capital, Tbilisi, during 2003–2005. Patients whose medical records and gastric biopsy slides were available were eligible for inclusion. Two pathologists reviewed hematoxylin and eosin–stained slides prepared from formalin-fixed, paraffin-embedded gastric biopsy specimens. Pathologists graded the amounts of *H. pylori*, acute and chronic inflammation, intestinal metaplasia, and atrophy according to the visual analogue scale of the Updated Sydney Classification System for Gastritis (*7*). Histologic characteristics were dichotomized as presence (grades ≥1) or absence (grade = 0) of a feature.

We conducted statistical analyses in SAS version 9.0 (SAS Institute, Inc., Cary, NC, USA). The human subjects committees at the National Center for Disease Control and Medical Statistics of ROG and the Centers for Disease Control and Prevention (Atlanta, GA, USA) approved the study.

We identified 90 eligible persons. Their median age was 62 years (range 6–81 years); 48 (54%) were male. Biopsy specimens were taken from the antrum in 89 (99%) persons and from the corpus in 1 person. *H. pylori* infection was diagnosed in 59 (72%) persons, acute inflammation in 81 (90%), chronic inflammation in 77 (87%), metaplasia in 29 (35%), and atrophy in 11 (16%). *H. pylori* was detected in 78% of patients who had gastritis, in 58% of patients who had peptic ulcer, and in 58% of patients who had dysplasia or gastric cancer (Table).

In a multivariable Poisson regression model, *H. pylori* positivity was strongly associated with acute inflammation (adjusted prevalence ratio [aPR] 1.4, 95% confidence interval [CI] 1.2–1.8) and chronic inflammation (aPR 1.5, 95% CI 1.2–1.9). Age ≥50 years (aPR 0.9, 95% CI 0.8–1.2) and male sex (aPR 1.0, 95% CI 0.9–1.2) did not confer increased risk for *H. pylori* infection.

H. pylori requires gastric mucus for growth, and mucus produced by the metaplastic and neoplastic cells is postulated to lack characteristics that sustain growth of *H. pylori*. When *H. pylori* has been observed in patients with ulcers, intestinal metaplasia, and adenocarcinoma, the bacteria usually are present in areas of the stomach that do not have these lesions.

In this cohort of patients, 14 (16%) had dysplasia or adenocarcinoma. Dysplasia and eventually cancer occur in a small group of susceptible persons with atrophy and intestinal metaplasia (*8*,*9*). Thus, *H. pylori* is now considered a type-1 carcinogen, and the ab-

Table. Histopathologic characteristics assessed in the biopsy specimens of study participants, by final pathologic diagnosis, Republic of Georgia, 2003–2005

Final pathologic diagnosis	No. *Heliobacter pylori* positive/no. tested (%), n = 82	No. with acute inflammation/no. tested (%), n = 90	No. with chronic inflammation/no. tested (%), n = 89	No. with intestinal metaplasia/no. tested (%), n = 84	No. with glandular atrophy/no. tested (%), n = 68
Gastritis* (n = 62)	45/58 (78)	55/62 (89)	55/62 (89)	19/60 (32)	10/50 (20)
Peptic ulcer (n = 14)	7/12 (58)	14/14 (100)	12/13 (92)	5/13 (38)	1/10 (10)
Dysplasia or cancer (n = 14)	7/12 (58)	12/14 (86)	10/14 (71)	5/11 (45)	0/8 (0)
Total (n = 90)	59/82 (72)	81/90 (90)	77/89 (87)	29/84 (35)	11/68 (16)

*According to the Updated Sydney Classification System for Gastritis (*7*).

sence of infection is predicted to result in at least 60% fewer cases of gastric cancer worldwide (*10*).

Our study has several limitations. First, because of the study's retrospective nature, pathologists reviewed archived hematoxylin and eosin–stained sections of biopsy specimens. The inability to use additional techniques (e.g., special staining, immunohistochemistry) commonly used to visualize *H. pylori* might have resulted in underdetection of the true rate of infection in the study population. Second, in 99% of cases, only 1 biopsy specimen was obtained from the antrum instead of the 5 antrum and corpus biopsy specimens recommended for appropriate diagnosis by the Updated Sydney Classification System (*7*). Third, the relatively small sample size precluded exploration of additional relationships between demographic characteristics and histologic grades.

