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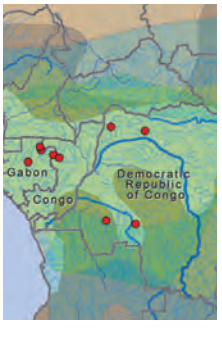

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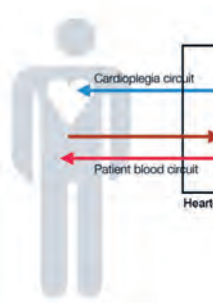

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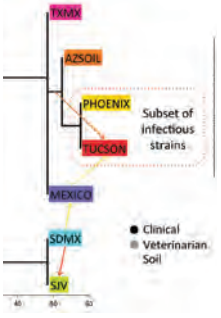

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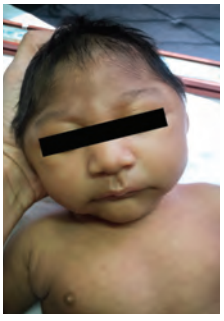

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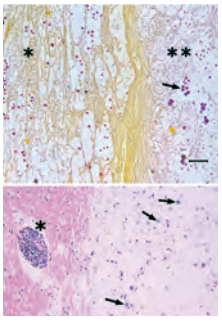

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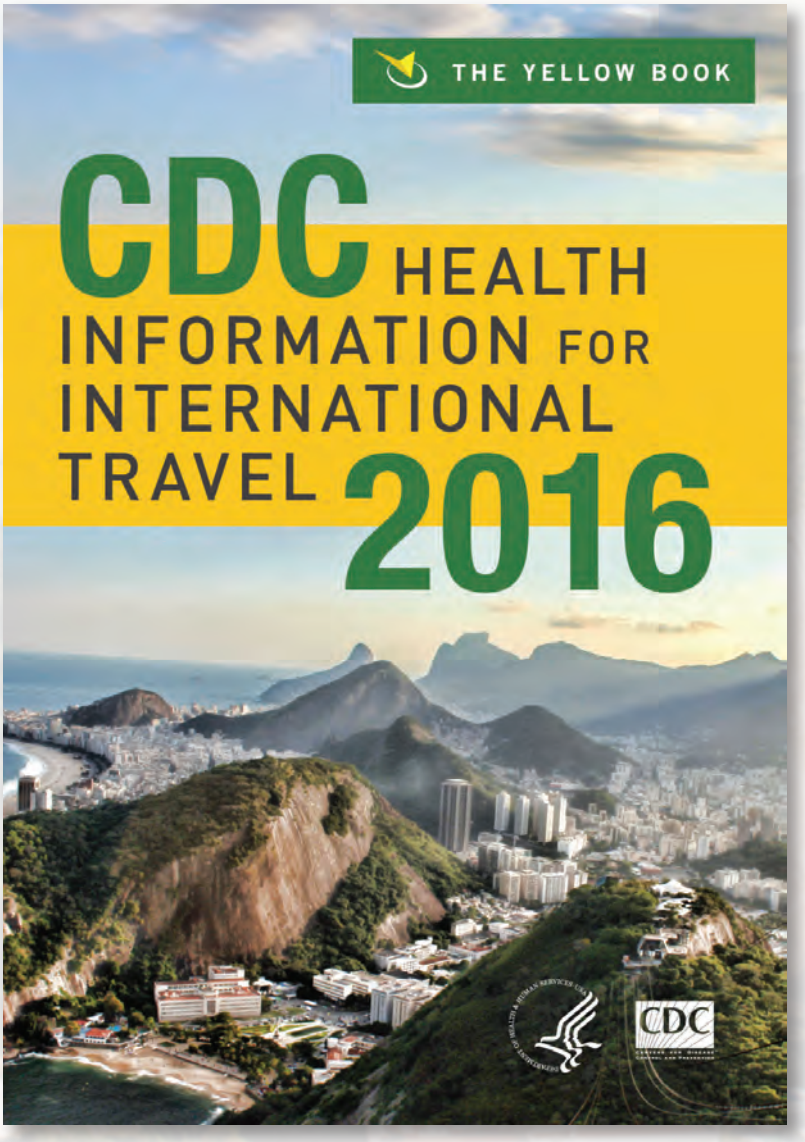

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Debate Regarding Oseltamivir Use for Seasonal and Pandemic Influenza

Aeron C. Hurt, Heath Kelly

A debate about the market-leading influenza antiviral medication, oseltamivir, which initially focused on treatment for generally mild illness, has been expanded to question the wisdom of stockpiling for use in future influenza pandemics. Although randomized controlled trial evidence confirms that oseltamivir will reduce symptom duration by 17–25 hours among otherwise healthy adolescents and adults with community-managed disease, no randomized controlled trials have examined the effectiveness of oseltamivir against more serious outcomes. Observational studies, although criticized on methodologic grounds, suggest that oseltamivir given early can reduce the risk for death by half among persons hospitalized with confirmed infection caused by influenza A(H1N1)pdm09 and influenza A(H5N1) viruses. However, available randomized controlled trial data may not be able to capture the effect of oseltamivir use among hospitalized patients with severe disease. We assert that data on outpatients with relatively mild disease should not form the basis for policies on the management of more severe disease.

A lively, and sometimes heated, debate has recently been conducted in the popular press (*1*) and medical literature (*2*–*4*) about the effectiveness of oseltamivir, its usefulness in treating seasonal influenza, and the need for it to be stockpiled for use in a future influenza pandemic. Oseltamivir, manufactured by F. Hoffmann-La Roche (Roche) (Indianapolis, IN, USA), is the market leader of the neuraminidase inhibitors (NAI), the first class of antiviral drugs designed specifically to treat influenza.

Oseltamivir came on the market in many countries in 2000 after clinical studies had been conducted among influenza virus–infected patients with uncomplicated illness. Trial data from outpatient studies have been summarized in a recent meta-analysis of individual patient data, including published and unpublished studies, which confirms that oseltamivir will reduce the duration of symptomatic laboratory-confirmed influenza in otherwise healthy adults from 5 days to 4 days (*2*), a result consistent with those of a previous systematic review by the Cochrane group (*3*).

After the drug's market release, oseltamivir use for treatment of seasonal influenza was modest in most countries, except for Japan, where widespread use of the drug was adopted. However, governments began to consider antiviral drug administration as a key component of their planned pandemic response after human infections with avian influenza A(H5N1) virus increased in 2003 and were associated with a case-fatality risk of >50% (*5*). Suitable vaccines would not be available at the start of a pandemic; thus, use of antiviral agents was seen as a critical part of a pandemic response.

Because the mode of administration for oseltamivir was simpler (oral) than that for zanamivir (inhalation) and because the systemic effect of oseltamivir was expected to be appropriate for treatment of highly pathogenic viruses, oseltamivir was suddenly in high demand, apparently driven by warnings from Roche that preemptive stockpiling was the only way that governments could be assured of drug availability (*6*). Since 2005, governments of middle-income and high-income countries around the world have spent billions of dollars (estimated) stockpiling oseltamivir (*7*). However, by November 2015, the influenza A(H5N1) virus that initiated stockpiling had caused only 844 human cases of infection and 449 deaths (case-fatality risk 53.2%) across 16 countries worldwide, with only 7 countries reporting >10 cases (*8*).

The first pandemic of the 21st century occurred unexpectedly in 2009 after the global spread of a novel virus—influenza A(H1N1)pdm09—of swine (rather than avian) origin. In response, many countries activated their stockpiles of antiviral agents or accessed existing community supplies. This was the first time that specific antiviral drugs were available in a pandemic. In the United States during 2009, 8.7 million oseltamivir prescriptions (28.4 prescriptions/1,000 persons) were dispensed from community pharmacies, not from the stockpile, at a cost of US $905 million (*9*).

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Although the number of deaths due to the 2009 pandemic was lower than initially anticipated, a unique opportunity was provided to review the effectiveness of oseltamivir in the pandemic setting and to determine the benefit of oseltamivir for patients who were hospitalized with confirmed influenza A(H1N1)pdm09 virus infection. Such observational data were valuable to ascertain the effect of oseltamivir in severely ill or hospitalized patients given the continued absence of data from placebo-controlled, randomized controlled trials.

Questioning whether oseltamivir is useful for treating serious illness and whether it should be stockpiled has extended the debate on the effectiveness of oseltamivir in the community. We believe that these issues should be considered separately.

Oseltamivir Treatment of Seasonal Influenza

After partially successful efforts to retrieve unpublished data from Roche (*10*,*11*), the Cochrane group conducted a meta-analysis of the effectiveness of oseltamivir in treating uncomplicated community-acquired influenza. Their findings led the group to conclude that oseltamivir had no specific antiviral effect, even though the drug had been specifically designed to achieve exactly that (*3*). The Cochrane systematic review, which focused only on an intention-to-treat analysis, confirmed that oseltamivir reduced symptom duration in the intention-to-treat group by <24 hours. Earlier, the Cochrane group had noted, "We are unsure of the generalizability of our conclusions from seasonal to pandemic or avian influenza" (*12*).

As noted previously, a subsequent meta-analysis (funded by an unrestricted grant from Roche) also confirmed a ≈1-day reduction in symptoms among adults and adolescents who had laboratory-confirmed influenza and were treated within 48 hours of symptom onset (*2*). This analysis included outcomes of both intention-to-treat and intention-to-treat-infected groups. Benefit was found for the intention-to-treat-infected group, but no benefit was found for patients with influenza-like illness who did not have laboratory-confirmed influenza (the intention-to-treat but not infected group) (*2*). Given that the benefit of oseltamivir was confined to symptomatic patients with laboratory-confirmed infection, the authors concluded that the effect of oseltamivir was due to its effect on the influenza virus, rather than a nonspecific antiviral effect, as had been suggested by the Cochrane group (*3*).

Secondary analyses from the Roche-sponsored meta-analysis suggested the following: a 63% (95% CI 19%–83%) decreased risk in hospitalization for any cause, based on 9/1,591 (0.6%) oseltamivir treated vs 22/1,302 (1.7%) placebo-treated patients; and a 44% (95% CI 25%–58%) decreased risk of antibiotic prescription for lower respiratory disease in patients with laboratory-confirmed influenza, based on 65/1,544 (4.2%) oseltamivir-treated vs 110/1,263 (8.7%) placebo-treated patients. However, hospital admissions were all cause and not confined to those that may have been associated with influenza infection; also, no formal diagnostic criteria existed for lower respiratory tract infection (*2*,*13*). We consider these secondary analyses less convincing than the analyses of primary endpoints. The latter were largely in agreement with those of the Cochrane group. Yet, despite this agreement and the arm's length funding mechanism, the Roche-sponsored meta-analysis has been criticized as being influenced by the manufacturer (*14*).

Oseltamivir Treatment of Severe Influenza

Although necessary to consider oseltamivir's effect on more serious infections, no randomized controlled trials exist that can be included in a meta-analysis. The Cochrane group chooses only to conduct meta-analyses of randomized controlled trials, which are generally accepted to be the highest level of evidence. Thus, the Cochrane group could not review severe outcomes of laboratory-confirmed influenza. Evidence is instead derived from observational studies on the use of oseltamivir to treat complications of influenza virus infection, as in hospitalized patients or in those who died. These studies are subject to uncontrolled bias. For instance, sicker patients may be more (or less) likely to be treated, thus attenuating (or exaggerating) the effect of the intervention. Also, a serious outcome may occur soon after the treatment was initiated in a severely ill patient, so that the treatment has not had a chance to succeed. Similarly, patients who receive early treatment are more likely to benefit from treatment than patients who receive late treatment. To minimize bias, researchers conducting observational studies have attempted to adjust for time from disease onset to treatment and time from treatment to outcome. Some observational studies have also adjusted for propensity to be treated as well as patient coexisting conditions and disease severity, which may affect treatment decisions and outcomes.

Observational studies that enrolled adults have often used death as an outcome, given its ease of definition. However, many observational studies fail to control completely for potential biases, including time biases. Among studies that have attempted to control for these biases, a decreased risk for death after oseltamivir treatment has been reported, and early treatment appears to be critical (*4*,*15*,*16*). For instance, in a retrospective cohort study from Israel of 449 patients hospitalized with influenza A(H1N1) pdm09 infection, all patients were treated with oseltamivir, and 189 (42%) were treated within 48 hours. This observational study controlled for propensity to treat and patient coexisting conditions and demonstrated the odds of death increased by 2.2 times (95% CI 1.4–3.5 times) if treatment was started late (>48 hours after symptom onset) (*16*).

In an attempt to overcome the criticisms of design and analysis of the observational studies, Roche chose to fund a patient level meta-analysis of individual data from 78 different observational studies, which included >29,000 patients. By adjusting for time, propensity to treat, and patient coexisting conditions, and comparing the effect of treatment with no treatment in patients infected with influenza A(H1N1)pdm09, researchers found that the odds of death were reported as 0.50 (95% CI 0.37–0.67) for adults whose treatment was initiated within 48 hours, compared with the odds of death for untreated adults (*4*). However, in a series of exchanges published in the British Medical Journal and The Lancet Respiratory Medicine, even this carefully designed study has been criticized on methodologic grounds (*17–20*). A more recent meta-analysis of individual patient data from the same group of investigators examined the potential effect of oseltamivir on influenza-related pneumonia among >20,000 patients with laboratory-confirmed influenza A(H1N1)pdm09 (*21*). However, this study has many of the predictable methodologic problems associated with retrospective reviews and does not add to the evidence base. There is scant other evidence for the benefit of oseltamivir on reducing the risk for death to help resolve residual uncertainty. The few potentially informative observational studies report on human infection with avian influenza strains (*22*) and seasonal influenza (*15*,*23*), including an unpublished review sponsored by Roche (*24*,*25*).

In a review of individual patient data for 308 patients from observational studies conducted in 12 countries, based on data from a patient register funded by Roche, oseltamivir treatment was reported to decrease the risk for death from influenza A(H5N1) virus by 49% (95% CI 23%–66%). The analysis of risk for death was restricted to 258 patients from 7 countries; mean values were substituted for missing data (*22*).

Two prospective observational studies of hospitalized patients with laboratory-confirmed seasonal influenza have shown oseltamivir treatment decreases the risk for death. In a prospective observational cohort study from Hong Kong, which enrolled 754, mostly elderly, hospitalized patients with co-existing conditions during 2007–2008, oseltamivir treatment was associated with a reduced risk for death (adjusted hazard ratio 0.27, 95% CI 0.13–0.55; p<0.001), with a further reduction associated with earlier treatment (*15*). A small prospective observational study of patients hospitalized with laboratory-confirmed influenza in the 2005–06 season in Ontario, Canada, found the adjusted odds ratio of death among oseltamivir-treated patients was 0.21 (95% CI 0.06–0.80; p = 0.03), based on 22 (10%) of 219 deaths in the untreated group compared with 4 of 103 deaths in the treated group (*23*).

Also supporting the conclusion that oseltamivir use has a beneficial effect on reducing the risk for death were findings from a large review from the Ingenix Research Data Mart (*24*), apparently sponsored by Roche. We have only been able to find an abstract of the study with an associated commentary (*25*). The observational study found that oseltamivir use decreased the risk for death in patients of all ages with influenza (1 death/39,202 patients) compared with untreated patients (56 deaths/136,799 patients; p = 0.02). However, the commentary raised several issues related to study design, which could not be resolved without further detail (*25*).

We have not been able to find an analysis of oseltamivir effectiveness for treating infections with avian influenza A(H7N9) virus in China, a virus that, since its emergence in 2013, has caused a greater number of annual cases and deaths than influenza A(H5N1) virus. Such a study might also contribute to the evidence base.

Oseltamivir Policies for Seasonal and Pandemic Influenza

The evidence from randomized controlled trials is clear that oseltamivir treatment decreases the duration of symptoms by up to 1 day in adolescent and adult patients with laboratory-confirmed seasonal influenza whose infections are able to be managed in the community. Oseltamivir provides no benefit to patients who have influenza-like illness not caused by influenza virus (*2*). Reviews of observational data regarding patients hospitalized with influenza A(H1N1)pdm09 or influenza A(H5N1) infections found that risk for death is cut in half if treatment is initiated within 48 hours of symptom onset (*4*,*22*). Small prepandemic observational studies, although they generally have controlled less for potential biases, also support the conclusion that risk for death is decreased with oseltamivir treatment (*15*,*23*).

These 2 lines of evidence may appear inconsistent. How would an intervention that has a modest effect on symptom duration in patients whose uncomplicated influenza was treated after a visit to a general practitioner be able to cut in half the risk for death among hospitalized patients?

It should not be surprising that treatment for uncomplicated influenza will only produce a modest effect because influenza virus replication precedes symptoms by 1–2 days. This means that the viral load in the patient may have peaked by the time the patient begins antiviral treatment. Given that most antiviral drugs, including oseltamivir, act by reducing viral replication, the effect of treatment will therefore be minimal in otherwise healthy persons when immune responses are already reducing viral titers. It is therefore plausible that community-based randomized controlled trials are not capturing critical information about the mode of action of oseltamivir that is beneficial to severely ill patients.

It is possible that benefit to severely ill patients may be related to the increased duration of viral shedding and higher viral loads found in this group of patients (*26*). Elevated cytokine levels, sometimes referred to as a cytokine storm, have been detected for patients infected with highly pathogenic A(H5N1) virus (*27*) and for severely ill patients infected with influenza A(H1N1)pdm09 and seasonal influenza viruses (*28*,*29*). A randomized controlled trial study of 117 healthy adults experimentally infected with seasonal influenza virus A(H1N1) reported that oseltamivir treatment significantly reduced interleukin-6, interferon-γ, and tumor necrosis factor-α cytokine responses in patients compared with responses in placebo-treated patients (*30*). Although these results do not clarify whether the decreased cytokine response was the result of effective viral treatment or a (postulated) immune modulatory effect of oseltamivir, ferret studies conducted in our laboratory suggest that oseltamivir treatment consistently reduces peak temperatures and improves ferret activity/wellness but often does so in the absence of any significant effect on viral load (*31*). The difference in outcome for severely infected patients treated with oseltamivir may relate to decreasing the adverse outcome associated with a cytokine storm, which would not be expected in patients with mild disease.

Implications for Stockpiling

Data from the randomized controlled trials of patients with mild influenza and the observational data from severely ill patients demonstrate the clear clinical benefits of initiating treatment as early as possible after infection (*32*). NAI use in Japan is so widespread that almost every confirmed influenza case is treated, which is likely to have led to the extensive and rapid delivery of the drugs in the 2009 pandemic. For instance, in a study of Japanese children hospitalized with influenza A(H1N1)pdm09 virus infections, >98% (984/1,000) were treated with an NAI, and for those for whom the treatment time was recorded, 89% received NAIs within 48 hours and 70% within 24 hours. Only 1% of the hospitalized children ultimately required mechanical ventilation, and 1 death was recorded (*33*).

Similar ecologic data were observed among pregnant women in Japan, a group of patients at increased risk for hospitalization and death when infected with influenza A(H1N1)pdm09. Pregnant Japanese women were treated prophylactically after close contact with an infected person, and if infected and hospitalized, >90% were given NAIs within 48 hours of symptom onset. In comparison to the high mortality rates among pregnant women in many countries around the world (*34*), no maternal deaths occurred in Japan during the pandemic (*35*).

Ecologic data from Japan, although regarded as the weakest form of epidemiologic evidence, suggest that rapid access to stockpiled NAIs in a pandemic is necessary to achieve the greatest benefit from their use. Rapid access during the 2009 pandemic in Japan was possible because rapid access represented routine care for seasonal influenza. In other countries, the 2009 pandemic confirmed that centralized stockpiles did not facilitate rapid distribution (*36*) and that decentralized stockpiles would be more efficient. Stockpiles in hospitals, for example, would facilitate rapid treatment of ill patients in a pandemic but might also allow the periodic use of some material for the treatment of interpandemic seasonal influenza to avoid wastage due to an expiring stockpile (*36*).

The Way Forward

There is general agreement derived from randomized controlled trials about the modest effectiveness of oseltamivir against relatively mild illness in otherwise healthy persons, but several lines of evidence from observational studies suggest that oseltamivir decreases the risk for death (*37*). However, within the next 5–10 years, we do not expect to see clarifying new evidence from trials of patients recruited from the community who have an endpoint of severe influenza. Severe outcomes from influenza are uncommon, as shown in the meta-analyses of community trial data, and randomized controlled trials that recruit healthy persons would require extremely large patient numbers and need to be conducted over multiple seasons to account for potentially different outcomes by influenza type and sub-type.

On the other hand, a randomized controlled trial that recruited only patients with severe influenza, although feasible from a design perspective, could not ethically evaluate active treatment versus placebo treatment because oseltamivir treatment is the standard of care for patients with severe influenza virus infections. In a study that overcame the ethical issue, a randomized controlled trial of patients with severe influenza that examined single-dose versus double-dose oseltamivir found no difference in outcomes between the 2 treatment arms (*38*). We do not anticipate that this trial would be repeated.

Existing observational evidence on the benefits of oseltamivir for the treatment of influenza in hospitalized patients, including assessing the risk for death, accrues from ecologic data from Japan, weak secondary analyses from randomized controlled trials of laboratory-confirmed influenza initially managed in the community, and the systematic reviews and analyses of observational studies of patients with confirmed infections caused by influenza A(H1N1)pdm09 and influenza A(H5N1) viruses. Methodologically less robust, small observational studies conducted before the pandemic support the finding that oseltamivir decreases the risk for death. However, well-designed prospective observational studies may provide the most informative data in the next 5–10 years.

We reached several conclusions regarding the use of oseltamivir and the considerations that will be necessary

Table. Conclusions of study evaluating the use of oseltamivir for seasonal and pandemic influenza and wisdom of stockpiling*

Summary conclusions
1. Although debate continues, there is general agreement from meta-analyses of RCTs that oseltamivir reduces symptoms in healthy adults and adolescents with influenza by up to 1 day. There is disagreement on the mechanism. On 1 side of the debate, the Cochrane group maintains that there is a nonspecific effect of oseltamivir, whereas, on the other side, investigators sponsored by Roche maintain that oseltamivir has a specific anti–influenza virus effect.
2. There have been no RCTs that can be meta-analyzed to summarize the effect of oseltamivir on severe outcomes of influenza virus infection. Evidence derived from observational studies of serious outcomes consistently suggests that oseltamivir reduces the risk for death in severely ill patients with documented influenza infection.
3. The apparent discrepancy between a modest drug effect for healthy persons and a substantial effect on number of deaths remains unexplained. Currently, oseltamivir is the only licensed drug available for all ages.
4. Based on available evidence, oseltamivir should be used for treatment of hospitalized patients with laboratory-confirmed seasonal influenza and stockpiled for the treatment of patients with severe laboratory-confirmed pandemic influenza, whether hospitalized or not. These stockpiles should be widely distributed to facilitate rapid use when needed.
5. Without a mechanism for rapid distribution of the drug in an emergency, any potential benefit of such large-scale stockpiling will not be realized. Rapid distribution in an emergency is only likely if a mechanism exists for routine rapid distribution. In countries where such a mechanism does not exist, we see no place for stockpiling oseltamivir for widespread community use during a pandemic.
6. It is unlikely that conventional RCT-level evidence to support antiviral treatment of severe laboratory-confirmed influenza in hospitalized patients will appear within the next decade due to the ethical constraints of evaluating oseltamivir vs placebo, when oseltamivir is the current standard of care for the treatment of severe influenza infection. New studies should be pragmatic trials or high-quality prospective multisite observational studies and employ methods to minimize bias to the greatest extent possible.
7. Studies designed for assessing interventions for seasonal influenza should be readily adaptable to studies of pandemic influenza on very short notice. Because of the ethical and design constraints of RCTs, prospective observational studies are more feasible than RCTs in an emergency response situation. In addition to data on outcome, such as risk of ICU admission and death among adults, or length of stay among children, these observational studies should also record time from disease onset to treatment and time from treatment to outcome to minimize bias. Sequential data on markers of immune function in at least a subset of recruited patients would also be valuable.

*RCT, randomized controlled trial; ICU, intensive care unit.

for future studies (Table). Nguyen-Van-Tam et al. recently outlined a list of covariates that would need to be collected to help strengthen the evidence that oseltamivir treatment benefits hospitalized patients with influenza (*39*). These include standardized data on illness onset and progression, comorbid conditions, disease severity, treatment, duration of hospital stay, the need for critical care, and influenza-related mortality. In addition, a review by the UK Academy of Medical Sciences, sponsored by the Wellcome Trust, has offered a range of approaches to potential future studies and called specifically for "pragmatic or adaptive [randomized controlled trial] designs" of neuraminidase inhibitors in hospitalized patients (*37*). Our conclusions are in broad agreement with those of this report.

The details of study design may be best accomplished by an experienced group of international researchers, focusing on standardized recruitment procedures and covariate and outcome definitions with a clearly defined analysis plan designed to minimize bias, as far as possible. Funding for such studies could come from the public or private sectors, with prior safeguards on perceptions of conflicts of interest. The studies should be adaptable to evaluate new antiviral medications, including intravenous forms of the NAIs, newly licensed non-NAI antiviral agents that are currently in late-phase clinical trials, or adjunctive therapies and immunomodulatory agents. Combinations of novel drugs with existing NAIs will likely be a useful approach and will require evaluation. Plans for ensuring broad availability and regulatory approval for emergency use of unlicensed antiviral agents will need to be established on very short notice. If pragmatic trials or high-quality prospective observational studies are completed and published over the next decade, an improved evidence base may help clinicians and public health planners decide on the most appropriate use of oseltamivir and potential new influenza antiviral agents for patients with severe infections caused by seasonal or pandemic influenza.