Our results indicate a high prevalence of *H. pylori* in patients with gastritis, peptic ulcer, and gastric cancer and suggest that *H. pylori* infection represents a serious public health problem in ROG. Studies are needed to explore demographic, socioeconomic, and behavioral risk factors that contribute to the high prevalence of *H. pylori* infection in symptomatic and asymptomatic persons living in ROG so that preventive measures can be identified.

This study was supported by the US Department of Health and Human Services' Biotechnology Engagement Program no. G-1195. The funding source played no role in designing the study; collecting, analyzing, or interpreting the data; writing the report; or deciding to submit it for publication.

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Spelling *Pneumocystis jirovecii*

To the Editor: Our 2002 article in Emerging Infectious Diseases about nomenclature changes for organisms in the genus *Pneumocystis* (*1*) has been widely cited and probably will remain a source for persons seeking information about this subject. Therefore, we need to correct an error in 1 of the species names presented in our article and in the 1999 article by Frenkel (*2*) on which our article was based. In the 1999 article, Frenkel proposed that the species of *Pneumocystis* found in humans be named to honor the Czech parasitologist, Otto Jirovec. The 1999 article was his second proposal for this change. In 1976, he first named the human pathogen *Pneumocystis jiroveci* (*3*), at which time it was classified as a protozoan and therefore named according to the International Code of Zoological Nomenclature. By 1999, it had become clear that the organisms in the genus *Pneumocystis* are fungi, which are named according to the International Code of Botanical Nomenclature (ICBN) (*4*). Differences between the International Code of Zoological Nomenclature and ICBN resulted in the realization of an error in the species epithet proposed by Frenkel in 1999, and our 2002 article contained this error. Frenkel's 1999 article should have modified the species epithet from "jiroveci" to "jirovecii," (ICBN Articles 32.7 and 60.11 and Rec. 60C.1b). The correct and valid name under ICBN is *Pneumocystis jirovecii*. Redhead et al. further explain the basis for this correction (*5*).

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Hypothetical *Pneumocystis jirovecii* Transmission from Immunocompetent Carriers to Infant

To the Editor: The recent dispatch article by Rivero et al. reports the transmission of *Pneumocystis jirovecii* from immunocompetent grandparents to their granddaughter (*1*). The authors' conclusion was based on 2 facts: the grandparents were carriers but neither the parents nor the child's brother was a carrier, and the *P. jirovecii* genotype observed in the grandparents was identical to that found in the infant. In our opinion, the data provided by the authors do not support the conclusion that transmission has occurred. First, the 2 markers used for typing show a small number of alleles and thus provide low discrimination among isolates (*2*). Consequently, the *P. jirovecii* isolates present in the grandparents and in the infant may have been epidemiologically unrelated. Second, the frequency of occurrence of the different genotypes obtained was not investigated. The presence of the same genotype in the grandparents and in the infant may result from a high frequency of this genotype in the geographic area where the family lived. In fact, the use of a validated typing method and the analysis of unlinked control patients have proven necessary in other studies to demonstrate transmission of *P. jirovecii* (*3–7*). We believe that the reported transmission event remains a hypothesis.

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In Response: We appreciate the comments from Hauser et al. (*1*) regarding our article that reported a case of *Pneumocystis jirovecii* transmission from colonized grandparents to their infant granddaughter (*2*). We agree with the authors that the 2 markers used for typing, which are described in our article, present a small number of alleles and thus provide low discrimination between isolates. However, these typing methods have been validated and have proven useful for molecular epidemiologic applications in *P. jirovecii* colonization studies (*3*,*4*). Unfortunately, other typing methods that can identify a high number of alleles, such as the sequence analysis of the internal transcribed spacer no. 1 and 2 gene regions, could not be used in our study because a low amplification rate has been observed for these regions when such methods are used to study colonized subjects (*5*). On the other hand, the multitarget single-strand conformation polymorphism method has been used only in patients with *Pneumocystis* pneumonia, and its usefulness for epidemiologic studies in colonized subjects has not been proven (*6*). For our study, we think that genotyping analysis of the mtLSU rRNA gene together with the dihydropteroate synthase (DHPS) gene provided sufficient epidemiologic information because this strategy allows identification of 24 different combinations of genotypes. However, no typing method is able to demonstrate interhuman *P. jirovecii* transmission conclusively because a common environmental source of infection cannot be ruled out in any case. Therefore, as we noted in our article, "We cannot exclude the possibility that the cases described were infected by the same environmental source," and we only hypothesized that "the infant was infected by *P. jirovecii* through close contact with her grandparents." However, we continue to think that the airborne transmission of *P. jirovecii* from the grandfather to the grandmother and the infant is the most probable explanation based on genotype data. Also, all persons in close contact with the infant were studied, and only her grandparents were colonized by *P. jirovecii*. Future research is needed to assess the importance of colonized subjects in the *P. jirovecii* transmission to susceptible hosts.