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Perspectives on West Africa Ebola Virus Disease Outbreak, 2013–2016

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The variety of factors that contributed to the initial undetected spread of Ebola virus disease in West Africa during 2013–2016 and the difficulty controlling the outbreak once the etiology was identified highlight priorities for disease prevention, detection, and response. These factors include occurrence in a region recovering from civil instability and lacking experience with Ebola response; inadequate surveillance, recognition of suspected cases, and Ebola diagnosis; mobile populations and extensive urban transmission; and the community's insufficient general understanding about the disease. The magnitude of the outbreak was not attributable to a substantial change of the virus. Continued efforts during the outbreak and in preparation for future outbreak response should involve identifying the reservoir, improving in-country detection and response capacity, conducting survivor studies and supporting survivors, engaging in culturally appropriate public education and risk communication, building productive interagency relationships, and continuing support for basic research.

In 1976, the investigation of concurrent outbreaks of a hemorrhagic fever syndrome (Ebola virus disease [EVD]) in Zaire (currently Democratic Republic of Congo) and Sudan (currently Republic of South Sudan) (*1*,*2*) led to isolation of 2 viruses now referred to as Ebola virus (EBOV) and Sudan virus, respectively, and to identification of a newly recognized viral hemorrhagic fever genus, *Ebolavirus* (family *Filoviridae*). Ebolaviruses now include EBOV, Sudan virus, Reston virus, Taï Forest virus and Bundibugyo virus. The other genus in the family *Filoviridae* is *Marburgvirus,* consisting of Marburg virus and Ravn virus (termed marburgviruses; MBGV), both of which are associated with severe disease (Marburg virus disease [MVD]) in humans (*3*,*4*). Before 2013, the largest Ebola outbreak was associated with Sudan virus in Gulu, Uganda, in 2000 that caused 425 cases (224 fatal) (*5*). The largest EVD outbreak associated with EBOV (the same virus responsible for the 2013–2016 outbreak in West Africa) was in Zaire (1976) and caused 318 cases and an associated case-fatality rate of 88% (*2*).

The EVD outbreak in Guinea, Liberia, and Sierra Leone was unprecedented in its sheer magnitude and the emergence of EBOV outside the Congo basin. The effect of the outbreak is profound; as of March 27, 2016, a total of 28,646 EVD cases and 11,323 deaths had been documented (*6*). Furthermore, this outbreak prompted an unparalleled international response: 7 US agencies operated 9 laboratories, and 11 international agencies operated 13 laboratories performing in-country diagnostic tests (Figure 1). The Centers for Disease Control and Prevention (CDC) supported ≈2,300 international deployments of ≈1,600 total personnel (both CDC and non-CDC staff) (*7*); and thousands of personnel from international aid agencies, e.g., World Health Organization, Médecins Sans Frontières, International Rescue Committee, International Finance Corporation, and Public Health England provided in-country support.

The EVD outbreak was not restricted to the 3 heavily affected West African countries; cases also occurred in Senegal, Nigeria, and Mali. In addition, EBOV-infected foreign aid workers were transported for treatment to Europe and the United States, and naturally imported cases (United States, Italy, United Kingdom) and domestic transmission (Spain, United States) were reported for the first time in several countries (*6*). The US EVD response included establishment of EBOV testing in the US Laboratory Response Network. As a result, 57 state, county, and local public health laboratories in 44 states currently are qualified to perform presumptive EBOV real-time quantitative PCR (qPCR).

This EVD outbreak highlights globalization, international social responsibility, and the importance of global health security. As the response to the outbreak progresses, the international research community must continue to address questions of EBOV emergence, pathogenesis, and transmission and advance therapeutic and vaccine development. National and international organizations must critically assess the details of this outbreak and the corresponding response to enable improved response and control of emerging viral outbreaks.

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Why Here? Why Now?

We lack precise answers to these questions. A spillover event is an exceedingly rare but high-consequence event that is likely the most critical initiating factor for an outbreak. Features of the virus phylogenetic analyses and putative reservoir species, and what we know about prior ebolavirus and MBGV spillover events, offer possible insight into "Why here? Why now?" All currently recognized EBOVs appear to share a recent common ancestor ≈50 years ago, probably because of a recent genetic bottleneck (*8*). Current EBOV lineages, first detected in northern Democratic Republic of Congo in 1976, appear to have spread across the Congo basin during this relatively short period and arrived to West Africa only in the past few years. Before the 2013–2016 outbreak, the only definitive evidence of ebolaviruses or the diseases they cause in West Africa was 1 nonfatal human case associated with Taï Forest virus, which caused illness and death in chimpanzees in Côte d'Ivoire in 1994 (*9*).

How EBOV spread across the Congo basin and whether this spread involved movement through bat, nonhuman primate, or other animal populations are unclear. EVD in humans has been linked to preparing and eating nonhuman primate (chimpanzee, gorilla, monkey) or duiker bushmeat (*10*). Contact with bats also has been identified as a putative source of EBOV spillover (*11*). However, the role of bats in virus maintenance and initiation of human disease outbreaks remains unclear. Evidence of bats involvement in the spillover event initiating the 2013–2016 outbreak is limited to anecdotal reports of interactions between bats and villagers in Guinea; no epidemiologic or genetic data associate a putative reservoir species with the current outbreak (*12*). Unlike MBGV, EBOV has yet to be isolated from bats, and no direct evidence links bats to EBOV infection in humans.

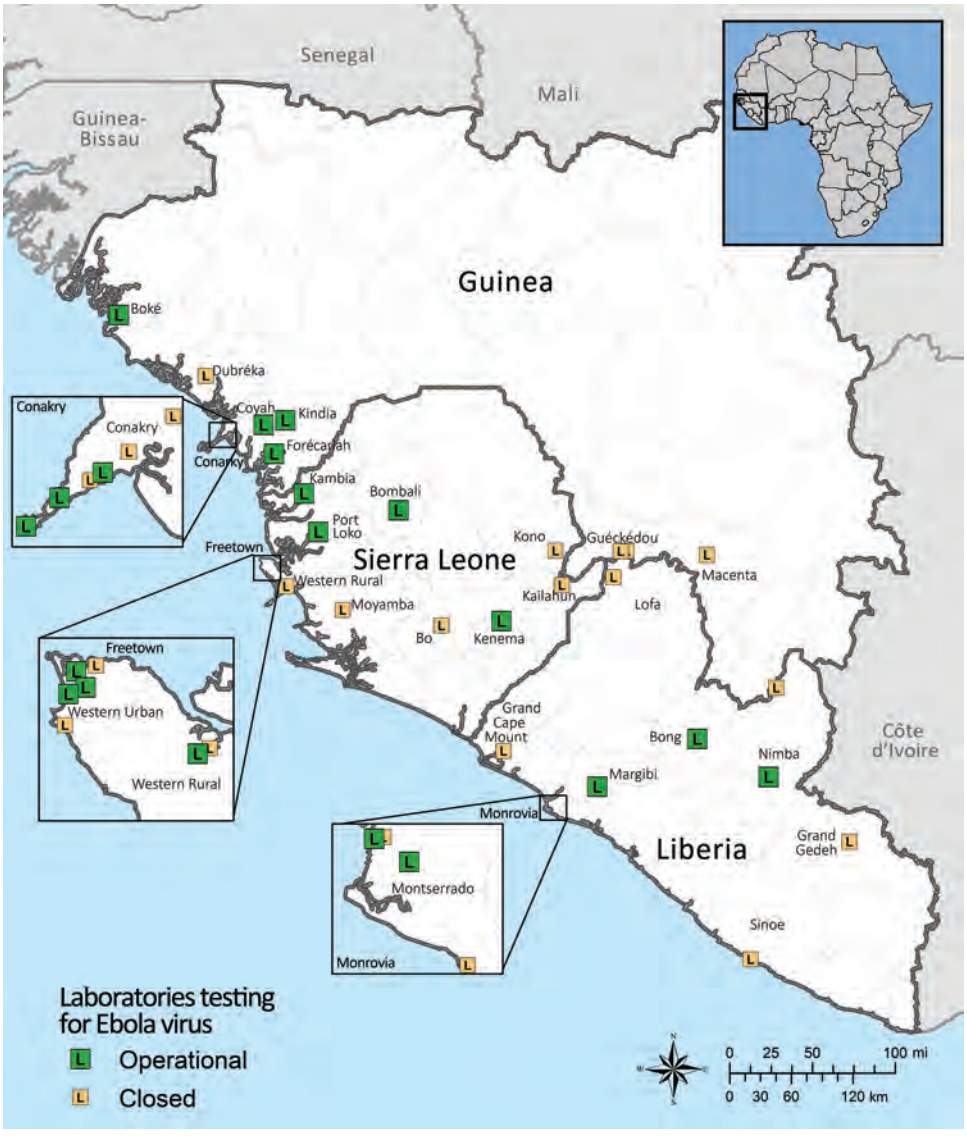

Figure 1. Geographic distribution of diagnostic laboratories currently or previously operational in West Africa during the 2014–2015 Ebola virus response, as of December 9, 2015. Data are from World Health Organization Ebola virus disease situation reports.

Regardless, epizootic spillover remains the most widely accepted theory for how the outbreak began.

Experimental infection studies with filoviruses indicate that the viral load in the carcass of an animal that died of EVD would be high (*13*,*14*), but the viral load in the carcass of a healthy reservoir species is probably much lower (*15*,*16*). However, the virus inoculum required to infect animal models of EVD by traditional experimental routes is very low. Thus, viral shedding through excreta or viral load in tissue (eaten or handled raw) of reservoir species might provide sufficient inoculum to initiate virus spillover. In spillover events involving MBGV, sequences from human isolates were ≈99% identical to virus isolates obtained directly from infected bats (*17*); thus, the EBOV spillover event most likely involved little or no virus adaptation. Most EBOV outbreaks appear to involve a single initiating spillover event followed by human-to-human transmission (*18*,*19*), whereas several MVD outbreaks have been associated with multiple spillover events (*4*,*17*). This dissimilarity might reflect a difference in the nature of human interactions with the different primary reservoir species of EBOV and MBGV.

Why So Big?

Some early speculation about the differences in magnitude between the 2013–2016 EVD outbreak and previous filovirus outbreaks was focused on the presence of a rapidly mutating, highly transmissible or highly virulent EBOV strain. Gire et al. reported a rapid accumulation of interhost and intrahost genetic variation in 99 EBOV genomes from 78 patients in Sierra Leone (*20*). However, later analysis of more EBOV full-length sequences indicated that the overall virus nucleotide substitution rate was consistent with rates observed in previous outbreaks in Central Africa (*8*,*21*). Pathogenesis studies also support that the size of the outbreak and characteristics of EVD in West Africa are not related to change in the virus but instead appear to be a result of factors extrinsic to the virus (*22*–*25*).

Although differences in the outbreak strain do not explain the magnitude of the outbreak, the situation in these West African countries in 2013 made them particularly vulnerable to a large outbreak in the event of the arrival of EBOV or spillover from wildlife. The West African outbreak occurred in an extremely resource-poor area that lacked basic infrastructure and was recovering from the effects of decades of civil war. The consequences of civil instability included the collapse of government institutions and schools, disruption of traditional societal values and structures, poor education standards, and struggling basic healthcare infrastructures (*26*–*28*). Unlike several other African countries, Guinea, Liberia, and Sierra Leone had no past experience in recognizing and managing filovirus outbreaks, and the outbreak occurred in a region with very high endemic levels of malaria that has a similar clinical presentation to EVD. Although these countries had experience with Lassa hemorrhagic fever, that experience most likely negatively affected the initial response: suspecting Lassa might have delayed identifying EBOV and enabled early EBOV transmission. Based on limited chains of human-to-human transmission Lassa virus appears to be less transmissible and requires less stringent use of personal protective equipment and containment to prevent healthcare worker infections.

Slow recognition of suspected cases, inability to accurately diagnose disease, and absence of appropriate surveillance for critical decision-making early in the outbreak severely hampered interruption of EVD spread at key points during the response. Distrust of government and outsiders hindered response efforts, and the spread of conspiracy theories among residents resulted in fear, superstition, and secrecy (*27*). In contrast to prior filovirus outbreak where treatment of cases and transmission occurred in major urban areas (e.g., Kinshasa, Zaire, in 1976; Nairobi, Kenya, in 1980; Kinshasa in 1995; Johannesburg, South Africa, in 1995; Luanda, Angola, in 2005; Kampala, Uganda, 2007; Kampala, Uganda, in 2014 [http://www.cdc.gov/vhf/ebola/outbreaks/history/chronology.html; http://www.cdc.gov/vhf/marburg/resources/outbreak-table.html]); the West African outbreak was the first to include multiple reintroductions to urban areas (such as Conakry) from human cases and extensive urban transmission. Porous borders and high population mobility within each country and into neighboring countries exacerbated widespread dissemination of disease from urban and rural transmission (*27*).

Future Priorities and Considerations

Identify the Reservoir

Predicting EBOV epizootics requires increased understanding of virus ecology. Epidemiology, serologic data, and detection of viral RNA support a role of bats and nonhuman primates in EBOV maintenance and spillover transmission from animal reservoirs to humans. However, EBOV has yet to be isolated in nature from any bat species or nonhuman primates. In contrast to EBOV, ecologic and experimental evidence confirms fruit bats (*Rousettus aegyptiacus*) as a reservoir for MBGV, and MBGV spillover events from bats to humans have been documented (*4*,*17*,*29*). Ecologic investigations of *R. aegyptiacus* fruit bats showed seasonal pulses of MBGV spillover events (*30*). MBGV has been isolated from naturally infected *R. aegyptiacus* fruit bats 20 times (*15*,*17*,*31*), and virus replication and oral shedding in the absence of clinical disease was observed in experimentally infected *R. aegyptiacus* fruit bats (*15*).

Although EBOV exhibits ecologic patterns similar to those of MBGV, confirming EBOV reservoir hosts by

virus isolation in nature remains elusive. One difficulty in obtaining EBOV isolates from bats, despite many attempts, appears to be identifying and sampling the appropriate bat species. Only 1, or a limited number of, bat species most likely can serve as hosts for each of the filovirus species, a phenomenon also seen with rodentborne hantaviruses and arenaviruses (*32*). In contrast to MBGV, no detectable viremia develops in *R. aegyptiacus* fruit bats experimentally infected with ebolaviruses (Sudan, Ebola, Bundibugyo, Taï Forest, and Reston viruses), and viral RNA detection was localized to the injection site (*33*), suggesting that *R. aegyptiacus* fruit bats are not a competent reservoir species for EBOVs. MBGV ecologic studies support the theory of 1, or a limited number of, host species because infection was found consistently in *R. aegyptiacus* fruit bats but not in *Hipposideros* spp. bats, despite their close interaction (*17*).

Although bat species involved in EBOV maintenance have yet to be discovered, limited detection of EBOV RNA and EBOV antibodies has implicated some frugivorous and insectivorous bat species distributed in areas of previous outbreaks, including the little collared fruit bat (*Myonycteris torquata*), hammer-headed bat (*Hypsignathus monstrosus*), Franquet's epauletted fruit bat (*Epomops franqueti*), straw-colored fruit bat (*Eidolon helvum*), and Angolan free-tailed bat (*Mops condylurus*) (*12*,*34*–*36*). Further investigation of these species as putative EBOV reservoirs is warranted; identifying EBOV reservoir species would enable predictive modeling based on distribution (Figure 2) and population dynamics (population size, reproduction, proximity to human populations). Tracking fruit bat migrations across their distribution ranges (Figure 2) including riverine highways and conducting reservoir population surveillance could identify high-risk disease foci before human population exposure, potentially mitigating another spillover and outbreak. In the case of MBGV, understanding the bat reservoir has led to risk reduction measures, such as identifying seasons at high risk for spillover, restricting access of miners or ecotourists to mines and caves with circulating MBGV, and constructing a safe viewing platform at a national park in Uganda.

Increase In-Country Surveillance, Diagnostic Capacity, and Epidemiologic Support

Curbing disease spread requires rapid identification of the initial human case or cluster after a spillover event to permit patient isolation and timely contact tracing. The payoff for investment in surveillance systems and diagnostic capacity to rapidly identify and respond to outbreaks is illustrated by efforts in Uganda and Democratic Republic of Congo, where the past several EVD and MVD outbreaks were quickly identified and restricted to a few cases. Effective contact tracing to interrupt disease transmission is personnel intensive and requires rapid organization and deployment after notification of suspected cases. In all EVD outbreaks to date, most transmission events involve close-contact human-to-human transmission. Because resources are often limited, case investigations should thoroughly rule out known sources of EBOV transmission before investigating speculated or new (e.g., airborne, environmental, dogs, or asymptomatic human) sources that have never been reported to be associated with disease in previous filovirus outbreaks. Accomplishing the aforementioned recommendations requires increased awareness at the community clinic level, improved quality of central laboratories and capacity for specimen transport (rapid collection and delivery, proper packaging, appropriate storage conditions), and more epidemiologists throughout the region. Finally, laboratorians and epidemiologists must work closely with well-trained clinical and infection control personnel to effectively identify and isolate infectious persons (*37*).

Test Appropriate Samples and Interpret Evidence-Based Data

Accurate diagnosis requires including EBOV in the differential diagnoses for febrile tropical illness and considering the possibility of co-infection with other frequent tropical diseases (e.g., malaria, typhoid). If appropriate diagnostic samples are not collected, cases might be missed at critical points in the response. Blood is the most sensitive diagnostic specimen early in EVD; oral swabbing, although less invasive, does not offer adequate sensitivity until late in the course of disease, but it is a sensitive and appropriate modality for testing postmortem specimens (*13*,*14*). Diagnostic data are critical in patient management and development of clinical recommendations and discharge policies. Interpretation of diagnostics should consider the sensitivity and specificity of the test and specimen type and what the test is detecting. For instance, qPCR is widely used, but a common mistake in interpreting results is that RNA detection is synonymous with the presence of infectious virus or viral shedding, which is not always the case (*38*). Although detectable viral RNA might indicate shedding, it does not equate with shedding, and this fact must be considered when transmission risk is evaluated on the basis of qPCR.

Investigate Viral Persistence and Physical and Psychological Sequelae in Survivors

The ≈17,000 survivors from the 2013–2016 EBOV outbreak might face major medical and social challenges after recovering from acute disease. Semen samples from EVD survivors in Kikwit, Democratic Republic of Congo, were positive for EBOV by RT-PCR up to 101 days after illness onset, and 1 sample obtained 82 days after disease onset yielded infectious virus (*19*,*39*). In addition, MBGV was isolated from semen of a convalescent patient and was the source of

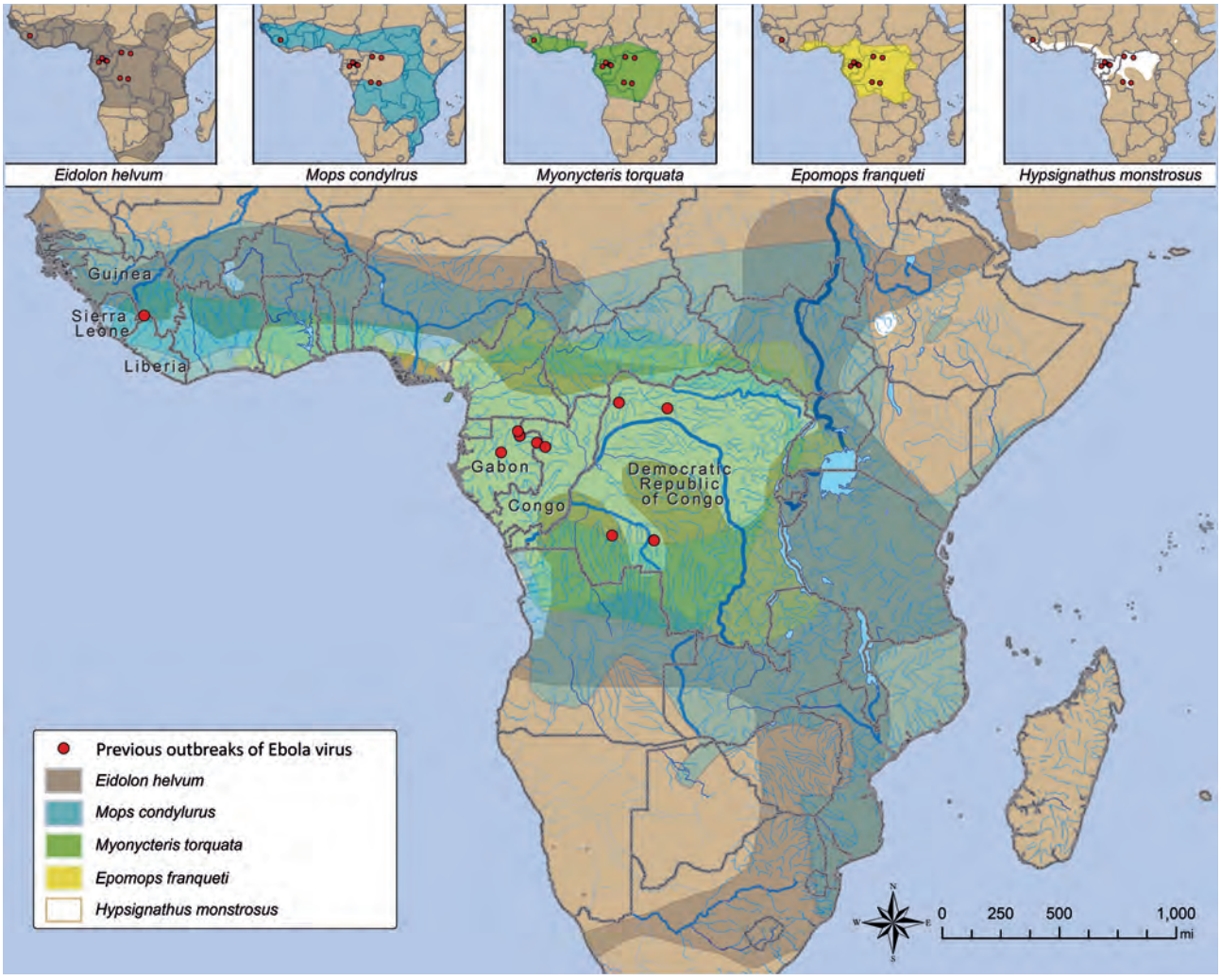

Figure 2. Relationship between location of index case in Ebola virus (*Zaire ebolavirus*) outbreaks and putative reservoir distribution. Ebola virus outbreaks (red dots) and distribution of *Eidolon helvum*, *Mops condylurus*, *Myonycteris torquata*, *Epomops franqueti*, *and Hypsignathus monstrosus* bats (insets) are shown. Data are from the Centers for Disease Control and Prevention and the International Union for the Conservation of Nature.

infection of a contact (*40*; reference *41* in online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/16-0021-Techapp1.pdf). Sexual transmission was also implicated in EBOV transmission recently in Liberia (references *42*,*43* in online Technical Appendix). This outbreak confirmed that EBOV can persist in immune-privileged sites and has highlighted the implications of these findings. EBOV has now been isolated from aqueous humor of the eye (reference *44* in online Technical Appendix), semen (*17*, reference *45* in online Technical Appendix), and cerebrospinal fluid (reference *46* in online Technical Appendix) of patients in whom the initial viremia cleared. The details and dynamics of EBOV shedding in body fluids after convalescence remain unclear and need to be investigated further, especially in fluids with higher potential for involvement in transmission events (e.g., semen and amniotic fluid) to clearly define specimens and behaviors with transmission risk.

Although understanding putative disease transmission from convalescent patients is essential to prevent EVD, clinicians and public health professionals also must investigate and address disease sequelae and social stigma associated with EVD in affected populations. Convalescent EVD patients reported arthralgia and myalgia more significantly than control patients during the 1995 Kikwit outbreak (*39*). In addition, 15% of the Kikwit survivors interviewed reported ocular sequelae, including ocular pain, photophobia, hyperlacrimation, and loss of visual acuity; all 4 patients reporting ocular sequelae had uveitis that responded to topical treatment (reference *47* in online Technical Appendix). EVD survivors of the Bundibugyo virus outbreak in Uganda in 2007 also had arthralgia and

ocular deficits; hearing loss, neurologic abnormalities, sleep disturbance, memory loss, and various other constitutional symptoms. Chronic health problems also were reported (references *48*,*49* in online Technical Appendix).

In addition to medical burdens associated with recovery, survivors are concurrently dealing with considerable psychological issues of fear, denial, and shame. In severe instances, the social stigma associated with disease can be profound, resulting in abandonment by family and friends (reference *50* in online Technical Appendix).

Increase Public Education and Risk Communication

The response to an EVD outbreak requires rapid, effective, widespread public education. In addition to increased potential for transmission, a lack of public education and knowledge can contribute to panic, anxiety, and psychosocial trauma; fear and distrust of treatment units and responders, sometimes to the point of violence; and isolation, stigmatization, and community ostracism of survivors and family members of patients (references *51*,*52* in online Technical Appendix). Despite extensive communication efforts during the current EVD outbreak, knowledge and understanding of EVD symptoms remained low, and fear of ill and recovered EVD patients and treatment units persists (reference *52* in online Technical Appendix). To be effective, public education must recognize community-specific risks and concerns. This education must balance culturally appropriate messaging in the context of scientifically founded risk reduction messages to minimize human exposure (references *52*–*54* in online Technical Appendix). Risky behavior must be identified, and messages about risky behavior, prevention strategies, and feasible alternatives must be communicated. Establishing in-country community partners should be integrated to health communication; these partners are often most effective at providing behavioral health education and overcoming language and cultural barriers (reference *54* in online Technical Appendix).