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Delinquent Mortgages, Neglected Swimming Pools, and West Nile Virus, California

To the Editor: Reisen et al. illustrated the potential relationship between environmental developments, such as those that result from major economic events, and increased risks for infectious diseases (*1*). The Institute of Medicine landmark report in 1992 on emerging pathogens (*2*) and the initiative of the Centers for Disease Control and Prevention in 1994 (which helped give rise to the Emerging Infectious Diseases journal) note the role of environmental phenomena in spawning some infectious diseases.

The Reisen et al. report also reflects the utility and limitations of ecologic studies and the legitimacy of simultaneously concluding that study findings are "hypothesis generating" from a scientific perspective yet sufficiently plausible to prompt public health interventions from a practical perspective. The report demonstrates the value of synthesizing multiple streams of surveillance data, observations from field investigations, and contextual awareness of community events to generate hypotheses that bear exploration—in this case, the hypothesis that increases in mosquito habitats resulting from abandonment of swimming pools cause increases in the incidence of West Nile virus (WNV) cases. Scientifically, this question was not definitively answered because the study could not directly assess the putative link between disease and exposures to WNV-infected mosquitoes that had bred in abandoned swimming pools in California.

Beyond a priori knowledge that abandoned swimming pools provide opportunities for mosquito breeding (and other hazards, such as drowning), this report raises other critically important questions, such as what action should be taken in response to such findings and how should the findings be communicated to the public? Practically, questions about the link between abandoned swimming pools and illness were sufficiently answered so that public and environmental health officials could respond, as manifested by Kern County's West Nile Virus Strategic Response Plan (*3*) and related strategies, including the Fight the Bite campaign, which calls for reporting of neglected swimming pools (*4*). Knowledge regarding the study's limitations about causality should not detract from these efforts, as has been recognized by other sectors, such as the mortgage industry (*5*).

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In Response: We thank Goodman and Buehler for their comments about our recent article drawing attention to linkages among neglected swimming pools, vector mosquito production, and West Nile virus (WNV) transmission (*1*). We agree with their comment that our "study could not directly assess the putative link between disease and exposures to WNV-infected mosquitoes that had bred in abandoned swimming pools in California." However, outbreaks of pathogens with complicated transmission cycles rarely afford isolation of any one factor for analysis. Notably, an update of events in Kern County during 2008 may support our previous conclusions. As in 2007, Kern County experienced continued drought conditions in 2008, and mosquito production was again confined largely to urban sources, including swimming pools. Avian abundance was low, and seroprevalence for WNV among selected bird species was similar to seroprevalence rates for 2007 (i.e., 7.8% of 192 house finches, 5.6% of 90 house sparrows, and 41.2% of 68 western scrub-jays were seropositive in 2007). However, early in the spring of 2008, unlike in 2007, the Kern Mosquito and Vector

Control District (MVCD) commenced aerial surveys, reassigned rural field crews to treat swimming pools (and other urban water sources), and immediately conducted ground-based adulticiding when surveillance detected enzootic activity. This aggressive approach resulted in 2,182 swimming pools treated (a 255% increase over the number treated in 2007), reduced enzootic transmission (i.e., 5% of 192 dead birds, 17 serum samples from 90 sentinel chickens, and 1% of 598 mosquito pools positive for WNV), and almost no evidence of human infection (only 1 case reported in October). Intervention by the Kern MVCD, coupled with continuing severe drought conditions that eliminated natural and agricultural-related mosquito production sites, effectively eliminated WNV epidemic transmission that had persisted during the 4 previous years (*2*).

Our study was supported by National Institutes of Health grant R01-AI55607 and the Centers for Disease Control and Prevention.

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Avian Influenza

Hans-Dieter Klenk, Mikhail N. Matrosovich, and Jürgen Stech, editors

Monographs in Virology, vol. 27, S. Karger AG, Basel, Switzerland, 2008

ISBN-10: 3805585012
ISBN-13: 978-3-8055-8501-9
Pages: 292; Price: US $249

Avian influenza, caused by influenza virus A (H5N1), continues to be a source of outbreaks among avian species and of sporadic human cases that result in a high case-fatality rate. These historically unprecedented outbreaks have raised serious global concerns for both animal health and human health. Significant progress in the research of avian influenza has occurred in the past decade, but unanswered questions remain. How does avian influenza cross species barriers and acquire transmissibility among humans? How can we minimize the risk of emergence of a pandemic virus? Will subtype H5N1 maintain its virulence in humans when it becomes a pandemic virus? This book helps readers understand what is known and what remains to be known about avian influenza.

The book contains 19 articles written by leaders in avian influenza research. The authors provide a comprehensive and updated review of current knowledge on avian influenza, with particular emphasis on H5N1. The articles cover various aspects of avian influenza, including its epidemiology and ecology as well as control strategies for potential outbreaks of avian influenza in Asia and Europe. Some articles describe the molecular mechanisms of interspecies transmission and virulence in birds and humans. Both interspecies transmission and virulence are determined by many molecular changes in different genes, but the mechanisms for interspecies transmission and virulence are not completely understood. Other articles address timely and important issues such as vaccine development and antiviral resistance.

All pandemic influenza viruses in humans originated from avian influenza viruses. Understanding how an avian virus can become a pandemic virus that causes devastating effects on human health is critical. This book is a valuable reference for scientists and public health specialists who work in either animal health or human health.

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Forgotten People, Forgotten Diseases: The Neglected Tropical Diseases and Their Impact on Global Health and Development

Peter J. Hotez

American Society for Microbiology Press, Washington, DC, USA, 2008

ISBN: 978-1555814403
Pages: 215; Price: US $39.95

Forgotten People, Forgotten Diseases is an interesting and highly informative book about the global status of neglected tropical diseases (NTDs). Author Peter Hotez introduces NTDs by describing them in general, their historical importance and global impact, and their shared characteristics. According to Hotez, NTDs are among the most common infections from antiquity and occur in the world's poorest people. Their distribution and health and economic effects are similar to those of AIDS, malaria, and tuberculosis. NTDs, however, are much less well known than these diseases and frequently display high rates of illness but few deaths, promote poverty, and create profound social stigma.

Twelve well-illustrated chapters address the important NTDs, including soil-transmitted helminth infections, schistosomiasis, filariasis, onchocerciasis, trachoma, mycobacterial infections, trypanosomiasis, leishmaniasis, dengue, leptospirosis, and rabies. Hotez discusses what these diseases are, where they occur, and who they affect. The final chapters focus on prospects to prevent and control NTDs and the need for additional advocacy. Hotez emphasizes the need for new safer and more effective drugs, as well as for so-called "anti-poverty vaccines," which by promoting health will open doors to economic advancement and stability, goals that have been all but impossible in developing countries, largely because of NTDs.

Few people are more qualified to write such a book than Hotez, president of the Sabin Vaccine Institute and a pioneer in hookworm molecular genetics, physiology, immunology, and pathogenesis. This easy-to-read and up-to-date text undoubtedly will prove useful to graduate students, volunteers, advocates, healthcare professionals, and others interested in global health and equality. Forgotten People, Forgotten Diseases is an essential read for every serious student of tropical medicine and global infectious diseases.