Risk communication can prevent or greatly reduce transmission. EBOV transmission occurs through close contact with symptomatic EVD patients. Familial and social networks play a major role in transmission, particularly through caregivers' contact with infectious fluids from ill persons at home and in healthcare facilities and through contact with deceased persons during funeral rites. Viral transmission is relatively inefficient compared with other highly infectious agents (reference *55* in online Technical Appendix). An exception is the proposed contribution of EBOV superspreaders (reference *56* in online Technical Appendix): persons who infect disproportionally more secondary contacts. For EBOV, superspreaders fall into 2 categories: biologic superspreaders, who shed more virus, and situational superspreaders, who solely because of circumstances or behavior potentially expose more persons, for example, persons who travel extensively, have occupations that interact closely with many persons (e.g., traditional healers), or deceased patients who had a highly attended funeral. Although biologic superspreaders appear to occur in EVD outbreaks (references *57*,*58* in online Technical Appendix), situational superspreaders more notably elicit transmission events, which successful community education on EVD can greatly reduce.

Promote Productive Interagency Relationships

Overall outbreak prevention and response will benefit greatly from continuing efforts to develop relationships with nongovernment organizations operating in the region and encouragement of constructive reform of national and international response agencies. We believe the very large and complex nature of the outbreak made communication within and among agencies exceptionally difficult during the outbreak. Frequently, well-intended centralized decision-making did not translate into appropriate application in the field. During the outbreak, partnering among agencies evolved in an effort to improve communication and the outcome of collaborative efforts, but further improvements are possible. Delegating roles among agencies in accordance with their strengths and abilities to acquire the necessary resources for epidemiologic investigations, diagnostics, clinical care, media relations, public education, and logistics might improve efficiency.

Continue Support for Basic Research

Pathogenesis studies and development of diagnostic tests, therapeutic drugs, and vaccines are the foundation of the public heath response. The international scientific community must continue to prioritize research on EBOV and viral pathogens that have yet to manifest into large outbreaks but have the fundamental characteristics to do so: viruses causing high rates of illness and death that are capable of person-to-person transmission and lack therapeutic drugs, vaccines, and other interventions (e.g., Nipah virus and Crimean-Congo hemorrhagic fever virus). The development and study of new tools toward the end of an outbreak is more likely to be hampered by a lack of patients, as we observed with the EBOV vaccine trials and new diagnostic test evaluations. Thus, future vital research projects should be poised to deploy at the start of new outbreaks, which will require prioritization and substantial regulatory forethought and preparation. However, research projects should not detract from outbreak response. The benefits of research investigations and fundamental response efforts must be balanced appropriately. In response to the outbreak, EBOV researchers worked together in a remarkable effort to advance research and address questions from the field in real time. Interagency collaborations and the open communication of data should continue after the outbreak

to develop vaccines and therapeutic drugs and to address key questions on EBOV and other high-consequence, high-containment hemorrhagic fever viruses.

Conclusions

The large size and long duration of the West Africa EVD outbreak and the resulting enormous national and international response efforts yielded many lessons for improved prevention and control efforts for emerging viral diseases. Although the current outbreak comes to a close and other health crises emerge in the news headlines, we must not forget that many features of this tragic outbreak strongly reinforce the benefit of continued investment in global health security efforts.

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SYNOPSIS

Human Infection with Influenza A(H7N9) Virus during 3 Major Epidemic Waves, China, 2013–2015

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Learning Objectives

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

- Describe changes in laboratory-confirmed human infections with H7N9 across 3 epidemic waves in 2013–2015 in mainland China, and other epidemiologic features, based on a surveillance study.
- Identify changes in hospitalized cases of H7N9 across 3 epidemic waves in 2013–2015 in mainland China.
- Determine possible reasons for the observed changes in laboratory-confirmed and hospitalized cases of H7N9 across 3 epidemic waves in 2013–2015 in mainland China.

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Since March 2013, a novel influenza A(H7N9) virus has caused 3 epidemic waves of human infection in mainland China. We analyzed data from patients with laboratory-confirmed influenza A(H7N9) virus infection to estimate the risks for severe outcomes after hospitalization across the 3 waves. We found that hospitalized patients with confirmed infections in waves 2 and 3 were younger and more likely to be residing in small cities and rural areas than were patients in wave 1; they also had a higher risk for death, after adjustment for age and underlying medical conditions. Risk for death among hospitalized patients during waves 2 and 3 was lower in Jiangxi and Fujian Provinces than in eastern and southern provinces. The variation in risk for death among hospitalized case-patients in different areas across 3 epidemic waves might be associated with differences in case ascertainment, changes in clinical management, or virus genetic diversity.

More than 3 years have passed since novel influenza A(H7N9) virus infections were first detected among humans in mainland China (*1*). The first epidemic of human infections occurred in the spring of 2013; 134 cases were laboratory confirmed through September 2013 (control measures in combination with environmental factors led to a lull in incidence in the summer of 2013) (*2*,*3*). However, a second epidemic of infections occurred in the winter of 2013–14 (*4*), and a third epidemic occurred in the winter of 2014–15. A fourth wave is ongoing, and as of March 3, 2016, in mainland China, 730 laboratory-confirmed human cases of influenza A(H7N9) virus infection have been reported, most associated with severe disease; 295 of the infections were fatal. Low pathogenicity of influenza A(H7N9) infections in chickens has been well established (*5*), and most human infections can be attributed to close contact with infected chickens, particularly in live poultry markets (*1*,*6*,*7*).

The objectives of this study were to compare the epidemiology of human cases of influenza A(H7N9) infection across the 3 epidemics and, in particular, to examine whether the severity of disease among hospitalized case-patients has changed over time. To do this, we estimated the risks for death, use of mechanical ventilation, and admission to an intensive care unit (ICU) among hospitalized patients with severe infections caused by influenza A(H7H9) virus.

Methods

Source of Data

All laboratory-confirmed cases of avian influenza A(H7N9) virus infection in mainland China are reported to the Chinese Center for Disease Control and Prevention (China CDC) through a national surveillance system. Case definitions, surveillance for identification of cases, and laboratory assays have been described (*8*,*9*). Demographic, epidemiologic, and basic clinical data on each confirmed case-patient were obtained on standardized forms and entered into an integrated database at China CDC. We based our analyses on the version of this database existing on June 15, 2015; we retrieved information about patient age, sex, place of residence, occupation, underlying medical conditions, potential exposure to live poultry, dates of illness onset, hospital admission, ICU admission, mechanical ventilation, death, and recovery or discharge.

Ethical Approval

The National Health and Family Planning Commission ruled that the collection of data for laboratory-confirmed cases of influenza A(H7N9) virus infection was part of a continuing public health investigation of an emerging outbreak. The study was therefore exempt from institutional review board assessment.

Statistical Analysis

We analyzed data on the severity of laboratory-confirmed case-patients who were hospitalized for medical reasons (rather than for the sole purpose of isolating them from the community) on the basis of clinical judgment (e.g., those exhibiting complications such as pneumonia). After excluding a small number of case-patients who had mild respiratory symptoms and had been hospitalized only for the purpose of isolation, we estimated the risks of ICU admission, mechanical ventilation, and death after hospitalization (*8*). The number of such cases was small, and inclusion of these cases in analyses did not have any effect on the conclusions. We also excluded case-patients for whom clinical outcomes were not reported from the analysis of severity. We estimated the risk for ICU admission, mechanical ventilation, and death following hospitalization by dividing the number of case-patients who were admitted to ICU, treated with mechanical ventilation, or died by the number of all case-patients with definite clinical outcomes. We derived binomial 95% CIs for each point estimate of severity among hospitalized case-patients.

We examined epidemiologic time-to-event distributions using kernel density methods as described (*9*) and conducted a logistic regression analysis to investigate potential factors affecting the risk for death among hospitalized patients infected with influenza A(H7N9) virus in 3 waves. We estimate adjusted odds ratios and 95% CIs for potential risk factors, including age, sex, place of residence, underlying medical conditions, and time delay from symptom onset to hospital admission. We performed all statistical analyses by using R version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Three major epidemics of human influenza A(H7N9) virus infections have occurred since the first human case was identified in March 2013: spring 2013, winter–spring of 2013–14, and winter–spring of 2014–15 (Figure 1; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-1752-Techapp1.pdf). The median age of confirmed case-patients in each of the 3 epidemics was 61 years, 57 years, and 56 years, respectively. Most patients with laboratory-confirmed cases were men, and a substantial proportion of case-patients had underlying medical conditions (Table 1). Approximately half of the laboratory-confirmed cases in the first wave were detected in municipalities or provincial capital cities such as Shanghai, Hangzhou, and Nanjing; in the second and third waves, most of the case-patients were from smaller cities or rural areas (Table 1; online Technical Appendix).

Although human cases in the first epidemic were concentrated in the Yangtze River delta (Figure 2, panel A), human cases in the second and third epidemics occurred across a broader swathe of the country (Figure 2, panels B and C). A separate study that used virus sequence data showed that the viruses in the different parts of China had diverged by the start of the second epidemic, forming 3 separate clades (Figure 2, panel D) (*10*).

We previously divided the first epidemic into 2 parts—wave 1A for case-patients hospitalized before April 1, 2013, and wave 1B for case-patients hospitalized from April 1 to September 30, 2013—because of higher risks for among case-patients hospitalized before March 31, 2013, the date when the first confirmed human cases of influenza A(H7N9) virus infection were officially announced in China (*11*). We then estimated clinical severity among hospitalized case-patients as measured by hospitalization fatality risk, mechanical ventilation fatality risk, and ICU fatality risk over 3 waves (with wave 1 divided into 2 parts) (Figure 3). Apart from wave 1A, which included mainly retrospective detection of severe cases, some evidence indicated that the severity of hospitalized case-patients increased over time, with statistically significantly higher risk for death among hospitalized patients in wave 3 than for case-patients in wave 1B among those <60 years of age and ≥60 years of age (Figure 3).

We then examined whether these differences in risk for death could be explained by changes in the characteristics of patients across the 3 waves. In a regression analysis, we found that hospitalized case-patients in wave 2 or 3 had a higher risk for death than those in wave 1B after adjusting for patients' demographic characteristics and underlying medical conditions (Table 2). The higher risk for death observed in waves 2 and 3 remained significant after further adjustment for patients' residence and delay from symptom onset to hospital admission (Table 2). Patients ≥60 years of age had a higher risk for death, and rural patients were less likely to die than urban patients.

We conducted a further analysis to investigate the risk for death among patients in different geographic locations where research suggested that circulating influenza A(H7N9) viruses belonged to different genetic clades (Figure 4) (*10*). We found that hospitalized case-patients detected in Jiangxi and Fujian in wave 3 had a lower risk for death than case-patients reported in eastern China, including Shanghai, Zhejiang Province, and Jiangsu Province, particularly in the third wave, as well as in Guangdong Province in southern China. However, the severity of infection in hospitalized case-patients was similar in patients detected in waves 2 and 3 in Jiangxi and Fujian Provinces (Figure 4).

We found estimates of the incubation period (Figure 5, panel A) and the time from illness onset to laboratory confirmation (Figure 5, panel C) consistent across the 3 waves. The time from illness onset to hospital admission

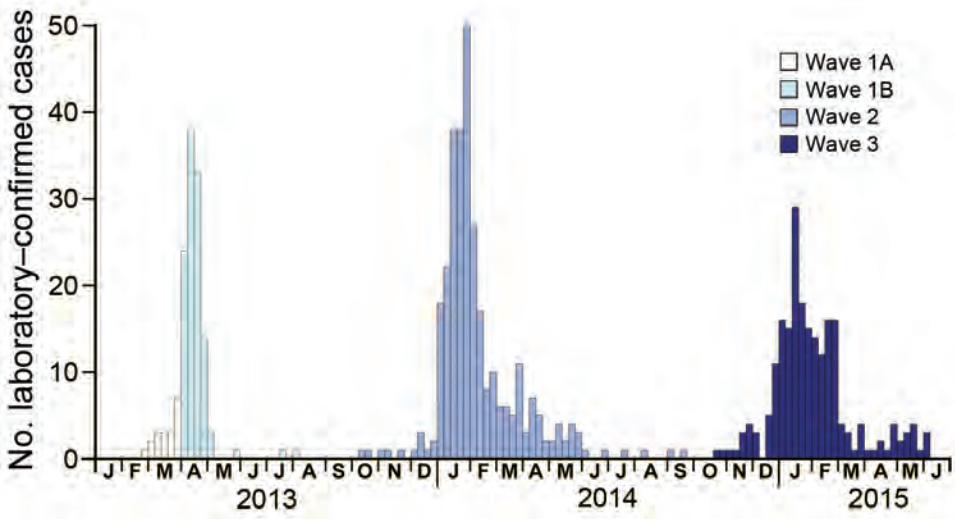

Figure 1. Weekly hospital admissions of human case-patients with laboratory-confirmed influenza A(H7N9) virus infection in 3 epidemic waves, China, 2013–2015.

Table 1. Characteristics of laboratory-confirmed influenza A(H7N9) cases detected in 3 epidemic waves, China, 2013–2015

Characteristic	No. (%) cases			
	Wave 1A, Jan 2013–Mar 2013, n = 19	Wave 1B, Apr 2013–Sep 2013, n = 115	Wave 2, Oct 2013–Sep 2014, n = 306	Wave 3, Oct 2014–Mar 2015, n = 215
Age group, y				
0–15	1 (5)	6 (5)	19 (6)	17 (8)
16–59	8 (42)	47 (41)	151 (49)	113 (53)
60–74	8 (42)	37 (32)	89 (29)	58 (27)
≥75	2 (11)	25 (22)	47 (15)	27 (13)
Median	60	61	57	56
Male sex	13 (68)	81 (70)	212 (69)	154 (72)
Residence*				
Provincial capital or municipality	10 (53)	58 (50)	57 (19)	22 (10)
Other cities	6 (32)	23 (20)	131 (43)	100 (47)
Rural areas	3 (16)	34 (30)	118 (39)	93 (43)
Presence of ≥1 underlying medical condition†	10 (53)	42 (37)	91 (30)	64 (30)
Onset to hospital admission, d				
0–2	2 (11)	12 (10)	41 (14)	32 (18)
3–6	8 (44)	62 (54)	151 (52)	84 (46)
≥7	8 (44)	41 (36)	96 (33)	65 (36)
Poultry exposure				
Any exposure to poultry	15 (79)	91 (83)	165 (81)	116 (74)
Occupational exposure to live poultry	1 (5)	6 (5)	21 (7)	22 (10)
Visited live poultry market	12 (63)	62 (54)	132 (61)	105 (67)
Exposure to sick or dead poultry	0 (0)	3 (3)	3 (1)	7 (5)
Exposure to backyard poultry	6 (35)	48 (49)	34 (20)	4 (2)

*For more information see the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/22/6/15-1752-Techapp1.pdf).
†Only underlying medical disorders associated with a high risk for influenza complications were counted here, including chronic respiratory disease, asthma, chronic cardiovascular disease, diabetes, chronic liver disease, chronic kidney disease, immunosuppressed status, and neuromuscular disorders.

was relatively shorter in more recent waves than in previous waves (Figure 5, panel B). The mean time from illness onset to laboratory confirmation was 8.0 days in wave 1B, 9.0 days in wave 2, and 8.4 days in wave 3 (analysis of variance, $p = 0.44$). The time from final hospital admission to death was longer for patients detected in the third wave than for patients from wave 1B and wave 2, whereas the distribution of the interval was similar for wave 3 and 1A (Figure 5, panel D). The time from hospital admission to discharge was generally similar across different epidemic waves (Figure 5, panel E).

Discussion

In this study, we found some evidence that the estimated risk for severe outcomes in hospitalized patients with influenza A(H7N9) virus infection may have increased in some areas across the 3 epidemic waves over time. Although hospitalized patients in the first part of wave 1 (wave 1A) had more severe cases (perhaps because of ascertainment biases) (*4*), hospitalized patients in the main part of the first wave (wave 1B) generally had less severe cases (Figure 3). The risk for death among hospitalized patients in the second and third waves was higher than the risk for younger and older persons in wave 1B (Figure 3), and this finding could not be fully explained by differences in age, prevalence of underlying medical conditions, or urban/rural residence (Table 2). This difference occurred despite a faster time to admission and similar time to laboratory confirmation of cases (Figure 5).

This observed increase in estimates of severity of cases among hospitalized patients could be real and indicative of an increase in pathogenicity of the virus in humans, or an artifact of case ascertainment biases. In the first hypothesis, apart from potential changes in the virus, an increase in pathogenicity in hospitalized case-patients would also arise if management and treatment of patients differed between the waves (*4*). Infections in the winter in waves 2 and 3 rather than the spring in wave 1B (Figure 1) might be more severe if other pathogens that could cause secondary or co-infections among infected patients were more prevalent. On the other hand, it is possible that prioritized repeating laboratory testing in the early wave of influenza A(H7N9) virus infections and increased laboratory capacity in testing for the virus, particularly among patients with more severe cases over the past 2 years might have led to an artifactual increase in severity of cases among hospitalized patients (*4*). We also observed that a relatively lower proportion of hospitalized patients in the second and third waves were transferred to major regional referral hospitals than in the first wave, which might contribute to a potential increase of clinical severity among hospitalized case-patients if different hospitals were assumed to vary in their capacity for managing these patients.

The relatively lower severity of cases among hospitalized patients estimated in Jiangxi and Fujian Provinces in contrast to the higher severity of hospitalized cases in eastern China and Guangdong Province might be driven

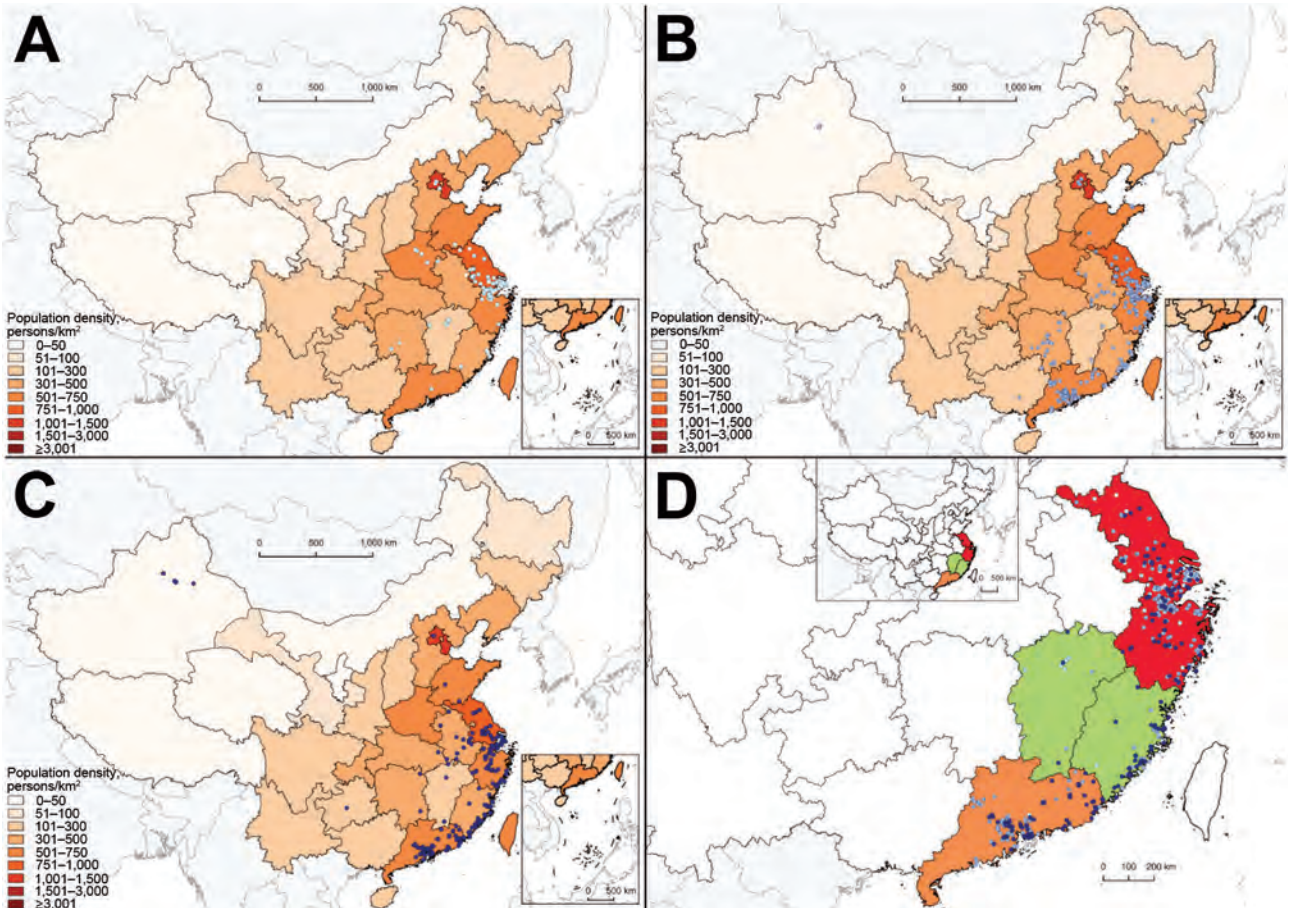

Figure 2. Geographic distribution of human cases of laboratory-confirmed influenza A(H7N9) virus infection, China, 2013–2015. A) Cases detected in wave 1A (white dots) and wave 1B (light blue dots); B) cases detected in wave 2 (medium blue dots); C) cases detected in wave 3 (dark blue dots); D) cases detected in eastern China (red), Jiangxi and Fujian Provinces (green), and Guangdong Province (yellow).

by different factors, although the geographic distribution in severity of cases among hospitalized patients was largely consistent with the 3 genetic clades detected in similar areas (*10*). Infections in Jiangxi and Fujian Provinces might be associated with 1 of the 3 clades identified in wave 2 (clade W2-C), whereas the other 2 virus clades originated from provinces in eastern China (clade W2-A) and Guangdong Province (clade W2-B) (*10*), possibly implying a change in virus pathogenesis. Another explanation for the potential increased severity cases among hospitalized patients in these provinces, other than potential viral changes, is that clinical management may have improved in other provinces that acquired more experience in treating these infections. However, this difference may also be an artifact of differential case ascertainment rather than real differences in severity of cases in hospitalized patients, and we did not have access to individual virus sequence data to confirm that each of the cases in Jiangxi and Fujian Provinces was associated with clade W2-C viruses.

Across the 3 epidemics, the declining median age of case-patients might result from population-level behavioral changes in exposure to live poultry, particularly in potential high-risk groups such as the elderly, as indicated in previous studies (*9*,*12*). Population contact with live poultry decreased in influenza A(H7N9) virus–affected and –non-affected areas after cases were detected in China, although exposure to live poultry in urban and rural areas remained high in the country (*12*). Live poultry markets in China, particularly in cities, have been closing either temporarily or permanently since the first wave in 2013 (*2*,*13*). Some cities severely affected by influenza A(H7N9) virus in the Yangtze River Delta permanently closed all live poultry markets in 2014, which led to a substantial decline in population exposure to the virus and the risk for infection. A relatively higher proportion of rural cases were anticipated in later waves than in earlier waves because contact with backyard poultry instead of commercial live poultry in markets accounted for most poultry exposure for residents of rural and semiurban areas (*14*). The similar geographic

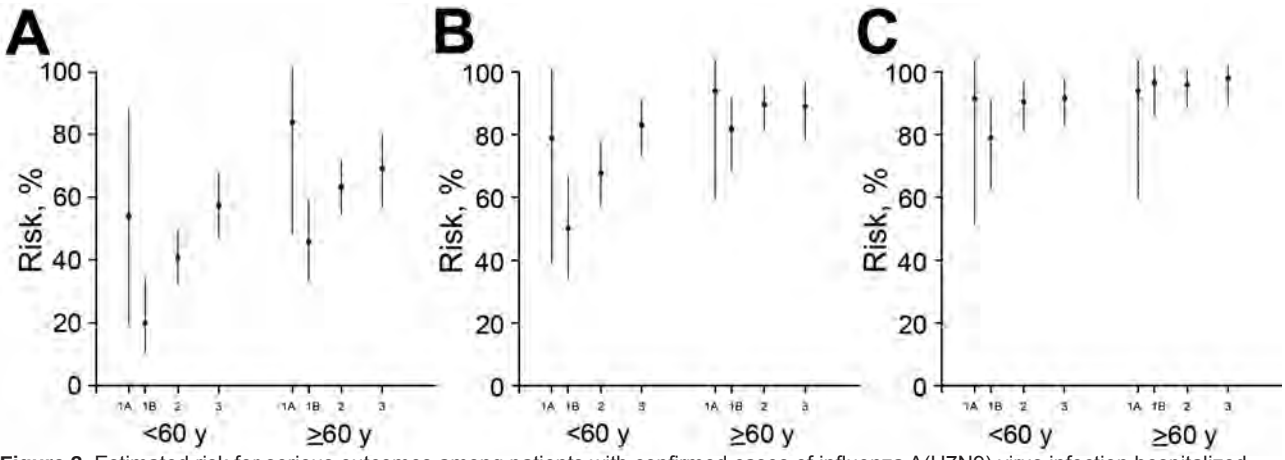

Figure 3. Estimated risk for serious outcomes among patients with confirmed cases of influenza A(H7N9) virus infection hospitalized for medical reasons and 95% CIs, by age and epidemic wave, China, 2013–2015. A) Risk for death; B) risk for death or mechanical ventilation; C) risk for death or mechanical ventilation or intensive care unit admission. The periods covered by waves 1A, 1B, 2, and 3 are shown in Figure 1.

dispersion of case-patients in wave 2 and 3 matches the areas with the highest poultry density in eastern and southern China (*15*) and is also consistent with virus genetic findings that new virus clades originating from eastern China in wave 2 might have been well established and become endemic locally in southern China or other areas (*10*).