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Travel Medicine, 2nd Edition

Jay S. Keystone, Phyllis E. Kozarsky, David O. Freedman, Hans D. Nothdurft, and Bradley A. Connor, editors

Elsevier, Philadelphia, Pennsylvania, USA, 2008

ISBN: 978-0-323-03453-1
Pages: 640; Price: US $169

The second edition of this valuable textbook highlights a dynamic specialty that integrates many disciplines. Edited by 5 internationally renowned leaders in travel medicine, the book assembles a fine group of authors and chapters. The print version is hard cover and comes with online access that is easy to use and fully searchable. Both versions include useful maps, tables, and figures, which can be exported from the online version to PowerPoint presentations to assist in teaching. The online version also links to abstracts of references, allowing readers easy access to the abstracts simply by clicking on linked references.

The book consists of 57 well-organized chapters that begin with fundamental topics such as epidemiology, travel clinic management and resources, and basic disease prevention. The chapters then progress to more specialized topics such as special hosts (e.g., immunocompromised travelers), special itineraries (e.g., expatriates and persons on expeditions), health problems while traveling, and posttravel care. This edition also features a new chapter on cruise ship travel. The chapters vary greatly in length and content: chapters on jet lag and sun-associated problems are brief; chapters on topics such as immunization of healthy adults are detailed and lengthy (i.e., 36 pages). The immunization chapter covers a large amount of material that is challenging because it addresses international variations in vaccine licensure and guidelines. For instance, tick-borne encephalitis vaccine and cholera vaccine, which are described in this chapter, are available in Europe but not in the United States. However, the global nature of travel medicine necessitates practitioners' knowledge of these vaccines.

As the field of travel medicine matures and its evidence base increases, topics such as environmental aspects of travel, psychological aspects of travel, and travel injuries are gaining greater recognition. If a new edition is considered, additional topics that would be helpful to clinicians include travelers' precautions for eye care (especially regarding certain environmental exposures), breastfeeding among travelers (currently an abbreviated section in a chapter that discusses pregnant travelers), and collaboration among practitioners of travel medicine and public health professionals. Specific policies and procedures for travel clinic management could also address the triage of posttravel illnesses.

This book is a comprehensive reference on travel medicine. It is rich in information, pleasant to read, and practical. It has been updated to recommend current best practices in travel medicine. The editors have assembled an excellent textbook, and I recommend it enthusiastically to health professionals interested in this growing specialty.

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Fantastic Voyage: Influenza

Julian W. Tang

Time to go! Time to go!
An influenza virus,
Hiding in saliva, buried in a cell,
Antibodies and T-cells are coming.
Get out! But how?
Induce a sneeze? Kick-start a cough?
Ah yes, here we go …
Aahhh-choo! Freedom at last!

Where are we?
A quick look around
A hospital? I see children,
Very thin, sick children,
Must be the cancer ward.
No B cells or T cells—a virus paradise!
Let's travel, find a breeze and float.
Where's my next victim?

My previous young doctor host
Walking quickly away
Head down, embarrassed
Scolded by a nurse—where was his mask?
Too busy and careless, poor fool
He still serves me well, dragging me into his wake.
Here I go, following and floating
A door nearby opens—negative pressure!

In I go, but on a cancer ward,
This should be positive pressure!
To keep bugs out, not draw them in.
I cannot complain
All good for me, for now I can see
A young girl with leukemia
Sitting in bed, watching TV
Laughing, inhaling, bringing me close.

But wait, what's this? Her mother!
Opening the toilet door,
Even greater negative pressure in there.
And worse, wet surfaces glistening inside,
Cleaned by her mother with chlorhexidine.
No! Not yet! Not now! I'm so close!
Being pulled in. No escape. Falling, falling …
The young girl is laughing. Now her mother is too.

Dr Tang is a clinical and academic virologist with a special interest in aerosol-transmitted viral infections, particularly influenza. His research interests include viral molecular epidemiology and investigations into the factors involved in the aerosol transmission of viral infections.

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Liubov Popova (1889–1924) The Traveler (1915). Oil on canvas (142.2 cm × 105.4 cm)
Courtesy of Norton Simon Art Foundation, Pasadena, CA, USA

Tango with Cows

Polyxeni Potter

Farm animals engaged in a sophisticated dance is how poet Vasily Kamensky represented the incongruous entanglement between Russia's rural past and sweeping modernism. In his daring book Tango with Cows, he abandoned syntax for a spatial arrangement of words on old wallpaper to explore visual poetry. Political oppression, industrial development, and rapid urbanization between the revolutions of 1905 and 1917 shook the foundation of society and promoted experimentation in literature, music, and art. Part of sprouting radical movements known as Russian avant-garde, Liubov Popova made her mark as a leading artist of the 20th century.

Popova was born near Moscow into an affluent family approving of her talent. She traveled widely, within Russia for the architecture and hagiography and abroad to Italy and France. She studied Cubism at the Académie de la Palette under Henri Le Fauconnier and Jean Metzinger. While she admired Giotto and other masters of the Renaissance, her work moved steadily away from naturalism toward a personal style drawn from the flat linearity of Russian icons, the principles of Cubism, and revolutionary ideas. "Representation of reality—without artistic deformation and transformation—cannot be the subject of painting," she wrote.

In its origins with Picasso and Braque, Cubism was a formal style applied to traditional subjects to depict space and volume through multiple viewpoints and shifting planes. With time, others saw in its geometric precision the potential to capture modern life and its increasing reliance on machines. In Italy, a group called the Futurists used it to express in art what Albert Einstein defined in 1905 in his theory of relativity, a new sense of time, space, and energy. "We wish to exalt aggressive movement," read the Futurist manifesto, "feverish insomnia, running, the perilous leap, the cuff, and the blow." From her travels, Popova brought home these influences, which she integrated with folk and decorative elements in shaping the development of combined Cubism and Futurism in Russia.

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She joined major art studios and worked with Vladimir Tatlin, advocate of constructivism: the exploration of geometric form in two and three dimensions, not for art's sake but as service to society. She became increasingly devoted to abstraction, and in 1916 she joined the Supremus group, organized by Kazimir Malevich: "The artist has rid himself of everything which pre-decided the objective ideal structure of life and 'art,' " he wrote, "He has freed himself from ideas, concepts and representations in order to listen only to pure sensibility."

Popova turned exclusively to dynamic geometric forms and experimented with texture, rhythm, density, and color in works she called "painterly architectonics." Unlike the painters of European Cubism and Futurism, who never abandoned recognizable form, she was able to develop a completely nonrepresentational idiom through layered panels of color.

The "Artist Builder," as she became known, proposed that "Form transformed is abstract and finds itself totally subject to architectonic requirements, as well as to the intentions of the artist, who attains complete freedom in total abstraction, in the distribution and construction of lines, surfaces, volumetric elements and chromatic values." Popova participated in many exhibitions and became very successful.

In 1921, she joined other artists in rejecting studio painting in favor of industrial design: textile, book, porcelain, ceramic, theater set. As a designer of women's fabrics at the First State Cotton-Printing factory, she was called upon to "unite the demands of economics, the laws of exterior design, and the mysterious taste of the peasant woman from Tula," a task she reportedly did not resent, "Not one of her artistic successes ever gave her such deep satisfaction as the sight of a peasant woman and a worker buying lengths of her material."

Popova's precipitous rise to artistic prominence was marred by infectious disease catastrophes. Her husband of 1 year, an art historian, died of typhoid fever. Infected herself, she survived but only briefly. She died at age 35 of scarlet fever caught from her son, who died days before she did. Her untimely demise cut short a brilliant artistic career. One obituary read, "This spring, the women of Moscow … the cooks, the service workers—began dressing themselves up. Instead of the former petite bourgeois little flowers, there appeared on the fabrics new and unexpected strong and clear patterns."

The Traveler, on this month's cover, was painted when Popova was committed to abstraction but still maintained in her work recognizable forms. At first glance, the image appears a jumble of planes, triangles, cylinders, and semicircles arranged aggressively across the canvas to the very edge. But a closer look yields clues to an image possibly shattered and reconstructed from its fragments.

At the center of the composition, a yellow necklace draws the eye to a hidden female form. Nearby, a collar follows the curve of a cape against a cochleated armrest. The neck, head, and part of a hat are discernible. A green umbrella, firmly clutched, takes front center, its generous flaps against the passenger's legs and feet below. The seated figure delineated, the viewer can make out passing scenery: a glimpse of railing, a flag, some green. Letters are stenciled over the image forming shop signs and guideposts: "dangerous zone," "…magazines," "natural gas."