Although we have focused on the severity of cases among hospitalized patients, it is also possible to characterize severity in other ways, such as the risk for severe disease among persons with symptomatic influenza A(H7N9) virus infections (*4*,*8*) or the risk for mild and severe disease among all infections. The latter could be estimated if serologic data were available, but few population-based serologic studies of influenza A(H7N9) virus infections have been published (*16*,*17*).

Our study is limited by potential underascertainment of hospitalized case-patients, particularly because of the insufficient capacity of health care facilities to deal with a sudden

Table 2. Comparison of risk of death among patients with laboratory-confirmed influenza A(H7N9) virus infection detected in 3 epidemic waves, China, 2013–2015

Characteristic	Laboratory-confirmed H7N9 deaths, adjusted odds ratio (95% CI)	
	Model 1	Model 2
Wave		
1A	4.88 (1.64–14.53)	5.07 (1.67–15.46)
1B	1.00	1.00
2	2.39 (1.46–3.91)	3.48 (2.00–6.06)
3	3.93 (2.30–6.72)	4.84 (2.66–8.80)
Age group, y		
0–15	0.56 (0.05–5.78)	0.50 (0.05–5.43)
16–59	1.00	1.00
60–74	2.05 (1.38–3.04)	2.09 (1.34–3.26)
≥75	2.88 (1.75–4.74)	2.49 (1.44–4.30)
Sex		
F	1.00	1.00
M	1.00 (0.68–1.46)	0.94 (0.61–1.45)
Underlying medical conditions		
No underlying medical disorder	1.00	1.00
≥1 underlying medical condition*	1.28 (0.88–1.84)	1.22 (0.82–1.81)
Residence		
Residence in a provincial capital or municipality	–	1.00
Residence in other cities§	–	0.52 (0.30–0.89)
Rural residence	–	0.53 (0.31–0.91)
Onset to final hospital admission, d		
0–4	–	1.00
5–7	–	1.21 (0.77–1.90)
≥7	–	1.19 (0.72–1.95)

*Only underlying medical disorders associated with a high risk for influenza complications were counted here, including chronic respiratory disease, asthma, chronic cardiovascular disease, diabetes, chronic liver disease, chronic kidney disease, immunosuppressed status, and neuromuscular disorders.

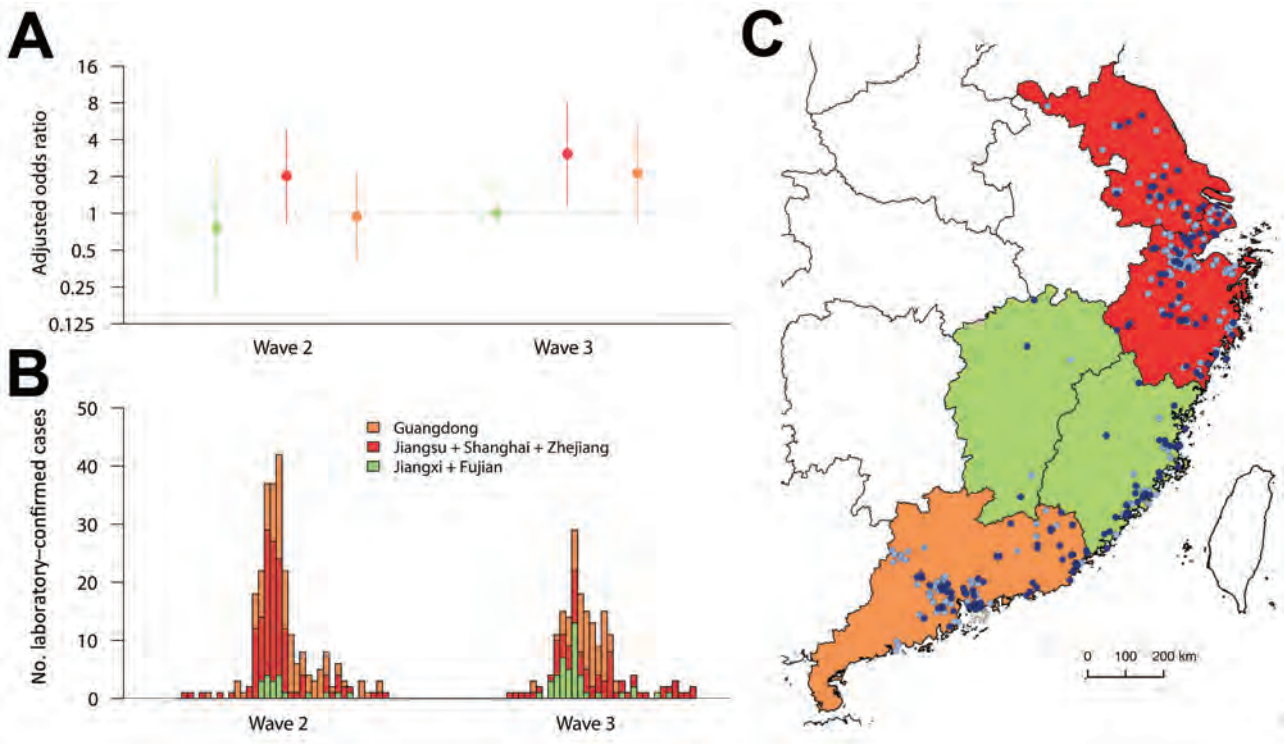

Figure 4. Comparison of risk for death among hospitalized patients with laboratory-confirmed influenza A(H7N9) virus infection detected in 3 areas of China where circulating influenza A(H7N9) viruses might belong to distinct genetic clades, 2013–2015. A) Odds ratios for death, adjusted for age, sex, patient's residence, underlying medical conditions, and delay from onset to hospital admission; B) symptom onsets of case-patients detected in 3 areas; C) geographic distribution of cases detected in 3 areas. The periods covered by waves 2 and 3 are shown in Figure 1.

increased need for diagnosis and treatment of patients with severe cases or a decreasing vigilance on influenza A(H7N9) virus infection in population or health care settings. Ascertainment of hospitalized case-patients could also be potentially affected by changes in clinical management or disease surveillance, particularly of severe acute respiratory infections or unexplained pneumonia through which many cases of influenza A(H7N9) virus infection were detected. However, no major changes were identified in clinical and surveillance practice during the study period. The estimates of the risks of serious outcomes among hospitalized patients could be affected by case ascertainment; limited access to laboratory testing, especially in rural areas; and self-reported exposure data by patients that could be subject to reporting and recall bias.

We did not have detailed information on clinical management such as oseltamivir use, and therefore we could not explore whether differences in treatment were associated with differences in risk for death. Using a composite endpoint to measure severity of cases in hospitalized patients might provide more information on severe outcomes related to this infection than death only, although use of ventilation and admission to ICUs can be limited by hospital capacity and availability of resources. Inaccuracies in the exact dates of hospitalization and uncertainty about a small proportion (5%) of final outcomes might lead to small biases in our estimates but should not change the overall conclusions of this study.

In conclusion, our study explored the epidemiology of human infections with H7N9 virus in mainland China across 3 epidemic waves in 2013–2015. Laboratory-confirmed H7N9 case-patients were younger and more likely to be from small cities and rural areas in wave 2 and wave 3 than in wave 1. Hospitalized H7N9 patients had an increasing risk for death across 3 waves. The increased risk in waves 2 and 3 might imply a changing pathogenesis associated with genetic clades of H7N9 virus that appeared in later epidemic waves or differences in clinical management in different provinces, although case ascertainment bias could not be ruled out.

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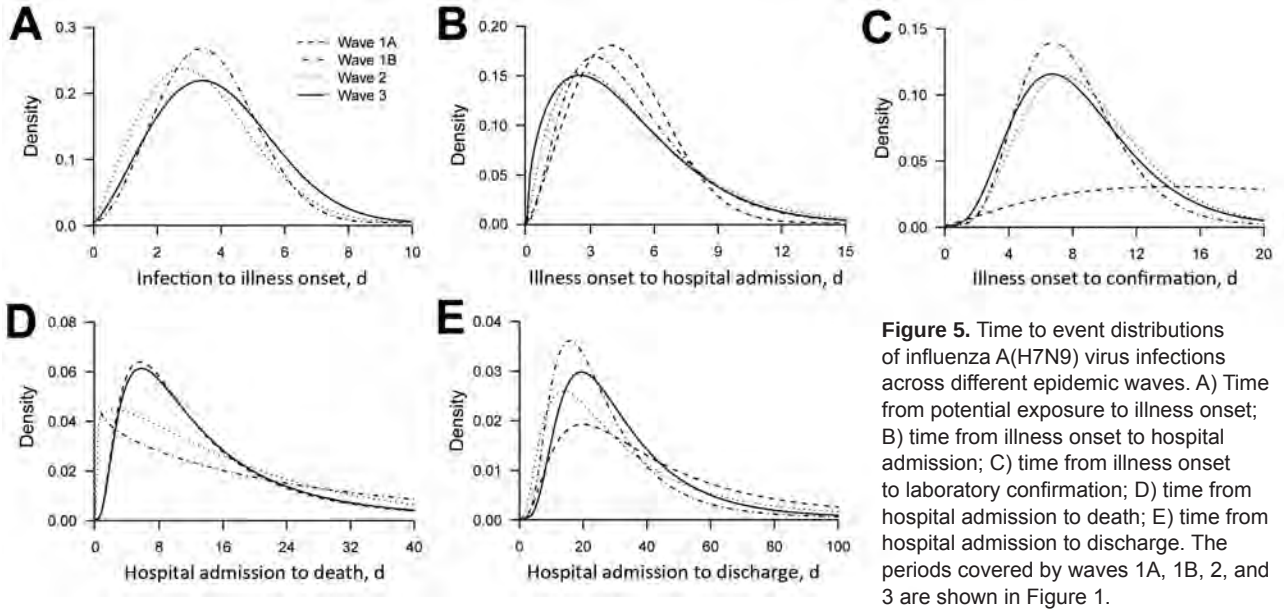

Figure 5. Time to event distributions of influenza A(H7N9) virus infections across different epidemic waves. A) Time from potential exposure to illness onset; B) time from illness onset to hospital admission; C) time from illness onset to laboratory confirmation; D) time from hospital admission to death; E) time from hospital admission to discharge. The periods covered by waves 1A, 1B, 2, and 3 are shown in Figure 1.

where human A(H7N9) cases occurred for providing assistance with field investigation, administration and data collection. We thank Bingyi Yang for technical assistance with Figures 2 and 4.

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Integration of Genomic and Other Epidemiologic Data to Investigate and Control a Cross-Institutional Outbreak of *Streptococcus pyogenes*

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Single-strain outbreaks of *Streptococcus pyogenes* infections are common and often go undetected. In 2013, two clusters of invasive group A *Streptococcus* (iGAS) infection were identified in independent but closely located care homes in Oxfordshire, United Kingdom. Investigation included visits to each home, chart review, staff survey, microbiologic sampling, and genome sequencing. *S. pyogenes emm* type 1.0, the most common circulating type nationally, was identified from all cases yielding GAS isolates. A tailored whole-genome reference population comprising epidemiologically relevant contemporaneous isolates and published isolates was assembled. Data were analyzed independently using whole-genome multilocus sequencing and single-nucleotide polymorphism analyses. Six isolates from staff and residents of the homes formed a single cluster that was separated from the reference population by both analytical approaches. No further cases occurred after mass chemoprophylaxis and enhanced infection control. Our findings demonstrate the ability of 2 independent analytical approaches to enable robust conclusions from nonstandardized whole-genome analysis to support public health practice.

The reported annual incidence of invasive group A *Streptococcus* (iGAS) infection in industrialized countries is ≈3 cases per 100,000 persons per year (*1*–*3*). Incidence is 3-fold higher among persons ≥70 years of age, particularly among the very elderly (*1*,*2*,*4*). Older persons have also been shown to have higher case-fatality rates, including in studies considering particular clinical syndromes, suggesting that age is a risk for death independent of the clinical form of iGAS (*1*,*5*,*6*). Population-based study data estimate incidence among long-term care facility (LTCF) residents as 3.4-fold (*7*), 6.0-fold (*8*), and 7.8-fold (*9*) higher than that among elderly persons living outside institutional settings. Annual incidence estimates among LTCF residents range from 27 (*7*) to 74 (*9*) cases per 100,000 persons. Case-fatality rates are also higher among LTCF residents (*7*–*9*).

Although iGAS mainly occurs sporadically, outbreaks have been recognized in hospitals, particularly in association with surgery and maternity care (*10*,*11*), and in LTCFs, where they appear to be increasing (*12*,*13*). Many iGAS outbreaks go unidentified. Investigations that reviewed residents' medical records in 7 LTCFs in which 1 case of iGAS was reported identified missed outbreaks in 4 of the facilities (*4*). Furthermore, a surveillance study reported that LTCF staff members were unaware of hospital-diagnosed iGAS among residents other than through study feedback (*7*). Three surveillance studies, including 2 that used bacterial subtyping (*7*,*8*), identified iGAS clusters that had not been identified as outbreaks outside the surveillance scheme (*7*–*9*). The delayed identification of outbreaks in LTCFs (*14*,*15*) and clinical geriatric care settings (*16*,*17*) has also been described. Prospective surveillance in Ontario, Canada, identified 20 hospital outbreaks that were of smaller average size, often shorter duration, and more often outside the surgical and maternity settings than was expected based on findings in the nosocomial outbreak literature (*10*). Thus, sporadic disease may include many unidentified small outbreaks. In an LTCF surveillance study (*8*), 40 of 383 isolates were members of 18 clusters of indistinguishable strains, and in another study (*7*), 34 of 134 isolates were associated with 13 clusters, suggesting that 10%–25% of culture-confirmed cases in LTCFs may be associated with outbreaks.

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Genomic data are increasingly available to support the identification and investigation of outbreaks (*18*,*19*), including 2 iGAS outbreaks associated with maternity units (*20*,*21*). In 1 of these studies, isolates collected on 2 consecutive days at a hospital were highly similar, and they were distinguishable from 2 other isolates of the same M protein gene (*emm*) type collected at a later date from 2 other hospitals (*21*). In the second study (*20*), genome sequencing confirmed the relatedness of isolates from 2 patients on a maternity ward with fatal disease and isolates subsequently obtained from another patient, household contacts, and healthcare workers; the study also discriminated these isolates from 9 epidemiologically and geographically separated isolates of identical *emm* type. Genome sequencing may, therefore, separate *Streptococcus pyogenes* isolates with close epidemiologic relationships from the background population, as suggested from findings from some other species of bacteria (*19*).

The purpose of this study was to integrate genomic data with other epidemiologic data in the investigation and control of a cross-institutional outbreak of *S. pyogenes* infection. We assessed approaches to enable robust inferences in the absence of standard analytical methods.

Methods

We used standard epidemiologic and microbiologic approaches to investigate clusters of GAS infection in 2 managerially independent but closely located LTCFs (home A and home B) in Oxfordshire, United Kingdom, in 2013. We also applied genomic sequencing to available isolates, analyzed the data using 2 independent approaches, and performed a systematic literature review to describe evidence for the efficacy of different control strategies.

Literature Search

On February 21, 2014, we searched PubMed, using the terms: ("pyogenes" OR "group A streptococc*") AND ("care" OR "nursing" OR "residential") AND ("home" OR "homes" OR "facility" OR "facilities" OR "setting" OR "settings"). One author (N.D.M.) reviewed the 131 abstracts retrieved by this search to identify those that referred to outbreaks or LTCFs (27 papers) and to population-level surveillance (3 papers). Identified papers were reviewed, references were searched for similar papers, and information was extracted on the control methods used, outcomes, and whether reported outbreaks were each due to a single strain of *S. pyogenes* or involved multiple strains.

Epidemiologic Investigation of GAS Cases in LTCFs

We reviewed medical records for all possible cases of GAS infection among residents of the 2 LTCFs in 2013. In addition to cases already notified to public health authorities, additional possible cases of GAS were identified for review through interviews by public health staff with senior LTCF staff. Healthcare staff used case definitions from the UK national guidance (*22*) to assess residents or staff with symptoms suggestive of GAS infection. Clinically indicated samples were obtained, as were skin and soft tissue infection samples and throat swab samples from residents or staff reporting sore throats. A web-based survey of staff enabled anonymous reporting of symptoms and infection-control practices.

Microbiology

Samples from residents and staff were cultured to detect *S. pyogenes* using standard methods. The Public Health England National Streptococcal Reference Laboratory (Bacterial Reference Department) performed *emm* gene sequence typing on each isolate obtained as previously described (*23*,*24*) using DNA prepared by using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA); quality was determined by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), and quantity was determined by using a Qubit 3.0 Fluorometer and quantitation assays (Thermo Fisher Scientific, Waltham, MA, USA). For sequencing preparation, we used a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and for sequencing, we used a HiSeq 2500 System (Illumina) and the 2 × 100-bp paired-end mode. As a reference dataset to represent the background population, we used published genomes (*20*) and contemporaneous isolates of the same *emm* type from 3 clusters in other areas of the United Kingdom (Table).

Bioinformatic Processing

We used 2 independent approaches to process 39 genome sequences, of which 6 were for isolates from the LTCFs, 9 were for isolates from contemporaneous confirmed cases, and 24 were published genomes (*20*). We used Burrows-Wheeler Aligner software (http://bio-bwa.sourceforge.net/) (*25*) to map reads to *emm* sequence type 1.0 (*emm*1) of *S. pyogenes* MGAS5505 (GenBank accession no. NC_007297-2). Single-nucleotide polymorphisms (SNPs) were discovered by using GATK2 software (Genome Analysis Toolkit v2) (*26*) and filtered by using the following parameters: genotype quality ≥40, ratio of consensus/nonconsensus base ≥0.8, distance to nearest SNP >15, root mean square of the mapping quality of the reads ≥50, number of reads with 0 mapping quality = 0. Reads were independently assembled by using the Velvet algorithm package (*27*), and loci were annotated with the genome annotation of *S. pyogenes* MGAS5005, which resulted in identification of 1,514 non-paralogous genetic loci with sequence data, enabling whole-genome multilocus sequence type (wgMLST) analysis (*28*).

Table. Clinical and demographic characteristics of patients with isolates sequenced in a study integrating genomic and other epidemiologic data to investigate and control a cross-institutional outbreak of *Streptococcus pyogenes**

Area, laboratory no.	Healthcare setting	Age/sex	Source of isolate	Clinical presentation	Outcome
1					
H131441217	Care home	95 y/F	Blood culture	Bilateral periorbital cellulitis, sepsis	Died
H131520646	Care home	91 y/M	Blood culture	Facial cellulitis	Died, ANP
H131640460	Care home	65 y/M	Nasal swab sample	Rash, fever	Recovered
H131620455	Care home	84 y/F	Arm wound swab sample	Arm cellulitis	Recovered
H131720333	Care home	101 y/F	Ear swab sample	Weeping ear	Recovered
H132060515	Care home (CW)	19 y/F	Throat swab sample	Sore throat	Recovered
2					
H131280521	Care home	94 y/F	Blood culture	Severe soft tissue infection	Died
H131100707	Care home	84 y/F	Blood culture	Fever, leg cellulitis, diarrhea, vomiting	NR
H131220725	Care home	93 y/F	Blood culture	Fever, severe cellulitis	Died
H131620436	Care home	78 y/F	Blood culture	Emergency room admission	NR
H131020872	Maternity service	7 d/F	Umbilical wound swab sample	NR	NR
3					
H131180727	Hospital	87 y/M	Blood culture	NR	NR
H130500483	Hospital	60 y/M	Blood culture	Rash, sepsis, suspected CAP	NR
4					
H130620575	Hospital	39 y/M	Pus swab sample	NR	NR
H130620574	Hospital	71 y/M	Cannula site swab sample	NR	NR

*ANP, acute necrotizing pneumonitis diagnosed postmortem; CAP, community-acquired pneumonia; CW, care worker, NR not recorded.

Bioinformatic Analysis

We constructed a maximum-likelihood phylogenetic tree by using RAxML (*29*), a multiple FASTA file of concatenated SNPs. We searched for 68 known superantigen and antimicrobial resistance genes, which were considered present if matches were found with >95% coverage and <10% SNPs difference from known variants.

We summarized allelic relationships by using the Genome Comparator tool within BIGSdb (*30*) and used pairwise differences to estimate a neighbor-joining tree by using MEGA5 software (*31*). The distribution of pairwise allelic differences among isolates was compared in each of 3 categories: within the current suspected outbreak, within an outbreak reported by Turner et al. (*20*), and comparing the current suspected outbreak isolates with all other isolates in the assembled dataset.

Results

Literature Review

Our literature review identified 72 LTCF-associated iGAS outbreaks from individual outbreak reports, summary data identified in surveillance studies, reviews, and laboratory studies (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/14-2050-Techapp1.pdf). These outbreaks included 31 clusters, which were defined as outbreaks on the basis of shared subtypes among ≥2 cases occurring at a facility within 1 year. Subtyping results were available for 22 other outbreaks that were identified by other means. A single or dominant strain was identified for 19 of these outbreaks; multiple strains were reported for the other 3 outbreaks.

Interventions were varied but encompassed 3 broad approaches and showed limited evidence of different outcomes. First, treatment restricted to patients with GAS or to patients with GAS and to their direct contacts with laboratory confirmed infection was associated with disease control in some outbreaks. However, in 1 outbreak, disease recurred after 2 rounds of this selective treatment, so mass chemoprophylaxis was administered within the LTCF. Second, in 2 reported outbreaks, all staff and residents were screened for GAS and if positive, they were offered chemoprophylaxis. This screening and treatment was associated with disease recurrence and repeated screening and treatment. Additional cases of iGAS were diagnosed between decisions to screen and provide chemoprophylaxis and to actually implement chemoprophylaxis. Third, in all identified reports, chemoprophylactic treatment of all staff and residents was associated with control of iGAS, although in 1 incident, persistent infection was shown in a resident who had a gastrostomy tube.

The literature review showed that screening detected carriage rates of <10% among LTCF residents, with 2 exceptions, for which carriage rates were 20% and 16%. As previously described, carriage rates were lower among LTCF staff than residents (*12*,*13*).

Epidemiology of Cases in Home A and Home B

After 1 iGAS case each was identified in homes A and B, advice was given to the LTCF managers by Public Health England on infection control and how to identify other GAS-compatible infections in residents and staff. Although the 2 LTCFs were geographically close to each

other, neither home could initially identify any links with the other. Mass chemoprophylaxis was initiated at home A the day after a second case was reported and at home B after GAS (not iGAS) was confirmed in another resident who had cellulitis (Figure 1). Further investigation identified 3 staff members who had worked in both of the managerially independent homes; 2 of these staff members had GAS-compatible symptoms. Of 41 staff who responded to the anonymous survey, 38 identified their roles as a healthcare assistant (18 persons), cleaner (6 persons), manager or administrator (6 persons), nurse (3 persons), or other (5 persons). One respondent reported working at both homes, and 1 reported working at home B and in the community. Of the 41 respondents, 32 reported no symptoms, 7 reported sore throats, 1 reported a rash on the neck, and 1 reported illness without specifying symptoms.

Twelve possible GAS infections were not confirmed by culture. One of the infections was on the scalp of a resident at home A; the patient, who had a temperature >39°C and hallucinations, was treated with antimicrobial drugs and tested negative on subsequent samples. Another possible infection was on the eyelid of a resident at home B. Eight possible infections were in persons who worked at home A and who reported symptoms consistent with pharyngitis; 1 of the 8 staff members also worked at home B. Another person who worked at both LTCFs reported having paronychia that was treated with antimicrobial drugs, and 1 staff member at home B reported a recurrent skin infection.

Microbiology

S. pyogenes isolates were obtained from 6 of 13 specimens from symptomatic residents and staff at homes A and B. Two patients in home A, 1 of whom died, had *S. pyogenes*–positive blood cultures and facial cellulitis. One patient in home B had periorbital cellulitis and GAS bacteremia. Two other patients in home B had confirmed GAS; 1 of these patients had cellulitis and systemic symptoms, including fever, but no sample from a normally sterile site, and the other patient had an outer ear infection. One staff member working in both institutions had GAS pharyngitis.

Isolate Relatedness, Antimicrobial Resistance, and Virulence Genes

All 6 isolates from the cross-institutional *S. pyogenes* outbreak were T-type 1 (phenotypic typing), *emm*1, and *sic* (streptococcal inhibitor of complement gene) sequence type 1.02. Nine isolates from 3 contemporaneous putative clusters in the United Kingdom were available for comparison. Whole-genome sequencing yielded, on average, 85-fold depth for these 15 isolates. All shared 7-locus MLST sequence type 28 with 24 published genomes from the United Kingdom (*20*). Analysis of all 39 genomes against the *S. pyogenes* MGAS5005 genome demonstrated 334 SNPs. Separation of the isolates from the cross-institutional *S. pyogenes* outbreak from all other analyzed isolates was strongly supported (98% bootstrap value). Isolates from the staff member and 3 residents of the 2 homes were positioned in the same monophylogenetic clade and, in each case, differed from the isolates from the remaining 2 infected residents by a single SNP (Figure 2). Gene-by-gene analysis (wgMLST) also clustered the LTCF outbreak (Figure 2). The 6 isolates showed pairwise differences from each other at 1–9 (median 4) of the 1,514 loci and pairwise differences from all other isolates at ≥13 (median 37) loci (Figure 3). The outbreak reported by Turner et al. (*20*) showed similar within-outbreak pairwise differences at 0–9 (median 5) loci. Putative contemporaneous clusters X and Y were also supported by genome sequencing.