Movement is achieved by overlapped planes denoting rapid succession. Shapes, tilted and angled into each other, are shaded and textured for depth and motion. Traveler and surroundings are one, gliding seamlessly in time and space.

Part and parcel of her tumultuous times, Popova recaptured in this painting not just the fragments of a broken image but also revolutionary concepts vital to science and public health. Her traveler, so directly connected with everything, carries with her everything, wherever she goes. And as she moves elegantly from place to place, she changes as does the landscape. She is faster or slower. She picks up things from one place and deposits them in another, ambivalent about past, present, or future.

Just as the rapid influx of technology produced radical art movements, an explosion of travel around the world has irrevocably globalized everything, dragging the rural cow into the metropolitan area to tango. The close meeting of different worlds, back and forth, from country to country and countryside to city, is making the old from the old environment ripe for emergence of the new in a new environment.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

The Status of Infectious Diseases in the Amazon Region

Oseltamivir-Resistant Influenza Virus A (H1N1) in Europe, 2007–08 Influenza Season

Exotic Small Mammals as Potential Reservoirs of Zoonotic *Bartonella* Species

Characterization of *Streptococcus pneumoniae* from Children with Acute Otitis Media

Animal Reservoir Hosts for Fish-borne Zoonotic Trematode Infections on Fish Farms

Enhancing Time Series Detection Algorithms for Automated Biosurveillance

Human Febrile Illness Caused by Encephalomyocarditis Virus Infection, Peru

Experimental Infection of Potential Reservoir Hosts with Venezuelan Equine Encephalitis Virus, Mexico

Hantavirus Pulmonary Syndrome, Brazil

Conjunctivitis with Episcleritis and Anterior Uveitis Linked to Adiaspiromycosis and Freshwater Sponges

Rapid Diagnostic Test for Syphilis in High-Risk Populations, Manaus, Brazil

Links between Climate and Malaria in Amazon Region

Simultaneous Chikungunya and Dengue Outbreaks, Gabon, 2007

Rift Valley Fever, Mayotte, France, 2007–2008

Complete list of articles in the April issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 2–3, 2009
Exploring the Dynamic Relationship Between Health and the Environment
The Center for Biodiversity and Conservation Milstein Science Symposium
American Museum of Natural History
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http://cbc.amnh.org/health
cbcsymposium@amnh.org

April 27–29, 2009
Twelfth Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/vaccine09

June 18–21, 2009
26th International Congress of Chemotherapy and Infection
Sheraton Centre Toronto Hotel
Toronto, Canada
http://www.icc-09.com

June 28–July 1, 2009
18th ISSTDR: International Society for STD Research
QEII Conference Centre
London, United Kingdom
http://www.isstdrlondon2009.com

August 10–21, 2009
11th International Dengue Course
http://www.ipk.sld.cu/cursos/dengue2009/indexen.htm

August 29–September 2, 2009
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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Clinical Risk Factors for Severe *Clostridium difficile*–associated Disease

CME Questions

1. All of the following were criteria for severe *Clostridium difficile*–associated disease (CDAD) in the current study, except:

A. One or more intensive care unit admissions in which *C. difficile* was a major contributor
B. Prolonged symptoms past 14 days requiring intravenous fluid replacement
C. Colectomy or other surgery directly attributed to *C. difficile*
D. Intestinal perforation in the setting of *C. difficile* infection

2. What was the prevalence of severe CDAD among all of the cases of CDAD in the current study?

A. <1%
B. 12%
C. 29%
D. 44%

3. Which of the following patient factors was most associated with an increased risk for severe CDAD on multivariate analysis of the current study?

A. Age >70 years
B. Chemotherapy use
C. Antimicrobial use
D. Previous hospital stay

4. All of the following laboratory factors were predictive of an increased risk for CDAD in the current study, except:

A. White blood cell count >20,000 cells/mL
B. Serum albumin <2.5 g/dL
C. Creatinine >2 mg/dL
D. Alanine aminotransferase >40 U/L

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

February 2009

Respiratory Infections

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 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 (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, 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. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

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. 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. 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. 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 a 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. 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.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online 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.