We determined the presence or absence of 68 virulence or antimicrobial drug–resistance–associated genes in the genomes sequenced for this study (online Technical Appendix Table). With the following exceptions, all isolates had the same genotype: *speC* was present only in isolates Y1 and Y2 (Figure 2), MF4 was present only in isolate TR7, and *spd*3 and *spy*1438 were present in all isolates except TR7. This varied presence of these 4 bacteriophage-associated genes may reflect phage mobility within *S. pyogenes* (*32*). Genes and mutations associated with macrolide and tetracycline resistance (homologs of *mefA*, *tetM*, and *tetO*) and fluoroquinolone-resistance mutations (in genes *parC* and *gyrA*) were not found in comparison with the fluoroquinolone-susceptible reference strain ATCC700294 (GenBank accession no. AF220946.1);

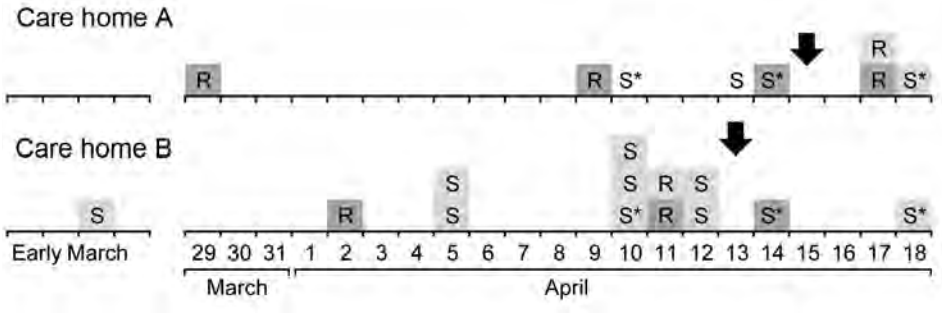

Figure 1. Onset dates of group A *Streptococcus* infection in 2 long-term care facilities in Oxfordshire, United Kingdom, 2013. Arrows indicate initiation of chemoprophylaxis; S, staff; R, resident; * indicates staff who worked in both homes. Dark gray shading indicates laboratory-confirmed infections; light gray shading indicates nonconfirmed infections.

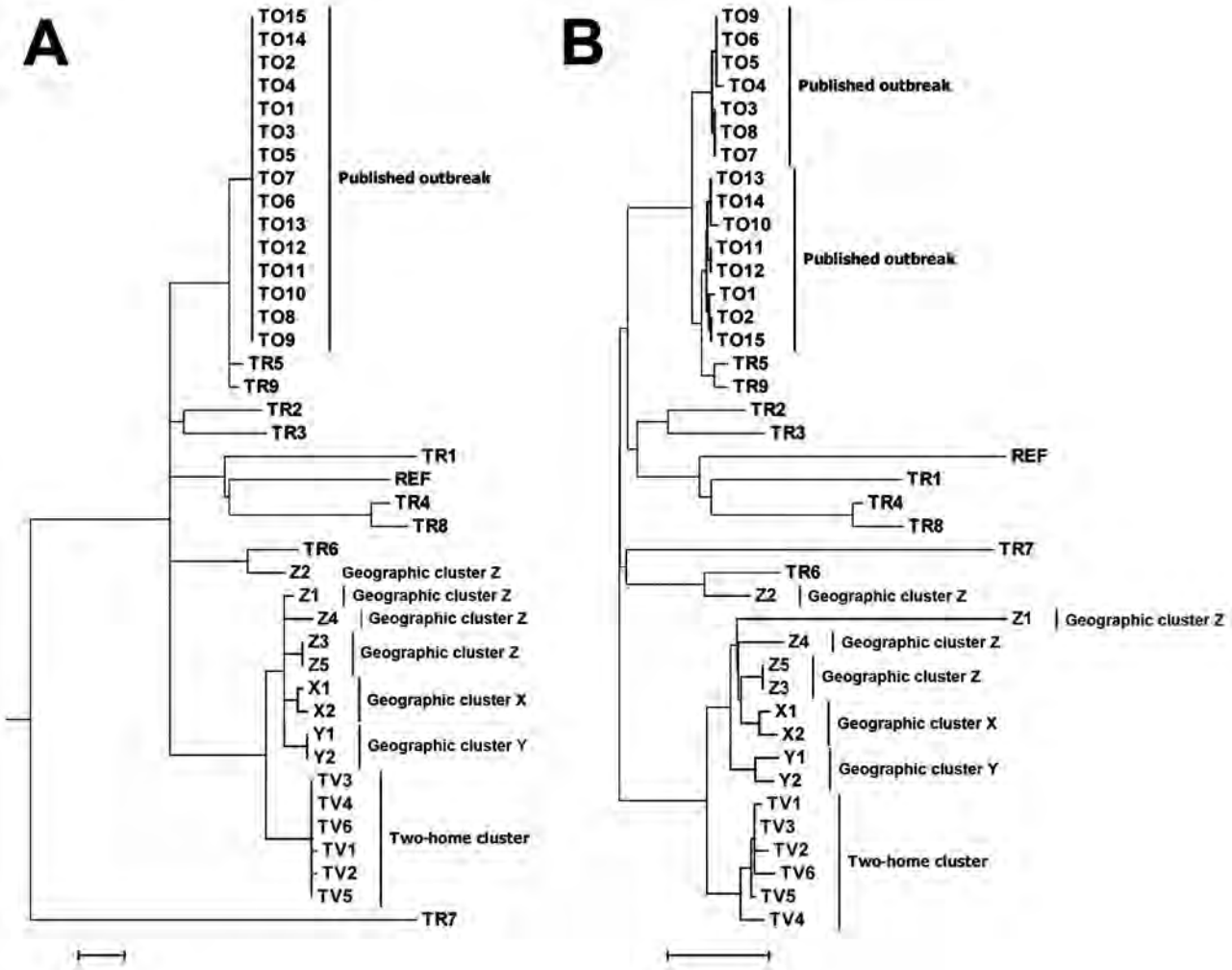

Figure 2. Genetic relatedness of isolates from a cross-institutional *Streptococcus pyogenes* outbreak in Oxfordshire, United Kingdom (indicated by TV plus isolate number); an outbreak described by Turner et al. (*20*) (indicated by TR and TO plus isolate number for reference and outbreak isolates, respectively); and 3 geographic outbreak clusters in the United Kingdom around the time of the TV outbreak (indicated by X, Y, or Z plus isolate number). Dendrograms are based on a single-nucleotide polymorphism maximum-likelihood phylogenetic tree constructed by using RAxML (*29*) (A) and on a neighbor-joining tree constructed from the allelic differences distance matrix (B). Scale bars indicate 10 single-nucleotide polymorphism differences (A) and 15 allelic differences (B).

however, a nonsynonymous SNP (T2393C) of the *par*C gene was identified in all strains. Mutations in covR/S (the *S. pyogenes* regulatory system), which are reported to be associated with strain hyperinvasiveness, were seen in all 15 isolates, in common with strain MGAS5005. No unique mutations were identified in any of the isolates.

Discussion

We have described an iGAS outbreak across 2 managerially independent LTCFs, in which some staff worked in both facilities on separate employment contracts. Clustering in the temporal and genomic dimensions supported GAS transmission within and between these settings. Shared staff across care settings without managerial awareness may be common in this sector, in which low pay and part-time working patterns are common. Loss of pay while absent from work has been recognized as a risk factor in LTCF outbreaks (*33*,*34*). Loss of pay when excluded from work in 1 setting may also contribute to spread of infection to other settings, unless staff members understand that this exclusion applies to similar work in other settings and comply with the exclusion from work in all settings.

We found 1 other report of an iGAS outbreak across 2 LTCFs; the article explored the use of subtyping but did not include epidemiologic details of the outbreak (*35*). Spread of infection between institutions, resulting in apparently sporadic cases or small outbreaks in each, may be difficult to identify as a single outbreak. Our genomic data, triangulated with other descriptive epidemiologic data, supported our conclusion that this was a single outbreak. However,

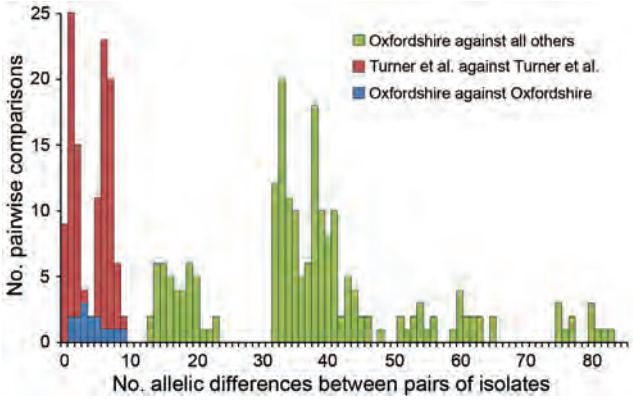

Figure 3. Pairwise allelic differences (across 1,514 genetic loci) among 6 isolates from a cross-institutional *Streptococcus pyogenes* outbreak in Oxfordshire, United Kingdom, and other isolates. Green indicates differences between each of the 6 Oxfordshire outbreak isolates and each of the other 33 isolates that occurred in other geographic areas in the United Kingdom around the time of the Oxfordshire outbreak or were reported by Turner et al. in 2013 (*20*). Red indicates differences between outbreak isolates from the cluster described by Turner et al. (*20*). Blue indicates differences between each isolate in the Oxfordshire outbreak compared with each of the other 5 isolates in the outbreak.

the application of these techniques is relatively untried: neither standardized methods nor extensive national, genome-sequenced reference populations are in place for *S. pyogenes*, and the population biology of this species at the whole-genome level is not fully described.

We dealt with the lack of an established analytical method, which would confirm that isolates are part of a single outbreak, and uncertain *S. pyogenes* population genomics by using 2 types of analyses (i.e., whole-genome multilocus sequence and single-nucleotide polymorphism analyses) and bioinformatics pipelines that did not rely on shared assumptions about the population biology of this species. The identification of core SNPs from a reference-based assembly, exclusion of SNPs that are nearby to avoid an excessive effect from single recombination events, and subsequent generation of an SNP-based phylogeny echo the techniques used in other analyses of this species (*20*,*21*). In our analysis of wgMLST data, we used a reference-free assembly method and assessed the number of shared and discordant alleles across the dataset without making assumptions on processes giving rise to allelic variation (*28*). The replication of clustering by different and independent approaches adds credibility to the epidemiologic inferences drawn. In the absence of extensive genome-sequenced reference populations relevant to the incident under investigation, we used a set of isolates that were identical to the isolates of interest by conventional *emm* sequence typing methods (*36*); some of the isolates from this set were also from the same time period and country as the isolates of interest. Discriminating isolates from other isolates of the same *emm* type can thus demonstrate discrimination from the population of *S. pyogenes* strains as a whole and support outbreak management when appropriate population-based, genome-sequenced reference datasets are unavailable.

Each approach clearly identified the outbreak cluster and differentiated the isolates in this outbreak from other contemporaneous isolates within the same *emm* gene sequence type (*emm*1) and MLST type, similar to findings by Turner et al. (*20*). SNP analysis indicated isolates from homes A and B were separated from all other clades by at least 14 SNPs, but they differed from each other by a maximum of 2 SNPs. Gene-by-gene analysis showed a median of 4 allelic differences in pairwise comparisons within the outbreak across 1,514 genetic loci; this finding contrasts with a median of 37 allelic differences between isolates from the current outbreak and 33 other genomes of the same *emm* type. Gene-by-gene analysis of data from the outbreak reported by Turner et al. (*20*) showed almost identical within-outbreak variation (median of 5 differences, range 0–9) (Figure 3). Thus a similar amount of allelic variation was present in the cross-institutional cluster and the cluster reported by Turner et al. (*20*). These findings contrast with much greater variation when compared with other isolates from the same *emm* type. These 2 incidents had epidemiologic data indicating likely transmission over a period of days to a few weeks. This range of variation may therefore be an estimate for expected variation in the transmission systems generating small, short-lived GAS outbreaks.

Additional well-characterized outbreaks with genome-sequenced isolates will enable fuller empirical validation of these results. More long-lived outbreaks may be associated with greater variation, and, indeed, multistrain streptococcal outbreaks can arise where a mobile genetic element can support the increase of several pathogenic lineages acquiring it (*37*). In contrast to the clear and similar discrimination of the cross-institutional outbreak isolates from other *emm*1 isolates by each analytical approach, no marked structure was shown by either analysis among the outbreak isolates. The limited extent of evolution occurring, as indexed and analyzed by our approaches across the available genome, does not enable inference on particular transmission pathways within this short-lived, single-clone outbreak.

Our literature review showed that most reported LTCF iGAS outbreaks have been caused by a single strain, and many outbreaks go unrecognized. The use of genome sequence analysis may distinguish epidemiologic clusters from background isolates, enabling detection of a large proportion of currently missed LTCF outbreaks. Given literature-based estimates that 10%–25% of apparently sporadic iGAS cases in LTCFs may belong to outbreaks (*7*,*8*), the integration of genome sequencing into iGAS surveillance in this population might be justified by this purpose alone.

Detection of missed outbreaks may enable identification of risk factors that contribute to the outbreaks. Genomic surveillance could also support detection and investigation of transmission events between LTCFs and other settings. The relatively low population-level incidence of iGAS (*1*–*3*) may make genome sequence surveillance financially feasible in countries that have microbiologic iGAS surveillance systems in place.

This outbreak was controlled after use of mass chemoprophylaxis and standard infection-control measures. Infection-control measures are widely reported as part of iGAS outbreak control methods; chemoprophylaxis shows more variation. There was, on average, better control in outbreaks in which mass chemoprophylaxis was undertaken, and there were reports of further cases during the wait for screening test results (*38*,*39*). However, the evidence base is limited and carriage rates are low among residents and staff (*12*,*13*), and some argue that mass chemoprophylaxis is inappropriate (*40*). The ability to identify large numbers of outbreaks early, as appears possible by genomic surveillance, may offer a sampling frame to generate more reliable data for the role of chemoprophylaxis by analyzing outcomes across a large number of outbreaks by the approach used or through trials of different approaches.

In summary, we investigated an iGAS outbreak in 2 institutions by integrating pathogen genomic epidemiology to infer epidemiologic relatedness. Independent bioinformatic and population genetic approaches enabled credible conclusions in the absence of a standardized approach. The excess burden of iGAS among elderly residents of LTCFs, the large proportion of cases that are associated with undetected outbreaks, and the consequent opportunity to improve the evidence base for control support consideration of genomic surveillance of iGAS.

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Infectious Disease Risk Associated with Contaminated Propofol Anesthesia, 1989–2014[1]

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Release date: May 11, 2016; Expiration date: May 11, 2017

Learning Objectives

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

- Assess the epidemiology of propofol-related infection.
- Identify the procedure associated with the highest number of propofol-related infections.
- Assess different sources of propofol-related infection.
- Evaluate other contributing factors in propofol-related infections.

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Administration of propofol, the most frequently used intravenous anesthetic worldwide, has been associated with several iatrogenic infections despite its relative safety. Little is known regarding the global epidemiology of propofol-related outbreaks and the effectiveness of existing preventive strategies. In this overview of the evidence of propofol as a source of

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infection and appraisal of preventive strategies, we identified 58 studies through a literature search in PubMed, Embase, and Lilacs for propofol-related infections during 1989–2014. Twenty propofol-related outbreaks have been reported, affecting 144 patients and resulting in 10 deaths. Related factors included reuse of syringes for multiple patients and prolonged exposure to the environment when vials were left open. The addition of antimicrobial drugs to the emulsion has been instituted in some countries, but outbreaks have still occurred. There remains a lack of comprehensive information on the effectiveness of measures to prevent future outbreaks.

Globally, propofol is the most frequently used intravenous (IV) anesthetic for the induction and maintenance of general anesthesia (*1*). The chemical in propofol, 2,6-diisopropylphenol, is insoluble in aqueous solutions, so the solution is formulated as a nonpyrogenic emulsion containing soybean oil, purified egg phosphatide, and glycerol. This anesthetic has several favorable characteristics as a hypnotic agent, including rapid onset and elimination times, predictability and ease of titration, and a strong overall safety profile (*1*). Despite these benefits, propofol has been associated with the occurrence of healthcare-related infections (*2*–*4*). The potential to cause infections has been attributed to the lipophilic nature of propofol formulations, a medium that strongly supports extrinsic bacterial growth at room temperature (*5*).

In 1989, the US Food and Drug Administration (FDA) approved propofol as an induction agent for general anesthesia. Since then, numerous reports of propofol-related infections have generated strong concern among public health officials (*6*), leading to the institution of strict aseptic handling protocols and, in some countries, the additional requirement of instilling antimicrobial additives to propofol formulations. In many countries, however, no such standards have been adopted, largely because of the additional costs involved and the argument that insufficient evidence exists for the effectiveness of such antimicrobial additives. In the United States, the American Society of Anesthesiologists and the Centers for Disease Control and Prevention have jointly recommended strict adherence to aseptic handling protocols of propofol (*7*).

There continues to be a lack of awareness of the occurrence of infections related to propofol use among healthcare providers (*6*). To our knowledge, no previous review has evaluated the characteristics of propofol-related outbreaks and the evidence supporting the use of existing preventive strategies. The aim of this article is to present an overview of the evidence of propofol as a source of healthcare-related infections.

Selection Criteria for Studies

We reviewed studies that reported on the occurrence of propofol-related infections in human subjects: single case reports, case series, retrospective chart reviews, cross-sectional studies, prospective follow-up studies, and registries published in the form of short communications or original contributions. We also reviewed laboratory studies reporting on propofol as a microbiological reservoir and studies evaluating the effectiveness of bacterial growth retardants in propofol formulations. Studies reporting on propofol-related infections in animals were excluded, as were reports found in newspaper articles and government Internet sites. The latter sources, because they are not peer-reviewed articles, provide insufficient evidence for the association between propofol and infectious events.

Search Methods for Identification of Studies

We identified appropriate articles by searching PubMed, Embase, and Lilacs for reports published during January 1, 1989–September 30, 2014. The search was limited to articles published in or after 1989, which is the year propofol was introduced to clinical practice. The electronic search strategy for PubMed was "(propofol OR Diprivan) AND (infection OR outbreak OR contamination)." The search strategy was translated in accordance to the other database Boolean operators and was not limited by language. For this study, outbreak was defined as ≥2 cases.

Study Selection

The titles and abstracts retrieved during the literature search were screened by 2 co-authors (A.Z.V., K.E.V.) independently for inclusion criteria. The full text of the selected studies was retrieved and related reference lists screened to identify additional publications. Disagreements on the selection of studies were solved by a third co-author (D.S.).

Data Extraction and Management

We stratified articles into 4 categories. 1) Features of propofol-related outbreaks worldwide (e.g., year, geographic localization, type of procedure, route of propofol contamination, type of microorganism isolated, number of cases, and number of deaths) were compiled for each outbreak during 1989–2014. Outbreak reports published more than once were occasionally encountered; only the most representative study was included to prevent data duplication. In addition, we took a conservative approach in extracting data from single cases if strong associations between propofol exposure and the infectious event were reported. 2) Laboratory-based evidence of propofol as a microbiological reservoir was retrieved regarding the frequency of contaminated propofol syringes, vials, or infusion lines used in operating rooms (ORs) or intensive care units (ICUs). 3) Epidemiologic evidence concerning

the risks of infections associated with propofol was confirmed by case-control, cohort, and clinical trial studies (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-0376- Techapp1.pdf). 4) Studies on propofol formulations were used for evaluation of the data suggestive of a reduction in microbial growth associated with specific propofol formulations.

We screened 465 abstracts and chose 53 to examine: 25 outbreak reports, 3 reports of single cases, 7 laboratory-based studies on propofol, 10 analytical studies that supported the healthcare effect of contaminated propofol in terms of the risk for infection, and 8 studies of the alternative propofol formulations (Figure 1). We retrieved an additional 5 articles by using references cited in 3 of the initial 53 articles to expand on specific points.

Worldwide Occurrence of Outbreaks Associated with Propofol-Based Anesthesia

The risk for postoperative infection depends on a variety of factors, including wound class (i.e., clean, clean-contaminated, contaminated, or dirty), the condition of the patient, type and length of surgery, use of antimicrobial drugs, and perioperative events. During the past 2 decades, several episodes of sepsis worldwide have been reported to be associated with propofol administered by syringe injection or used as a continuous infusion (*2–4,6,8–15*) (Table 1). These cases were reported in industrialized countries; no outbreaks have been documented in developing or low-income countries, such as those in Latin America, Africa, or Asia (Figure 2), likely as a consequence of deficiencies of surveillance programs and poor data acquisition regarding the frequency of contaminated propofol. Propofol-associated infections likely occur in developing countries with relatively higher frequency than in industrialized countries, related to the common problem of economic restraints and reduced use of universal precautions within the healthcare systems, leading to reuse of syringes and use of vials for multiple patients.

Since this anesthetic was introduced, 20 propofol-related infectious disease outbreaks have been reported worldwide, affecting 144 patients and resulting in 10 deaths; these outbreaks have lasted between 8 hours and 65 days (Table 1). However, many outbreaks related to propofol are likely undocumented and such reports do not reflect ongoing sporadic infections that are likely to be linked to propofol use.

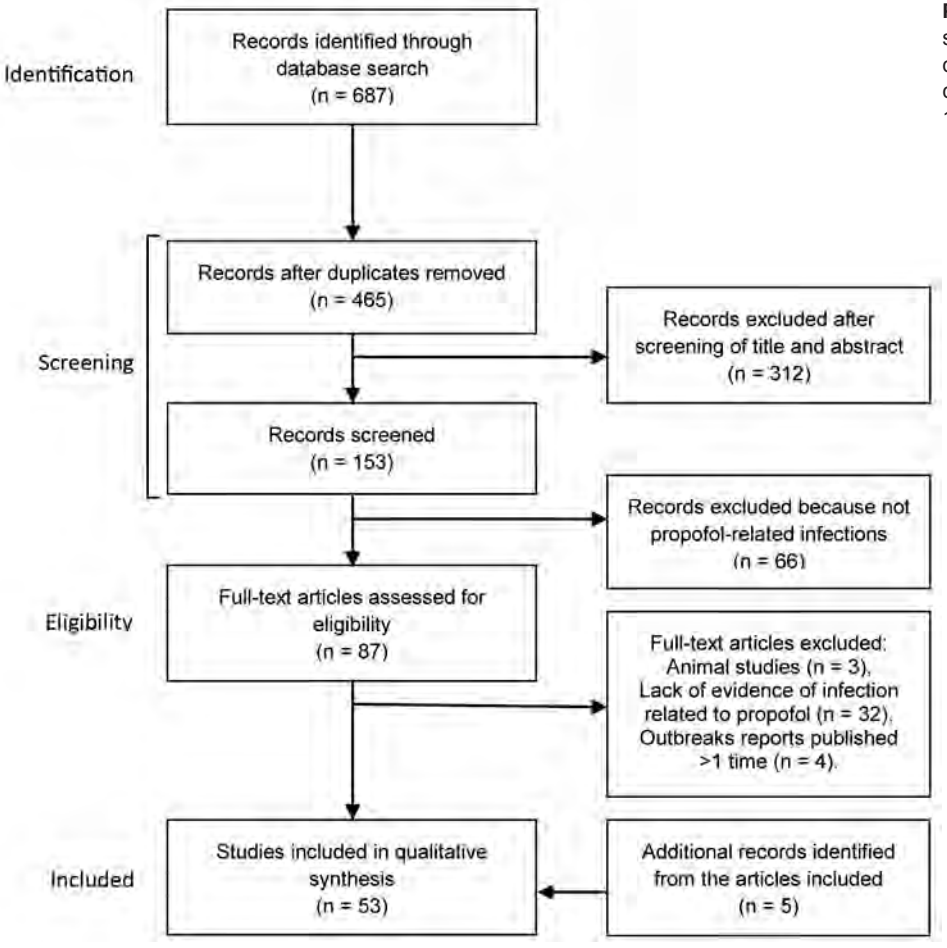

Figure 1. Flowchart of the selection of studies of infectious disease risk associated with contaminated propofol anesthesia, 1989–2014

Table 1. Summary data of iatragenic disease outbreaks associated with contaminated propofol reported worldwide, 1989–2014*

Location†	No. outbreaks	Duration, d‡	Year§	Type of infection	Type of surgery	Microorganism¶	No. cases	No. (%) deaths	Ref.
California, USA	1	8	1990	SSI	ND	*Staphylococcus aureus*	5	ND	(*6*)
Illinois, USA	1	5	1990	BSI, endophthalmitis	Endarterectomy, arthroscopy, dilation and curettage	*Candida albicans*	4	0	(*6*)
Maine, USA	1	2	1990	BSI	ND	*Moraxella osloensis*	2	0	(*6*)
Michigan, USA	1	14	1990	BSI, SSI	Orthopedics, gynecology, biopsy	*S. aureus*	13	ND	(*6*)
Houston, Texas, USA	1	65	1990	BSI, SSI, endophthalmitis	ND	*S. aureus*	16	2 (12.5)	(*2*)
United States	1	11	1990	BSI	General, urology, gynecology	*Enterobacter agglomerans*	4	0	(*2*)
United States	1	16	1992	BSI, SSI	Orthopedics	*Serratia marcescens*	6	0	(*2*)
United States	1	7	1992	ND	Gynecology	ND	4	0	(*2*)
Paris, France	1	0.33	1994	BSI	ND	*Klebsiella pneumoniae*	4	0	(*10*)
Atlanta, Georgia, USA	1	1	1997	BSI	Electroconvulsive therapy	*S. aureus*	5	1 (20)	(*8*)
Reggio Emilia, Italy	1	1	2001	Hepatitis C	Gynecology	HCV	5	0	(*11*)
Toronto, Ontario, Canada	1	ND	2001	BSI, SSI	Orthopedics, gastrointestinal, vascular, neurosurgery, pulmonary	*S. marcescens*	7	2 (28.6)	(*4*)
Berlin, Germany	1	ND	2002	BSI	ND	*E. cloacae*	4	2 (50)	(*3*)
Melbourne, Victoria, Australia	2	2	2003	Hepatitis C	Arthroscopy	HCV	6	ND	(*9*)
Las Vegas, Nevada, USA	1	2	2008	Hepatitis C	Endoscopy	HCV	9#	1 (11.1)	(*13*)
Alicante, Spain	1	ND	2010	Systemic candidiasis, endophthalmitis	Endoscopy	*C. albicans*	27	0	(*14*)
New York, USA	1	2	2010	Hepatitis C and B	Endoscopy	HCV, HBV	12	ND	(*12*)
Rotterdam, the Netherlands	1	2	2010	BSI, SIRS	Orthopedics, gynecology	*K. pneumoniae, S. marcescens*	7	2 (28.6)	(*15*)
Hsinchu, Taiwan	1	1	2013	Endotoxemia	Endoscopy, colonoscopy	ND	4	0	(*16*)
Total	20						144	10 (9.3)**	

*Outbreak, ≥2 cases; ND, not described in publication; BSI, bloodstream infection; SSI, surgical site infection; HCV, hepatitis C virus; HBV, hepatitis B virus; ref., reference; SIRS, systemic inflammatory response syndrome.
†Location where the outbreak emerged.
‡Duration of the outbreak.
§Year of publication.
¶Causative microorganism implicated in outbreak.
#Results of HCV tests of 60,000 persons (who underwent procedures requiring anesthesia at the same clinic from March 1, 2004 through January 11, 2008) are pending. The health department identified an additional 106 infections that could have been linked to the multi-dose vials of propofol. (http://www.cdc.gov/hepatitis/Outbreaks/HealthcareHepOutbreakTable.htm).
**Death rate was estimated taking into account only the published outbreaks with mortality data reported (n = 108).

Outbreaks have been associated with widely diverse types of procedures in both intensive care units (ICUs) and operating rooms (ORs) (Table 1). Although no specific clinical procedure has been causally related to propofol-related infectious outbreaks, endoscopic procedures have been the most frequently associated with propofol-related infections during the past 20 years.

Contrary to some healthcare perceptions, none of the reported outbreaks to date have been correlated with intrinsic batch-contamination of propofol. Nevertheless, some peer-reviewed reports of manufacturing deficiencies exist, as well as ≥2 outbreaks probably linked to intrinsic contamination; however, the latter were published on government Internet sites and therefore are not included in this review. A US

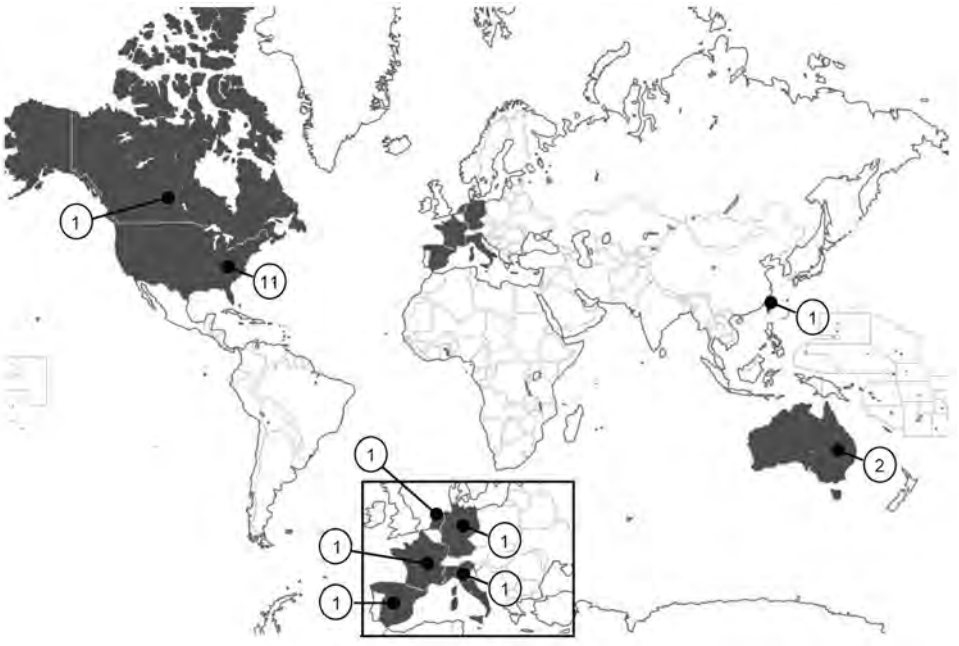

Figure 2. Geographic distribution of propofol-related infectious disease outbreaks worldwide, 1989–2014. Values indicate number of outbreaks for each country.

government report traced 41 cases of infection in 2009 to 1 contaminated batch (http://www.fda.gov/Safety/Recalls/ArchiveRecalls/2009/ucm172474.htm); 9 other possible cases were traced in Australia in 2014 (https://www.tga.gov.au/alert/propofol-provive-and-sandoz-propofol-1-emulsion-injection-all-sizes-and-all-batches-update-3).

Distribution of propofal-related outbreaks has been widespread geographically (Figure 2) and temporally (Figure 3). The United States has reported 11 outbreaks, the highest number of outbreaks during the assessed period, averaging 1 every 2 years and accounting for 55% of all reported outbreaks worldwide. According to the list of healthcare-associated hepatitis B and C virus (HBV and HCV, respectively) outbreaks reported by the US Centers for Disease Control and Prevention during 2008–2014, the coincident recent exposure to propofol was considered a factor leading to the screening of >60,000 patients (http://www.cdc.gov/hepatitis/Outbreaks/PDFs/HealthcareInvestigationTable.pdf).

In a 2003 study of the literature on hospital-acquired infections worldwide, Vonberg and Gastmeier calculated a mortality rate of 13.8% related to administration of propofol (*17*). The data we collected indicate an estimated mortality rate in propofol–associated infections of ≈9.3% (range 0%–50%) (Table 1). This value only summarizes the current published literature describing propofol–related outbreaks, and thus it may not represent the true magnitude of the problem (*2*). According to an inspection of data held by AstraZeneca, 345 cases of postoperative infections or febrile syndrome occurred after propofol use in the United States during November 1989–November 2004; unfortunately, data for these cases were archived and not published (*2*,*18*).

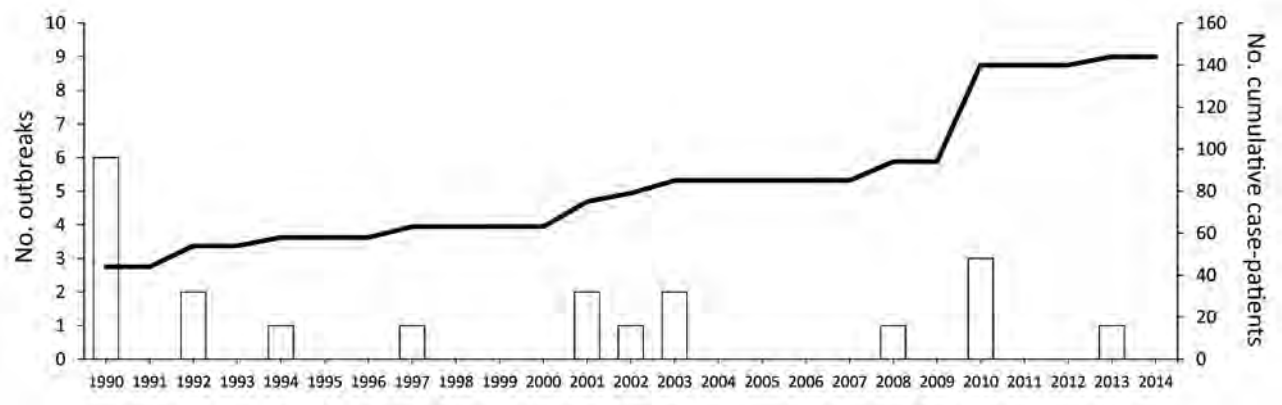

Figure 3. Timing of propofol-related infectious disease outbreaks worldwide during 1989–2014. An outbreak was defined as ≥2 cases. Dashed line indicates cumulative no. case-patients (secondary y-axis).

Mechanisms of Contamination of Propofol Formulations

Microbiological contamination of propofol lipid emulsions may occur from the environment either during manufacture (intrinsic) or after vial opening (extrinsic), the latter of which is the most frequent. The horizontal transmission of pathogens in anesthesia begins with the breach of handling precautions by anesthesia providers of devices or drugs. Frequently neglected precautions during the induction and maintenance of anesthesia include hand hygiene and protection against incidental propofol contact with the environment (*19*–*21*). In addition, surreptitious use of IV anesthetics by drug-addicted healthcare workers could raise the risk for extrinsic contamination (*22*). Other factors that may potentially affect the sterility of propofol in clinical use include preparation of multiple syringes for use throughout the day; re-use of vials, syringes, or infusion-pump lines on >1 patient; use of opened ampules longer than recommended by the manufacturer; and failure to wear sterile gloves during handling of propofol and to dispose of them after each contact.

The most common reservoirs associated with extrinsic contamination of propofol are syringes or micro-droppers, vials, and IV stopcock dead space. Syringes or micro-droppers have been implicated in most outbreaks (*23*–*27*). Propofol vials have been demonstrated to be a reservoir for microbes when contents are exposed to the environment (*28*). Delays in administration after propofol vials have been opened are a recognized risk factor; the degree of contamination of an opened vial may increase by 20%–26% after 12 hours (*29*). Propofol is available in vials of various volumes; a typical 20-mL vial contains 200 mg, and 50- and 100-mg vials are available. It is believed that administering a dose >200 mg to an adult in an OR will increase the probability of using an additional vial as a multi-dose vial for ≥1 patients. IV stopcock dead space has been shown to provide a potential route of entry for pathogenic, multidrug-resistant bacteria in infusion lines (*20*,*27*).

A number of microorganisms are associated with propofol in clinical- and laboratory-based studies at varying frequencies (Table 2). We report the specific pathogens that have been associated in several outbreaks (*2*–*4*,*6*,*8*–*10*,*12*–*15*,*30*,*31*), as well as all of the transmissible microorganisms that have been observed in contaminated propofol in

Table 2. Microorganisms identified in propofol anesthesia-related iatragenic infection outbreaks, single cases, or laboratory-based studies of syringes, vials, or infusion lines*

Category and microorganism	Type of infection	% Infections†	References
Gram-positive bacteria		27.08	
Staphylococcus aureus	BSI, SSI	27.08	(*2*,*6*,*8*,*30*)
S. epidermidis‡	–	–	(*23*,*27*,*30*,*31*)
MRSE§	SSI	–	(*31*)
Streptococcus salivarius‡	–	–	(*22*)
Enterococcus faecalis‡			(*32*)
Micrococcus sp.‡	–	–	(*23*,*25*,*27*)
Corynebacterium sp.‡	–	–	(*23*)
Bacillus sp.‡	–	–	(*23*,*25*)
Diphtheroids sp.‡	–	–	(*25*)
Kocuria sp.‡	–	–	(*27*)
Gram-negative bacteria		20.14	
Serratia marcescens	BSI, SSI	9.72	(*2*,*4*,*15*,*30*)
Enterobacter cloacae	BSI	2.78	(*31*)
E. agglomerans	BSI	2.78	(*2*)
Pseudomonas cepacia§	BSI	–	(*34*)
P. aeruginosa§‡	SSI	#	(*30*,*33*)
Escherichia coli§	BSI	–	(*35*)
Klebsiella pneumoniae	BSI	3.47	(*10*,*15*)
Moraxella osloensis	BSI, SSI	1.39	(*6*)
Acinetobacter sp.‡	–	–	(*27*)
Fungus		21.53	
Candida albicans	BSI, SSI	21.53	(*2*,*6*,*14*,*30*)
Viruses		22.53	
HCV	Hepatitis C	18.06#	(*9*,*11*–*13*)
HBV	Hepatitis B	4.17	(*12*)

*Outbreak, ≥2 cases; BSI, bloodstream infection; SSI, surgical site infection; MRSE, methicillin-resistant *Staphylococcus epidermidis*; HCV, hepatitis C virus; HBV, hepatitis B virus; dashes indicate no infections identified

†Percentage of infection estimated among the total of victims involved only in outbreaks in which a pathogen was identified (n = 131). In total, 9.03% of the patients reported in the outbreaks had no microorganisms identified, in part because the cultures were obtained after administration of antimicrobial drugs.

‡Microorganisms that have been identified by culture of residual propofol after clinical use but so far have not been involved in propofol-related outbreaks or infection associated with propofol.

§MRSE, *P. cepacia, P. aeruginosa*, and *E. coli* have been identified in case reports of infection and septic shock, but so far have not been involved in propofol-related outbreaks.

#*P. aeruginosa* and HCV have been implicated in outbreaks in Catalonia and Galicia, Spain. However, these reports appeared in newspapers and because of that were not included in this synopsis article (http://elpais.com/diario/2011/03/05/sociedad/1299279606_850215.html and http://elpais.com/diario/2011/03/09/sociedad/1299625207_850215.html).

syringes, vials, and infusion lines (*23*,*25*,*27*,*30*,*32*). We also describe 3 reported cases of septic shock related to propofol (*33*,*34*).

Propofol is an excellent medium not only for bacterial growth but also for fungal infections, which have been associated with propofol use particularly when poor hygienic standards are observed during the administration. Viral infections with HCV and HBV have also been demonstrated, possibly explained by the viral stability offered by propofol emulsions (*9*,*11*,*13*). Overall, ≈23% of the published infection outbreaks associated with propofol were caused by HCV (18.1%) and HBV (4.2%); 21.5% by *Candida albicans*; and 47.2% by bacteria (gram-positive 27.1%, gram-negative 20.1%). In the remaining reports, no microorganisms were identified, possibly as a consequence of concurrent antimicrobial drug therapy. The most frequent pathogens associated with propofol-related outbreaks, in order of frequency, were *Staphylococcus aureus* (39/144), *Candida albicans* (31/144), and HCV (26/144).

Laboratory-based (Table 3) and epidemiologic (online Technical Appendix Table 1) microbiological studies have demonstrated that the production of bacterial endotoxins is greatly enhanced by propofol solutions. Case reports of endotoxemia associated with the use of contaminated propofol have also been published (*5*,*34*,*35*).

Frequency of Contaminated Propofol Used in ICUs and ORs

Microbiological observations of opened propofol vials were reported in the first studies that identified bacterial growth in propofol, and observational studies on propofol formulations have determined the proportion of extrinsic contamination (Table 3). In 1994, Farrington et al. established an incidence rate of 6% (3/50) of contaminated propofol syringes in an ICU (*31*). Webb et al. retrospectively observed similar results in a different ICU setting with an incidence of 5.9% (18/302) (*23*). Soong observed a lower propofol contamination incidence (3.0%) in ORs and also noted an association between postoperative infections and vials from which multiple patients were medicated (*24*); Bach et al. found similar results (*30*). In 1995, McHugh and Roper reported an incidence of 6.3% (16/254) of infection when propofol was administered from vials but did not find that delays in the administration of propofol were associated in any increased likelihood of bacterial contamination (*25*). Cole et al. recorded the incidence of contamination as high as 17.3% (26/150) in propofol found in stopcock dead space (*27*).

The distribution of instructions for aseptic measures for handling propofol has shown to reduce the rate of contaminated propofol. Lorenz et al. reported that after a specific protocol for handling propofol was introduced and adhered to, a reduction in extrinsic contamination was achieved when compared with only adhering to the manufacturer's recommendations (8.8% versus 11.3%) (*26*). That protocol included additional aseptic precautions, such as refilling empty syringes for use on multiple patients by using a 3-way stopcock and replacing only the infusion line to the patient. Data from a study performed in a high-complexity hospital in Cali, Colombia, showed substantial microbial growth of

Table 3. Summary of studies of syringes, vials, infusion lines, and IV stopcock dead spaces for contamination after clinical use to administer propofol anesthesia*

Object† and study, year (reference)	Country	Antimicrobial agents‡	Hospital unit§	Crude % contaminated propofol (no. contaminated/no. tested)
Syringes				
Farrington et al., 1994 *(31)*	United Kingdom	No	ICU	6.0 (3/50)
Bach et al., 1997 (30)	Germany	No	OR	4.8 (8/168),¶ 5.1 (19/376)#
Webb et al., 1998 (23)	Australia	ND	ICU	5.9 (18/302)
Total				5.4 (48/896)**
Vials				
McHugh et al., 1995 (25)	New Zealand	No	OR	6.3 (16/254)
Soong et al., 1999 (*24*)	Australia	ND	OR	3.0 (3/100)
Zorrilla-Vaca et al., 2014 (*32*)	Colombia	No	OR	6.1 (12/198)
Total				5.6 (31/552)**
Infusion systems				
Bach et al., 1997 (*30*)	Germany	No	ICU	4.5 (10/224),¶ 1.6 (5/318)#
Lorenz et al., 2002 (*26*)	Austria	No	OR	11.3 (9/80),†† 8.8 (7/80)‡‡
Total				4.4 (31/702)**
IV stopcock dead spaces				
Cole et al., 2013 (*27*)	United States	Yes	OR	17.3 (26/150)

*ICU, intensive care unit; OR, operating room; ND, not described in publication; IV, intravenous.
†Clinical object from which residual propofol was taken to be cultured after clinical use.
‡Use of propofol formulations with antimicrobial additives.
§Hospital unit where the studies were conducted.
¶Results of a first study period during February 1–October 31, 1992.
#Results of a second study-period from December 1, 1994, through March 31, 1995.
**Total crude percentage of contaminated propofol for each kind of object (syringes, vials, infusion systems).
††Proportion of propofol contaminated, following the manufacturer's handling recommendations.
‡‡Proportion of propofol contaminated, following a modified propofol handling protocol. (i.e., refilling empty syringes and renewing only the infusion line to the patient).

6.1% (12/198) in residual propofol vials used in ORs at a tertiary hospital in Cali (*32*).

By collating the incidences of contaminated propofol in containers or devices, the data suggest that it is more common to encounter contaminated previously used vials of propofol (5.6%, 31/552) than in used syringes (5.4%, 48/896) and infusion systems (4.4%, 31/702). Similarly, analyzed by hospital location, the percentage of contaminated propofol is greater in ORs (7.3%, 103/1,405) than in ICUs (4.0%, 36/894), correlating well with the expected prevalence of opened vials and syringes used for bolus injections of propofol for multiple patients in the OR compared with those in the ICUs.

Risk for Infection Data Derived from Analytical Studies

After we reviewed the initial microbiological studies, our interest increased in ascertaining potential links between the use of propofol infusions and the incidence of infections and sepsis after surgery. We analyzed 10 epidemiologic studies (Table 4; online Technical Appendix Table 1); of these, 4 assessed the associations with infection when practitioners followed manufacturers' instructions for propofol handling, 5 assessed the association with infection when practitioners did not follow instructions, and 1 did not report on this issue. Studies following manufacturers' precautions stated such within the articles, but the degree of compliance of such precautions was not documented (online Technical Appendix Table 2). In 4 of 5 studies (80%) during which practitioners did not follow handling precautions, high infection risk was noted. In 4 studies in which precautions were followed, 2 (50%) scored above the significant risk threshold (online Technical Appendix Table 1). These findings underscore the controversy surrounding the utility of existing handling protocols and demonstrates the continued high potential of propofol as a causal factor of iatrogenic infection. Other authors have explain the susceptibility to infection because of the attenuation of the immunological activity caused by propofol infusions. More studies of specific handling protocols are required before a significant risk reduction is clearly observed. We developed an algorithmic approach that shows certain crucial measures to prevent future propofol-related outbreaks of infections (Figure 4); this approach was based on our analyses and summaries of the epidemiologic and clinical data selected.

Proposed Propofol Formulations without Risk for Infection

There are currently no propofol formulations without infection risk; however, several investigators have advocated the use of antimicrobial additives. As required by the FDA, the efficacy of such additives must retard microorganism growth to <10-fold at 24 hours after extrinsic contamination of propofol (*1*); many suggested antimicrobial agents have been rejected because of their poor efficacy, additional side effects, or higher costs (*1*). The fact that propofol is a lipid emulsion poses problems when additives are considered because admixture with other substances, especially charged species with differing

Table 4. Summary of epidemiologic studies analyzing the association between infectious conditions and contaminated propofol anesthesia*

Followed manufacturers' precautions, study, year	Type of study	Preservative-free propofol†	Other agents compared with propofol	Type of infection	Hospital unit‡	Association§
Yes						
Seeberger et al., 1998	Retrospective cohort	Yes	Thiopentone	Sepsis	OR	No
Shimizu et al., 2010	Cohort	ND	Sevoflurane	SSI	OR	Yes
Haddad et al., 2011	Nested cohort	Yes	ND	Multiple¶	ICU	Yes
Moehring et al., 2014	Case–control	ND	Fentanyl	BSI	ICU	No
No						
Bennett et al., 1995. (*2*)	Case–control and cohort	Yes	Sufentanil, alfentanil	BSI, SSI	OR	Yes
Henry et al., 2001 (*4*)	Case–control	Yes	ND	BSI, SSI	OR	Yes
McNeil et al., 1999	Cohort	Yes	Sufentanil, fentanyl, midazolam, vecuronium	Fungemia, endophthalmitis	OR	Yes
Sebert et al., 2002.	Case–control	ND	ND	BSI	OR	No
Muller et al., 2010 (*15*)	Retrospective cohort	ND	Fentanyl, midazolam	BSI, SIRS	OR	Yes
ND						
Kontopoulou et al., 2012	Case–control	ND	ND	BSI	ICU	Yes

*Complete data and full references are available in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/22/6/15-0376-Techapp1.pdf); OR, operating room; ICU, intensive care unit; BSI, bloodstream infection; SSI, Surgical site infection; ND, not described in publication; SIRS, systemic inflammatory response syndrome.
†Use of propofol without antimicrobial additives.
‡Hospital unit where the studies were conducted.
§Conclusion of the analytical study regarding the association between propofol exposure and infectious events.
¶Multiple infection types, including ventilator-associated pneumonia, urosepsis, BSI, catheter-related infections, and others.

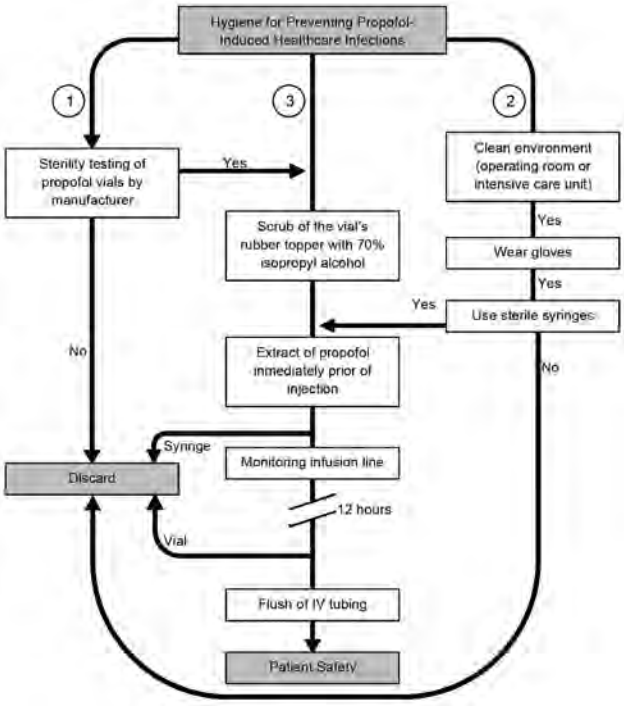

Figure 4. Algorithm for helping reduce the likelihood of infectious disease events when using propofol. To avoid intrinsic contamination, sufficient quality control during the manufacturers' process is required (*1*). Personnel must be aware of the importance of performing healthcare procedures in a clean environment and the use of gloves and sterile syringes for anesthetic procedures. Syringes and needles must never be reused (*2*). Also, the aseptic technique for administration of propofol includes cleaning of the rubber bung, if present, with isopropyl alcohol, leaving it to dry. Propofol should be drawn up immediately before its use and not left standing. Intravenous (IV) infusion lines and stopcock dead spaces should be completely flushed to ensure no residual propofol remains. Vials must be discarded after opening for single use, no matter the amount of the remainder (*3*).

partition coefficients, can interact with either the lipid phase or the emulsifying agents, resulting in emulsion destabilization. Older and newer advances on the use of additives are described in Table 5, which includes the scientific progress related to patients, clinical trials, original articles, and brief reports.

Propofol with EDTA

As an antimicrobial ion chelator, EDTA exerts its effect by removing divalent and trivalent metal cations, causing rupture of the microbial cell membrane by loss of control of osmotic pressure gradients. This combination is approved by the FDA; clinical trials demonstrated antimicrobial efficacy and safety in humans (*18*,*36*). For manufacturers, EDTA is the most broadly incorporated agent in combination with propofol formulations. Despite its widespread use, some controversy remains over its selection as the optimal additive (*37*). Moreover, in several developing countries, cost remains a considerable limiting factor for the use of EDTA-containing propofol formulations (A. Zorrilla-Vaca et al., unpub. data).

Fospropofol Disodium

A newly introduced agent, fospropofol disodium, is a water-soluble pro-drug of propofol that currently has a small evidence base for its use of 3 published clinical trials in the literature; in these studies, fospropofol was assessed for use in sedation for colonoscopies, bronchoscopies, and coronary artery bypass graft surgery, and showed an acceptable safety profile (*38*). The advantage of this drug in reducing the risk for infectious events is that it does not have a lipophilic formulation that would support bacterial growth. Nonetheless, the drug has some disadvantages that could discourage its use, such as transient paresthesias and pruritis in the perineal and perianal regions (*38*).

Anesthetic Mixtures

Pain on injection is a common side effect of propofol. Anesthesiologists use a variety of strategies to reduce this, such as the addition of 1–2 mL of lidocaine to the propofol before injection. Lidocaine, like other amide local anesthetics, has bacteriostatic properties, which could theoretically reduce the chances of infection (*29*). It is, however, not currently known whether lidocaine has sufficient antimicrobial effect to make a clinical difference in infection rates.

Other Additives

Benzyl alcohol at concentrations of ≤2% has been used as a preservative agent in propofol formulations (*39*). Despite its bacteriostatic activity, benzyl alcohol used in propofol formulations is limited by its toxicity and instability in the combination. Other additives, such as phenylmercuric nitrate, phenylmercuric acetate, chlorobutanol, and phenol have been studied experimentally with propofol; however, all of these agents were rejected because of their potential toxicity. The sodium metabisulfite–containing formulation, created originally with the aim of reducing the pain of propofol injection, has been shown to possess preservative properties. Unfortunately, it has a labeled pH from 4.5 to 6.4, which is different from the FDA requirement of a pH of 6–8.5 for propofol (*1*). The nonlipid nanoemulsion and Emul-Siv filter are the most recent alternative propofol formulations (*40*). In recent studies, these formulations of propofol have attained a level of antimicrobial activity above that observed with propofol with EDTA (*40*). Costs are currently a limiting factor for their use, but these 2 options seem to provide some promise for the future if production costs decline.

Table 5. Description of advantages and disadvantages of each formulation of propofol related to contamination and iatragenic infection*

Propofol formulation	Settings	Advantages	Disadvantages	FDA approval
Propofol with EDTA	Antimicrobial activity	This mixture with propofol at 0.005% wt/vol concentration has demonstrated microbial growth to be retarded to ≥1 log CFUs (*36*), of nearly 20 microorganisms, including 7 Gram-positive bacteria, 10 Gram-negative bacteria, and 3 yeasts (*18*). Further, incidence of propofol-related infection declined from 39 to 9 infections per year in the USA, after the introduction of EDTA into clinical use in 1996 (*18*).	Decreases serum ionized calcium levels, although statistically significant, has apparently no clinical effect (time to complete recovery, p = 0.77 [*37*]). Also, EDTA is nephrotoxic at high doses (2–3 g/d). Concern that use of an antimicrobial may cause health personnel to relax on aseptic handling practices (*1*).	Yes
Fospropofol disodium	Nonlipophilic preparation	Because of water solubility, eliminates some of the known lipid emulsion-associated disadvantages of propofol, including the risk for infection (*38*).	Minor side effects (e.g., paresthesia, hypotension). The prolonged onset of action of fospropofol (≈4–13 min, because of it must first undergo metabolism to propofol) compared with the prodrug propofol (≈40 s). Allergies caused by the accumulation of a phosphate-ester component (*38*).	Yes†
Propofol and lidocaine	Bacteriostatic activity	Experimentally causes loss of viability of several strains (*29*).	Has no sufficient retarding effect. Possibilities of micelle formation exist.	No
Benzyl alcohol	Antimicrobial activity	At low concentrations of ≥2%, has been used as a preservative agent.	Toxicity and presumed instability.	No
Sodium metabisulfite	Antimicrobial activity	Reduces the pain of propofol injection and has preservative properties.	Has a labeled pH of 4.5–6.4, which is different from the required pH for propofol (6–8.5) (*1*).	No
EmulSiv filter	Filter	Use of the 0.45 μm-rated filter is purported to provide protection from accidental microbial contamination, particulate contamination and entrained air while maintaining the integrity of the emulsion (*40*).	High costs, not currently available.	No
Nonlipid propofol nanoemulsion	Nonlipophilic preparation	Replaces soybean lecithin with polyethylene glycol 660 hydroxystearate as propofol carrier (*40*).	High costs, not currently available.	No

*FDA, US Food and Drug Administration.
†Approved for use by the FDA only for monitored anesthesia care; however, a decision from the US Drug Enforcement Agency could be scheduled (*38*).

Discussion

Contaminated propofol has been implicated in several episodes of iatrogenic infection in both the outpatient and inpatient settings, as well as in both surgical and nonsurgical patients (*2*,*6*,*11*,*12*,*15*). The risk for infection arises principally because the lipophilic nature of propofol supports microbial growth when the formulation becomes contaminated (*2*,*6*). In addition, the method of intravenous administration and the preservative-free preparations still used in many countries have been implicated in promoting infection with propofol use.

More than 2 decades have elapsed since the first outbreaks of contaminated propofol-related infection emerged in the United States (*6*), and incidents of contamination-related infections persist, despite the introduction of antimicrobial formulations. Contamination and infections associated with propofol have been most commonly reported in industrialized countries, but it is likely that this phenomenon is secondary to a lack of surveillance of propofol contamination in developing countries. Management of this risk for contamination and infection can be approached by continued medical education regarding patient safety. A lack of adherence to the manufacturers' guidelines appears to have been a causative factor in most of the episodes reported worldwide. The adherence to strict aseptic handling protocols is mandatory and more education efforts (e.g., the One and Only Campaign, http://www.oneandonlycampaign.org/) are needed to generate awareness in the healthcare community of the importance of proper propofol practices.

In summary, healthcare-associated infections linked with contaminated propofol constitute a complex public health issue that requires a multifaceted approach. Further efforts in surveillance and research are required to reduce the potential harm from contaminated propofol. Healthcare practitioners must focus on standard hygienic measures and the increased use of approved antimicrobial propofol formulations. Following these simple tenets, the risk for in-use contamination would be lowered and the safety use profile for propofol would greatly improve.

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Improved Global Capacity for Influenza Surveillance

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During 2004–2009, the Centers for Disease Control and Prevention (CDC) partnered with 39 national governments to strengthen global influenza surveillance. Using World Health Organization data and program evaluation indicators collected by CDC in 2013, we retrospectively evaluated progress made 4–9 years after the start of influenza surveillance capacity strengthening in the countries. Our results showed substantial increases in laboratory and sentinel surveillance capacities, which are essential for knowing which influenza strains circulate globally, detecting emergence of novel influenza, identifying viruses for vaccine selection, and determining the epidemiology of respiratory illness. Twenty-eight of 35 countries responding to a 2013 questionnaire indicated that they have leveraged routine influenza surveillance platforms to detect other pathogens. This additional surveillance illustrates increased health-system strengthening. Furthermore, 34 countries reported an increased ability to use data in decision making; data-driven decisions are critical for improving local prevention and control of influenza around the world.

After the threat of highly pathogenic avian influenza in 2004, the Centers for Disease Control and Prevention (CDC) began an international capacity-strengthening program with national governments across the globe. The program focused on strengthening 2 systems for preparedness: routine laboratory diagnostics to detect seasonal and novel influenza viruses and routine sentinel surveillance for influenza-like illness (ILI) and severe acute respiratory infection (SARI).

To foster sustainable development, the program prioritized the following principles: investing in routine national surveillance systems to ensure that capacities are regularly tested and used; providing long-term technical assistance driven by country performance and needs; and supporting development that builds on the existing World Health Organization (WHO) Global Influenza Surveillance and Response System. This latter principle includes alignment with WHO guidelines and recommendations for strengthening national laboratory capacities, a requirement for designation as a WHO National Influenza Center (NIC) and for implementation of the 2005 International Health Regulations, a legally binding framework for improving commitment to strengthening core aspects of an infectious disease preparedness and response system (*1*–*3*). Implicit in each principle is respect for the government as the decision-maker, implementer, and beneficiary of the capacity-strengthening process.

The 10-year program is managed through a cooperative agreement between CDC and a country's ministry of health or equivalent national health agency. The first 5 years of the program's phased approach focuses on capacity building; over the following 5 years, financial support from CDC is incrementally reduced. Reducing funding encourages transition of financial support for built routine surveillance systems to the countries. Through the cooperative agreement mechanism, the program provides support in 3 ways: providing funding for equipment, materials, and locally employed personnel; conducting hands-on training and long-term technical follow-up with staff within a country; and facilitating participatory, standardized assessments (http://www.cdc.gov/flu/international/tools.htm) of national influenza laboratories, surveillance systems, and core capabilities for influenza pandemic preparedness, each with targeted technical recommendations (*4*–*6*).

Evaluating outcomes of capacity building can be challenging for many reasons, including variation among countries, lag between capacity-building activities and performance outcomes, and methodologic challenges of collecting and analyzing data from multiple countries (*7*). Through systematic review of the funding opportunity announcements, we found the following 6 development areas to be the most emphasized: 1) achieving WHO NIC recognition; 2) improving weekly testing for influenza; 3) maintaining sentinel surveillance in ≥3 sites; 4) reporting weekly data to the WHO FluNet virus monitoring system; 5) sharing specimens with WHO collaborating centers for seasonal vaccine strain selection; and 6) increasing awareness of influenza disease by using national surveillance data to guide decision making for prevention and control strategies. These development areas are the focus of our evaluation.

During 2004–2013, a total of 39 countries participated in the program.We conducted a retrospective evaluation of the extent to which capacity was strengthened in the 6 focus areas after 4–9 years of countries' participation in the program.

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Methods

We collected data from external WHO sources (*8–10*; WHO, unpub. data) and a 2013 retrospective questionnaire that gathered information about capacity indicators from countries that began the program during 2004–2009. Analysis of the questionnaire indicators enabled systematic analysis of information unavailable from WHO sources, including partners' perspectives of the program. All countries that transitioned from capacity-building to the 5-year sustainability cooperative agreement and returned the completed questionnaire were included in our analysis.

WHO Data Sources

We analyzed the change in the number of countries designated as WHO NICs; an increase indicates a strengthened global surveillance network. NIC designation depends on several achievements, including the ability to monitor circulating influenza and isolate influenza viruses, a capacity that is key for selecting viruses for vaccines (*11,12*). We downloaded public data from the WHO Global Influenza Surveillance and Response System's FluNet, which monitors circulation of influenza viruses globally. For each country, we calculated the change in total number of specimens processed annually from the time the program started in a country through 2013; results served as a proxy indicator of a country's ability to collect, transport, and test specimens for influenza (*8*). For the descriptive program data, we calculated median values and interquartile ranges (IQRs). We also calculated the change in the number of countries reporting data on circulating viruses to FluNet for ≥90% of weeks in each year; this calculation served as an indicator of a country's ability to collect and share this information routinely on WHO's global platform (*8*).

In 2007, WHO developed a voluntary External Quality Assessment Project to test the quality of reverse transcription PCR (RT-PCR) diagnostics for influenza (*13*). We analyzed the change in the number of countries that participated in this project and, for participating countries, the number that scored 100% on all panels for each year during 2007–2013; these calculations served as indicators of progress made in the quality of influenza testing (*9*). During 2007–2011, two panels were available each year; during 2012 and 2013, only 1 panel was available.

Each year, the 5 WHO Collaborating Centers for influenza receive influenza specimens or viral isolates from NICs to analyze for seasonal influenza vaccine strain selection (*14,15*). Using the Northern and Southern Hemisphere vaccine strain selection information packages, we analyzed the change in the number of countries with NICs that shared specimens at the start of the program, compared with those sharing specimens in 2013; this change served as an indication of global contribution to vaccine strain selection. All data were analyzed by using Microsoft Excel (Redmond, WA, USA).

Questionnaire

The 2013 retrospective questionnaire was available in English, French, and Spanish in electronic and paper versions and was piloted in 3 countries before implementation. The pilot program included discussions of question interpretation with respondents to ensure consistency in attribute measurement. We analyzed the extent to which countries believed that the program contributed to their ability to collect and report data to WHO FluNet and to prepare for the 2009 influenza A(H1N1) pandemic (pH1N1). Responses used a Likert scale (i.e., critical, major, somewhat, little, none) and described qualitatively the contributions made. We inductively coded the main ideas mentioned and reported them by frequency of mention. To evaluate the growth in surveillance capacity, we analyzed the number of influenza sentinel sites conducting ILI or SARI surveillance and their geographic coverage during the first year of support and compared findings with those data for 2013. We also analyzed questions about additional pathogens that were added to the routine influenza diagnostic testing platforms and other types of syndromic surveillance conducted at influenza sentinel sites. Finally, we analyzed how countries ranked types of CDC program assistance (i.e., direct funding, technical and training assistance, objective assessments of capacity, and information exchange during meetings) on the basis of the programs' ability to improve functioning of the national surveillance system.

To assess internal validity of the questionnaire, we asked 2 questions for which we had externally validated data as a proxy test. One question asked if the country was reporting to WHO FluNet before starting the cooperative agreement with CDC. The other asked whether the country was sending specimens or viral isolates to WHO Collaborating Centers for influenza seasonal vaccine strain selection before starting the cooperative agreement with CDC. The accuracy of responses to those questions was >90%, indicating that for those 2 questions, history and maturation bias had little effect on the internal validity of responses. Data were double entered and analyzed by using Epi Info version 7.1.2 (CDC, Atlanta, GA, USA).

Results

Of the 39 countries that partnered with CDC to improve influenza surveillance capabilities, 36 (92%) transitioned to CDC's 5-year sustainability cooperative agreement, and 35 (97%) completed the questionnaire (Table 1). Among those responding to the questionnaire, 10% had worked in the country's national influenza programs for 1–3 years; 31% for 4–6 years; 31% for 7–9 years; and 27% for >9 years. No respondent had <1 year of experience.

Table 1. Countries that partnered with CDC in capacity strengthening for influenza surveillance, by start year and World Health Organization Region, 2004–2009*

Start year	AFR	EMR	EUR	AMR	SEAR	WPR
2004	–	Pakistan	–	–	India, Indonesia, Thailand	China, Mongolia, Philippines
2006	Angola Democratic Republic of Congo Cote d'Ivoire Ethiopia Nigeria Rwanda South Africa Republic of Tanzania Uganda	Afghanistan Morocco	Armenia Georgia Russia Ukraine	Brazil Mexico	Bangladesh	Vietnam Cambodia Laos People's Democratic Republic
2009	Madagascar Zambia	Egypt	Republic of Moldova	Paraguay	Nepal Sri Lanka	–
Total	11	4	5	3	6	6

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in our analysis. AFR, African Region; CDC, Centers for Disease Control and Prevention; EMR, Eastern Mediterranean Region; EUR, European Region; AMR, Region of the Americas; SEAR, South East Asian Region; WPR, Western Pacific Region; –, no countries partnered with CDC during that start year.

Improved Performance of Influenza Laboratories

The number of countries conducting routine virologic surveillance for influenza increased from 19 at the start of the capacity-strengthening program to 35 in 2013. The total annual number of specimens tested increased substantially, from 81,851 (median 37, IQR 0–2,411) at the start of the program to 542,235 (median 2,826, IQR 1,282–5,052) in 2013; most growth occurred during the year after the pH1N1 pandemic (Figure 1). Besides having influenza testing, 28 (80%) countries reported adding additional pathogens to the routine platforms that were developed or enhanced through capacity strengthening (Figure 2). Of 19 countries with no NIC at the start of the program, 12 (63%) fulfilled the needed criteria and received official NIC designation. The global influenza surveillance network was also enhanced by the designation of a fifth WHO Collaborating Center for influenza in China in 2009 after this country substantially enhanced the scope of its influenza surveillance system.

All national laboratories supported through the program now use real-time RT-PCR as the primary method to detect circulating influenza. Since WHO developed its EQAP quality assurance test for RT-PCR diagnostics for influenza in 2007, the number of countries using RT-PCR diagnostics increased from 11 in 2007 to 34 in 2013. The percentage of countries with no (0.0) error on the EQAP panels increased from 36% (4/11) in 2007 to 85% (30/34) in 2013.

Improved Weekly Reporting of Viruses

The proportion of countries reporting data to WHO FluNet for >90% of weeks per year increased considerably during 2004–2013 (Table 2). Among all countries, the median percentage of weeks reported per year increased from a baseline of 21% (IQR 0%–98%) to 100% (IQR 79%–100%) after 5 years of support, when all countries transitioned to the sustainability cooperative agreement. Of the 16 countries not reporting during the first year in the program, 14 provided data every week with 100% completion during 2013.

Thirty (86%) of 35 countries reported by questionnaire that the capacity-strengthening program played a critical

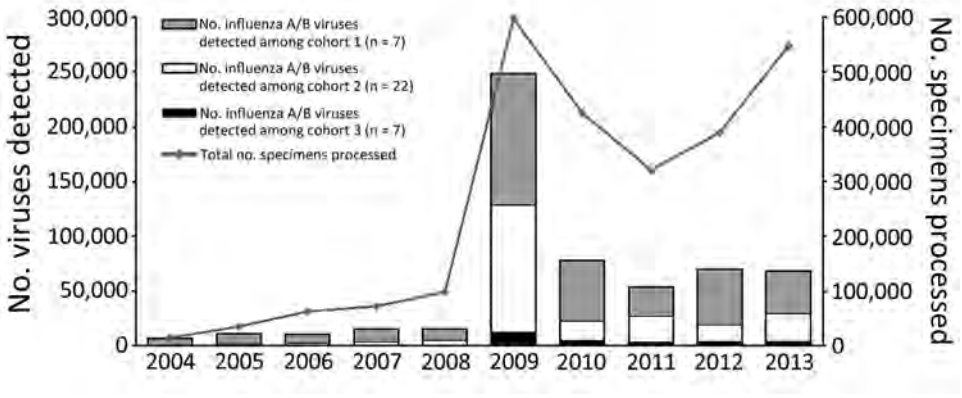

Figure 1. Changes in numbers of influenza specimens processed and in numbers of viruses detected per year among 35 countries that partnered with the Centers for Disease Control and Prevention to strengthen influenza surveillance capacity, 2004–2013. From a total of 39 participating countries, 35 responded to a 2013 questionnaire and are included in this analysis.

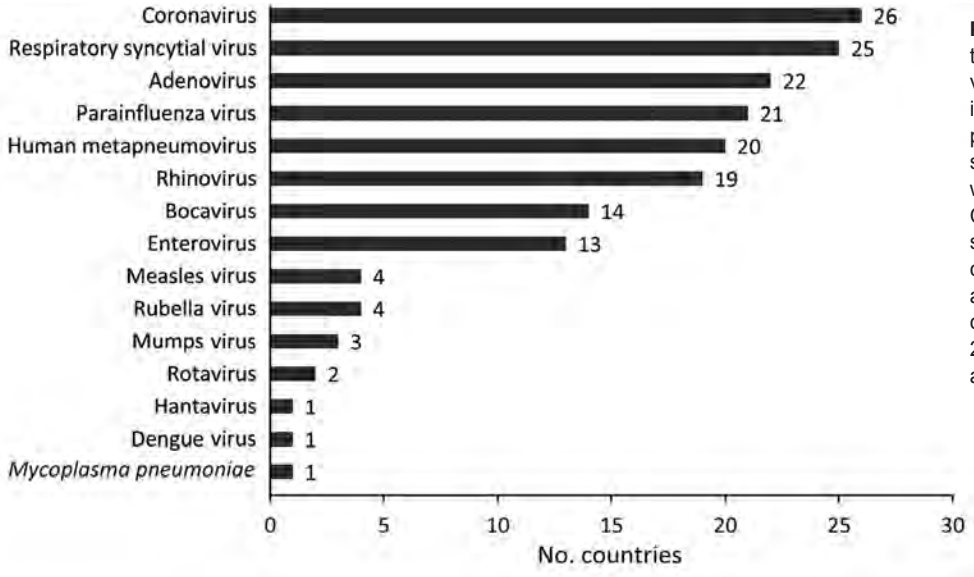

Figure 2. Number of countries that reported adding different virology testing assays to routine influenza laboratory testing platform by virus type from the start of the partnership program with the Centers for Disease Control and Prevention to strengthen influenza surveillance capacity, 2004–2013. From a total of 39 participating countries, 35 responded to a 2013 questionnaire; 28 reported adding tests for other pathogens.

(n = 10), major (n = 16), or small (n = 4) role in improving FluNet reporting. At the start of the program, median baseline reporting to FluNet was 0% among those countries reporting that CDC played a critical (IQR 0%–25%) and major (IQR 0%–74%) role. Those reporting that the program played a small (median 76%, IQR 30%–95%) or no (median 83%, IQR 60%– 94%) role had much higher baseline reporting than those countries reporting that the program played an important role in increasing FluNet reporting (Table 3).

Improved Participation in WHO Influenza Vaccine Strain Selection

The number of countries that contributed isolates or specimens for inclusion in global vaccine strain selection increased from 16 (42%) at the start of the CDC program to 28 (80%) in 2013. Questionnaire responses also showed progress in use of WHO selection criteria; a comparison of data for the start year and 2013 showed that an increased number of countries that selected viruses by age group (7 vs. 18), geographic area (9 vs. 26), phase of influenza season (10 vs. 22), or other high priority criteria for the country (7 vs. 14).

Growth of Influenza Sentinel Surveillance

On the basis of 35 countries' responses to the 2013 retrospective questionnaire, 32 (94%) countries established ≥3 surveillance sites since their start year. The number of sites capable of collecting weekly specimens and epidemiologic data from patients seeking healthcare for ILI or SARI increased from 446 at the start of the program to 2,075 in 2013 (Table 4). Moreover, 48% of countries that began the program with no influenza sentinel sites had 1,293 (median 7, IQR 5–14) functional sites in 2013. The number of provinces or districts with a functional influenza sentinel site increased in 29 (83%) countries.

Questionnaire responses for 29 (83%) countries indicated that influenza sentinel sites initiated surveillance for other diseases or syndromes (Figure 3). All 29 reported that program funds and technical assistance played a critical (n = 4), major (n = 16), or small (n = 9) role in capacity building for additional surveillance.

Improved Country Response to Influenza

Among the 35 responding countries, 26 started the capacity-strengthening program before onset of the pH1N1 outbreak; 25 (97%) of the 26 believed that capacity strengthening played a critical (n = 16) or major (n = 9) role in their pandemic response. In an inductive analysis of capacities that countries reportedly experienced as key to outbreak detection and pandemic response, the most common was establishment of routine sentinel SARI or ILI surveillance systems (Table 5). Also, 15 countries noted improvements

Table 2. Percentage of countries with data available in the WHO FluNet for 90%–100% of weeks per year by country start year in the program, 2004–2013*

	% Countries									
Start year	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
2004, n = 7	44	33	33	56	67	67	100	100	100	100
2006, n = 21	13	14	22	30	30	30	70	70	77	73
2009, n = 7	29	29	14	29	43	43	71	86	100	100

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in our analysis. CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.

Table 3. Most commonly reported ways that 35 countries used CDC program support to strengthen their ability to report national virologic data to WHO FluNet on a weekly basis, by rank order*

Methods used
1. Establishing reverse transcription PCR capabilities
2. Enhancing electronic database management (i.e., computer, internet, database, software, Web site)
3. Training database managers, laboratory managers, and laboratory diagnostic technicians
4. Developing a standardized weekly national report with indicators
5. Establishing a laboratory focal person to liaise with the sentinel network

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in this analysis. CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.

to influenza laboratory diagnostics, which made possible identification of pH1N1, highly pathogenic influenza A(H5N1), and influenza A(H7N9) viruses. Other key capacities described were the ability to understand seasonal trends, establishment of subnational diagnostic laboratories, and creation of systems for information sharing between laboratories and sentinel surveillance sites.

Improved Knowledge of Influenza for Local Decision Making

Among 35 responding countries, 29 reported that they have described the seasonality of influenza viruses in their country; 19 (66%) were described for the first time during the program (Table 6). Thirty-four (97%) countries reported an improved ability to use national influenza data in decision making in several ways: drive updates to national pandemic preparedness plans, create evidence-based vaccine guidelines, determine best use of antiviral medication, and determine need for community mitigation measures such as school closures. Countries in each of 6 WHO regions reported that they used their national surveillance data to support influenza vaccination programs.

Development and Ownership of Capacity Strengthening

Overall, 34 (97%) countries reported that they were mostly or very able to meet their countries' needs through the program; 32 (91%) mostly or always perceived that ownership of the capacity building was theirs. Countries had different perceptions of the program's impact on development of laboratory versus sentinel site systems. For 29 (81%) of countries, the top-ranked type of assistance for strengthening laboratories was financial assistance for laboratory equipment, materials, and reagents. For the remaining 6 (19%) countries, the most critical assistance was staff training and technical advice (n = 4) and the ability to exchange experience with colleagues during national or international meetings (n = 2). Objective assessments of the laboratory were ranked, on average, as the third most critical assistance.

Rankings regarding strengthening sentinel surveillance differed among countries. Financial assistance was ranked by 17 (49%) countries as most critical. The most critical assistance among the remaining 18 (51%) countries was trainings for staff and technical advice (n = 11), objective assessments of the surveillance system (n = 4), and the ability to exchange experience with colleagues during national and international meetings (n = 3). In the analysis of recommendations suggested in the questionnaire, the most common was to increase technical assistance for assessing, evaluating, and improving the sustainability of capabilities developed.

Discussion

In the context of the emergence and reemergence of severe acute respiratory syndrome and highly pathogenic influenza A(H5N1) virus, CDC's Influenza Division developed an international capacity-strengthening program that enabled countries to detect seasonal and pandemic influenza viruses and to make evidence-based decisions for risk reduction (*16–18*). Among 35 participating countries included in our evaluation, all indicators examined by using WHO data sources have shown dramatic improvement.

Table 4. Countries with sentinel sites capable of collecting specimens ≥1 time per week from patients screened for ILI or SARI in WHO regions*

	Start year		2013		
WHO region	No. countries with ≥1 site	Total no. sites	No. countries with ≥1 site	Total no. sites	Total increase in sites
AFR	3	174	11	231	57
EMR	3	126	4	176	50
EUR	0	0	5	94	94
AMR	2	60	3	807	747
SEAR	6	55	6	83	28
WPR	3	31	6	684	653
Total	17	446	35	2,075	1,629

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in our analysis. AFR, African Region; AMR, Region of the Americas; CDC, Centers for Disease Control and Prevention; EMR, Eastern Mediterranean Region; EUR, European Region; ILI, influenza-like illness; SARI, severe acute respiratory infection; SEAR, South-East Asian Region; WHO, World Health Organization; WPR, Western Pacific Region.

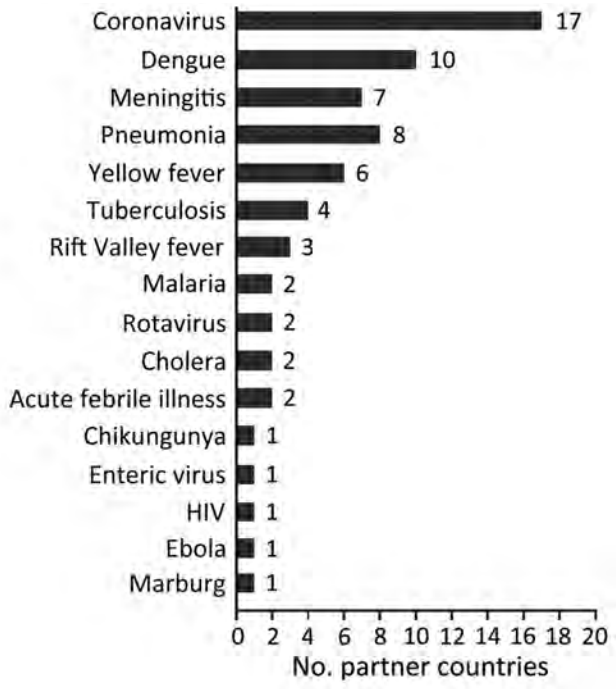

Figure 3. Number of countries that used influenza sentinel sites to initiate surveillance for other infectious diseases or syndromes since the start of the partnership program with the Centers for Disease Control and Prevention to strengthen influenza surveillance, 2004–2013. From a total of 39 participating countries, 35 responded to a 2013 questionnaire; 29 reported initiating surveillance for other diseases or syndromes.

The increase in influenza testing since the start of the program may be driven in part by the growth in ILI and SARI sentinel sites that collect weekly samples. Increases in influenza testing and number of surveillance sites call into question the notion of efficiency: how much surveillance and laboratory testing is enough, particularly in low-income countries where resources are scarce? In the United States, the Influenza Virologic Surveillance Right Size Project was launched in 2010 to help determine the optimal amount of surveillance needed to meet virologic surveillance objectives. This project and other such tools are critical for countries with limited resources and are important for the sustainability of influenza surveillance programs (*19*). As a step towards determining the optimal amount of needed surveillance, the capacity-strengthening program is helping partners clarify their national objectives and evaluate their influenza surveillance systems' data quality, flexibility, simplicity, stability, acceptability, and utility through training and technical assistance (*20*).

The program's role in supporting FluNet reporting was perceived as greater in countries that submitted reports during fewer weeks at the start of the than those that reported weekly or almost weekly. This emphasis on the program's role in increased reporting suggests a greater impact of capacity strengthening in countries with a lower baseline ability to report circulating viruses to FluNet.

Of 35 participating countries, 32 (91%) partly attributed their ability to respond to the pH1N1 pandemic to prior capacity strengthening; this perception of the role of capacity strengthening confirms the critical need for routine clinical, epidemiologic, and virologic influenza surveillance as a preparedness and response strategy. The value of routine surveillance capacity in supporting demands placed on systems during pandemics aligns with previous reports that showed significant progress in core capabilities for influenza pandemic preparedness among the same countries (*5*,*6*). In Bangladesh, enhanced surveillance of laboratory-confirmed pH1N1 infection facilitated a response weeks before the spread to the general population (*21*). In the African region, several countries were able to show the first introduction of pH1N1 virus within their countries (*22*,*23*).

Influenza viruses are constantly changing, requiring updates to the vaccine each year on the basis of which influenza viruses are infecting persons around the world, how those viruses are spreading, and how well the previous season's vaccine protects against those viruses. Therefore, the increase in the number of countries submitting specimens for seasonal vaccine strain selection is critical for selecting the most representative strains currently circulating.

The recent emergence of Middle East respiratory syndrome coronavirus in Saudi Arabia (*24*) and the devastating outbreak of Ebola in West Africa (*25*) have tested the flexibility of existing surveillance platforms in responding to emerging public health threats. Our findings provide preliminary evidence that existing health systems' strengthened influenza surveillance capacity, aimed at detecting clinical illness and prioritized for laboratory testing, has facilitated surveillance for other diseases, including Middle East respiratory syndrome and Ebola. More research regarding how influenza surveillance platforms are best leveraged is needed for the future.

Scientific data, such as laboratory-confirmed disease surveillance, aid countries in making evidence-based decisions about influenza preparedness, prevention, and control (*26*). This outcome was reported in 34 (97%) of 35 countries and shows the value of capacity strengthening. In India, surveillance data identified regional differences in the onset and length of influenza seasons; these differences affect vaccine formulation and timing (*27*). Similar evidence has been used in Southeast Asia countries, where progress in surveillance and viral typing has shown year-round circulation in some countries and biannual peaks of circulation in others; these findings informed vaccination recommendations and determination of appropriate timing for vaccination (*28*). Experience from the WHO Region of the Americas shows that the capacity for collecting and using accurate national data leads to more sustainable vaccine programs (*18*).

Table 5. Country perspectives on the role of surveillance system capacities during the 2009 influenza A(H1N1) pandemic in countries with 0–1 influenza sentinel sites at the start of the program*

WHO region	Perspective
South-East Asia	"The first fatal case during the pandemic was identified by our [influenza] network as part of hospital based surveillance activities. The network took a frontline role in providing diagnostics to the respective regions, actively participated in pandemic mitigation activities in coordination with the regional health authorities."
African	"The cooperative agreement has created awareness of influenza virus among health workers, policy makers and communities at large [and] laboratory capacity to test the virus: before there was no idea if the virus [was] in existence in the country, the types and subtypes, or the staff capacity to identify and respond to influenza." "The use of the case definitions for influenza-like illness (ILI) and severe acute respiratory infection (SARI) and the virologic analysis of the samples from cases has helped in identifying the onset of the pandemic flu H1N1 in the population and the period of dominance which informed the type of control measures put in place."
Eastern Mediterranean	"We routinely collect data on ILI and SARI cases. The sentinel sites send the epidemiologic data and specimen to [our] NIC [National Influenza Centre] for verification. We have certain examples of SARI outbreaks that the system easily detected and responded to."
European	"Sentinel sites are [now] located [along] bird migration routes and near the countries ['] points of entry. Established SARI case-based surveillance with lab confirmation is very helpful in order to provide timely detection and response to abnormal influenza."
Western Pacific	"Routine surveillance of epidemiology and viruses provided data on circulating strains with epi-clinical information which helped to detect abnormal influenza and thus helped to implement a plan."

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in our analysis. South East Asia Region had 1 sentinel site at the beginning of the program; all other regions had no sites at program start. CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.

The results of our evaluation have helped define future focus areas for the program. With the enhancement of influenza surveillance and situational awareness, CDC has developed a new program to support countries wanting to develop vaccination programs around such evidence. The challenge in answering questions about the burden of influenza disease and risk factors (Table 6) illustrates that another key next step will be to ensure that high quality surveillance data and capacity exist to help answer these questions.

That 32 (91%) countries felt ownership of capacity-strengthening offers encouraging evidence for the program's approach. The perception of increased effects of funding on laboratory strengthening, compared with increased effects on sentinel surveillance, is unsurprising, given the costs of maintaining a laboratory, a well-known barrier to routine surveillance. What is arguably more surprising is the perceived value of technical assistance beyond funding. Some responding countries perceived training and technical advice from experts, objective assessments of capacity, and the ability to share experience as having even greater effects than funding. This finding highlights the need for technical guidance, training, and partnership-building, all of which go beyond basic funding. A review of 8 Central America countries that reported a significant positive correlation between cumulative funding and technical assistance with pandemic preparedness progress supports this finding (*6*).

Strengthened influenza surveillance and detection help countries comply with WHO International Health Regulations and contribute to core competencies under the Global Health Security Agenda, which seeks to improve infectious disease detection, assessment, and response, particularly for novel influenza with pandemic potential (*29*).

The biggest limitation to this study was the reliance on retrospective data. Although WHO's externally validated data served to increase the validity of the findings, those data also have limitations. Because of the retrospective nature of the analysis, assessing lags in data availability on WHO FluNet each week was not possible, although this

Table 6. Progress in national estimates and recommendations tied to availability of routine national influenza surveillance data for 35 countries participating in CDC influenza surveillance capacity strengthening, 2004–2013*

Measures	Completed†		In progress‡	Not yet available
	Before	During		
Seasonality	10	19	4	2
Burden of influenza disease among sentinel sites	1	8	17	9
Risk factors for influenza disease	3	4	14	14
Burden of influenza disease in population	1	4	10	20
Antiviral recommendations	7	17	0	10
Vaccine recommendations	9	8	3	15
Vaccine acceptability	3	6	3	23

*A total of 39 countries partnered with CDC for influenza surveillance capacity strengthening during different years; 35 countries responded to a 2013 questionnaire and are included in our analysis. CDC, Centers for Disease Control and Prevention.
†Completed means that the measure was completed before or during the 10-year CDC capability-strengthening program.
‡In progress means that the measure was in progress during the 10-year program.

assessment assists in understanding the timeliness of monitoring. By their nature, retrospective questionnaires can be problematic because they rely on institutional memory and experience; however, respondents had a relatively high tenure in their national influenza programs. Most (31/35 [89%]) had ≥4 years of experience, and 20 (58%) had ≥7 years. The effects of influenza seasonal variation on increases in demand for testing need further elucidation and may be helped by projects such as determining optimal amounts of surveillance needed. Capacity-strengthening gains cannot be precisely attributed to the cooperative agreement because capacity strengthening is complex and involves many systems, organizations, and behaviors beyond the scope of this article.

In conclusion, considerable progress has been made in laboratory and sentinel surveillance capacities, which have proven to be essential building blocks for knowing which strains of influenza circulate globally, detecting and preparing for novel and pandemic influenza, understanding respiratory illness associated with influenza, and expanding public health surveillance beyond influenza. Countries are translating these capabilities into better decision making for their influenza prevention and control programs. Their ownership of capacity building makes this approach an important model for efforts to enhance global detection and response to emerging infectious diseases such as influenza.

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Reemergence of Dengue in Southern Texas, 2013

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During a dengue epidemic in northern Mexico, enhanced surveillance identified 53 laboratory-positive cases in southern Texas; 26 (49%) patients acquired the infection locally, and 29 (55%) were hospitalized. Of 83 patient specimens that were initially IgM negative according to ELISA performed at a commercial laboratory, 14 (17%) were dengue virus positive by real-time reverse transcription PCR performed at the Centers for Disease Control and Prevention. Dengue virus types 1 and 3 were identified, and molecular phylogenetic analysis demonstrated close identity with viruses that had recently circulated in Mexico and Central America. Of 51 household members of 22 dengue case-patients who participated in household investigations, 6 (12%) had been recently infected with a dengue virus and reported no recent travel, suggesting intrahousehold transmission. One household member reported having a recent illness consistent with dengue. This outbreak reinforces emergence of dengue in southern Texas, particularly when incidence is high in northern Mexico.

Dengue is an acute febrile illness that is common throughout the tropics and subtropics (*1*) and is a leading cause of febrile illness in travelers returning to the United States from these regions (*2*). From the late 1700s until the 1940s, dengue outbreaks occurred regularly in the southern United States (*3*) but did not occur after a campaign that rid much of the continental United States of *Aedes aegypti* mosquitoes, which transmit the 4 dengue virus types (DENV-1–4) (*4*). After the campaign ended, however, *Ae. aegypti* mosquito populations soon resurged (*5*), and dengue reemerged in southern Texas in 1980 (*6*). The 1980 dengue outbreak and subsequent outbreaks in 1999 (*7*) and 2005 (*8*) were associated with epidemics in northern Mexico. During the 1999 outbreak, despite a higher prevalence of mosquito-infested water containers in Texas, DENV infection was less frequent among residents of Texas than northern Mexico, partly because of the more prevalent use of air-conditioning, window screens, and other factors that limit human–mosquito contact in Texas (*7*). In line with these observations, infection during the 2005 outbreak was also associated with lower socioeconomic status (*8*).

During 2013, a dengue epidemic occurred in northern Mexico; >5,500 dengue cases were reported from the state of Tamaulipas (*9*), which shares a border with Texas. The first laboratory-positive dengue case in Texas was reported to the Texas Department of State Health Services in July, during the peak of the Tamaulipas epidemic. To identify and describe all suspected dengue cases in southern Texas (Cameron, Hidalgo, Starr, and Willacy Counties), enhanced dengue surveillance was conducted; this surveillance included case finding and additional diagnostic testing of specimens from suspected dengue patients. To describe the molecular epidemiology of the DENVs responsible for the outbreak, we performed molecular phylogenetic analysis. To identify persons with subclinical (either asymptomatic or symptomatic but not medically assessed) DENV infection and to describe demographic or behavioral factors associated with intrahousehold DENV transmission, we also conducted household investigations of patients with laboratory-positive dengue. This investigation proposal underwent review at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) and was determined to be public health practice and not research; as such, institutional review board approval was not required.

Methods

We identified dengue cases by compiling Texas Department of State Health Services surveillance case reports, retrieving positive and negative dengue diagnostic test results from 2 commercial laboratories, and conducting medical record reviews for patients for whom dengue diagnostic

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testing had been ordered. Specimens submitted to commercial laboratories were tested by IgM ELISA according to internal protocols. Available specimens from commercial laboratories were forwarded to CDC for testing by real-time reverse transcription PCR (rRT-PCR) (*9*).

For all specimens that were positive by rRT-PCR, we attempted amplification of the envelope glycoprotein gene (1,485 bp), followed by Sanger bidirectional sequencing; phylogenetic relationships were inferred by using previously described methods (*10*). We sequenced 5 DENV-1 isolates and 1 DENV-3 isolate. Bayesian maximum clade credibility trees were inferred to estimate genotype and phylogenetic origins.

Dengue case-patients and their household members were offered participation in household investigations. Interviews were conducted during November 2013–January 2014 and within 90 days of reported illness onset for the index case-patients. Household members completed a questionnaire that collected information on demographic, behavioral, and household characteristics; they also provided a blood specimen for dengue diagnostic testing by rRT-PCR and anti-DENV IgM antibody-capture (MAC) ELISA (InBios International, Inc., Seattle, WA, USA) performed at CDC.

We defined a suspected dengue case-patient as a patient with an acute febrile illness who sought medical care for which a clinician ordered dengue diagnostic testing. We defined a laboratory-positive case-patient as a patient with suspected dengue and a positive test result by rRT-PCR or by IgM or MAC ELISA. Because the day of specimen collection after illness onset was unknown for most patients tested at private laboratories, we defined a laboratory-negative case-patient as a patient with suspected dengue and negative IgM ELISA and rRT-PCR results for the same specimen or 2 negative IgM ELISA results for different specimens. We defined a laboratory-indeterminate case-patient as a patient with suspected dengue and a single negative IgM ELISA result. Intrahousehold DENV transmission (i.e., presumed mosquito-transmitted DENV infection within or near the home of a laboratory-positive case-patient) was defined by detection of DENV infection by rRT-PCR or MAC ELISA in a household member who had not traveled to Mexico in the past 90 days. Dengue and dengue hemorrhagic fever were defined according to the 1997 World Health Organization guidelines (*11*).

Results

Of 264 suspected dengue cases identified in southern Texas during 2013, a total of 53 (20%) were laboratory-positive; 24 (45%) of the laboratory-positive cases had been reported to the Texas Department of State Health Services. Suspected cases were identified throughout the year; about half (47%) occurred in October and November, when one third of suspected cases were laboratory-positive (Figure 1). A total of 112 serum specimens were forwarded from commercial laboratories to CDC for additional diagnostic testing by rRT-PCR; most of these specimens came from patients whose illness began during October–December. Positive rRT-PCR results were obtained for 14 (17%) of 83 IgM-negative specimens and 8 (28%) of 29 IgM-positive specimens. Of the 22 specimens positive by rRT-PCR, DENV-1 was detected in 19 (86%) and DENV-3 in 3 (14%).

All sequenced DENV-1 isolates belonged to the American-African genotype and diverged from a distinct lineage of Central American origin (Figure 2). The sequenced isolates clustered together in a subclade associated with contemporary sequences from viruses isolated in Nuevo Leon and the Yucatan Peninsula in Mexico, Nicaragua, El Salvador, and during an outbreak of travel-associated dengue in southern Arizona that occurred in late 2014 (*12*). Of the 5 Texas DENV-1 sequences, 4 diverged into a separate cluster within this subclade, and the fifth sequence was closely associated with a cluster from Nuevo Leon, Mexico, in 2012. The DENV-3 isolate belonged to the Indian subcontinent genotype, which commonly circulates in the Americas, and clustered with other contemporary sequences from viruses isolated in Nicaragua and El Salvador (data not shown).

Of the 53 laboratory-positive case-patients, most (70%) resided in Cameron County, 15 (28%) in Hidalgo County, and 1 (2%) in Willacy County. Median case-patient age

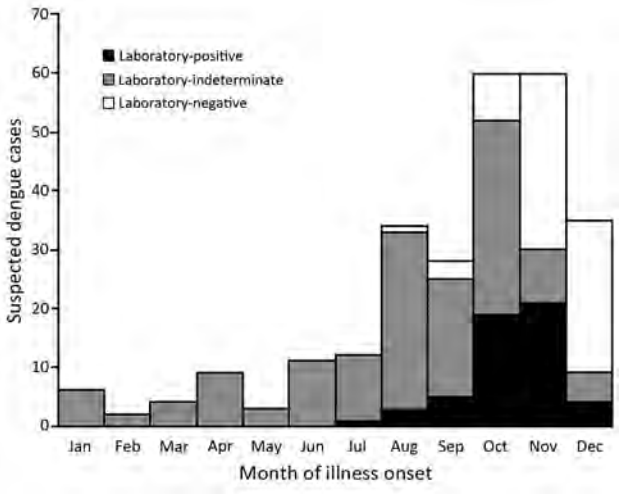

Figure 1. Suspected dengue cases identified by the Texas Department of State Health Services, 2013. A total of 264 suspected dengue cases were reported along with IgM ELISA diagnostic test results obtained from commercial diagnostic laboratories. A subset of 112 available specimens was forwarded for confirmatory diagnostic testing by real-time reverse transcription PCR and anti–dengue virus IgM ELISA. Black, positive result (n = 53); gray, laboratory-indeterminate result (n = 127); white, laboratory-negative result (n = 84).

was 28 years (range 1–85 years), and more than half (58%) of case-patients were female. Of 49 case-patients who reported their travel history, 26 (53%) did not report travel outside of Texas in the 2 weeks before illness onset and the remainder reported recent travel to Mexico. The case definition for dengue was met by 52 (98%) cases, and the definition for dengue hemorrhagic fever was met by none. More than half (29 [55%]) of the 53 laboratory-positive case-patients were hospitalized.

Participation in the household investigations was agreed to by 22 (42%) laboratory-positive dengue case-patients from 22 households and by 51 (54%) of their 95 household members, none of whom had been reported as a suspected dengue case-patient. Evidence of recent DENV

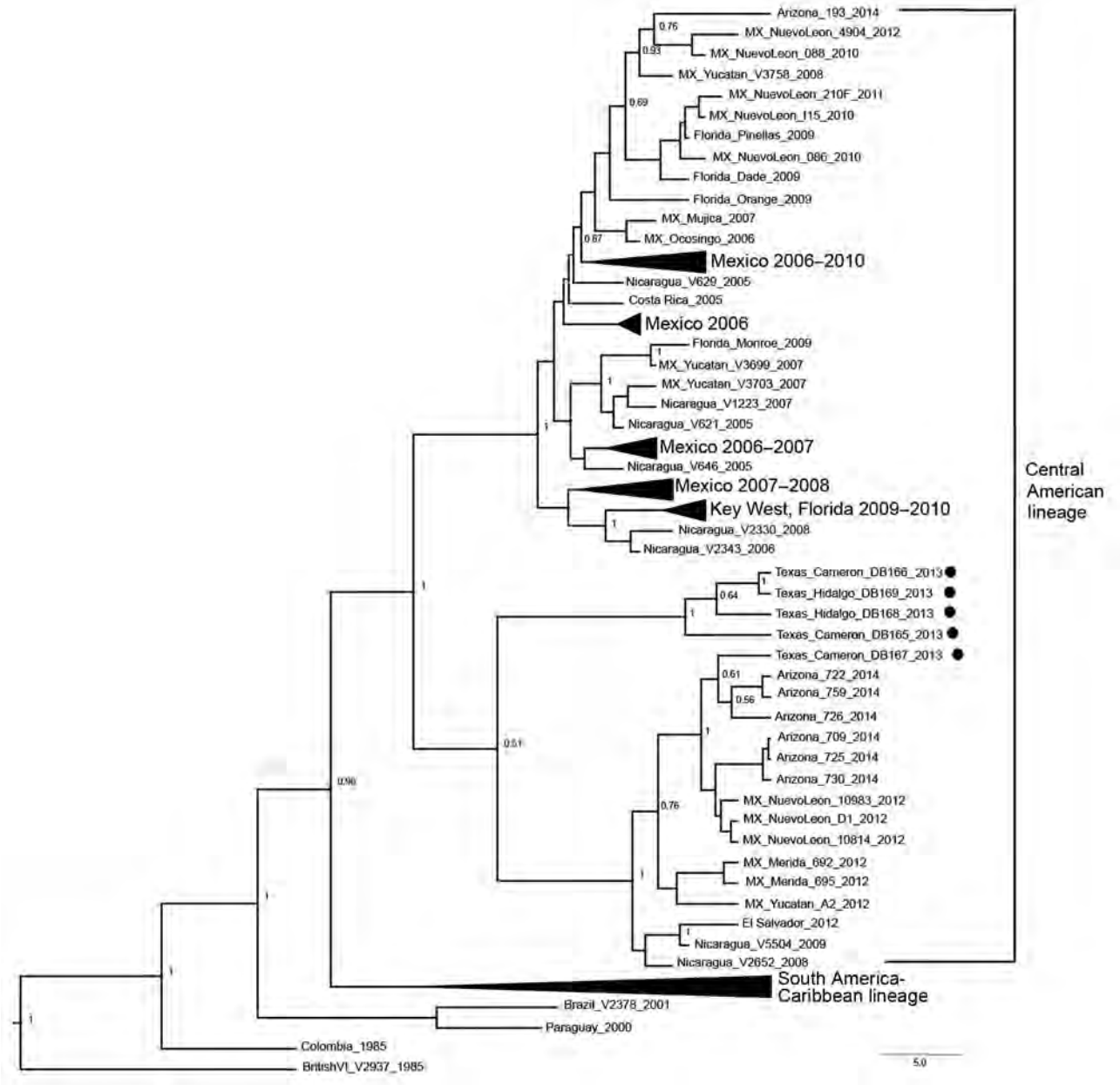

Figure 2. Phylogenetic tree of the 5 dengue virus type 1 isolates obtained from dengue case-patients in southern Texas, 2013. Maximum clade credibility tree inferred from 90 envelope glycoprotein gene sequences: 5 from Texas in 2013 (solid circles), and 85 from GenBank. BEAST version 1.8.2 (http://beast.bio.ed.ac.uk/) was used with strict molecular clock constant population size and 10 million Markov chain Monte Carlo iterations; effective sample size >200. Posterior probabilities >0.50 are shown in major nodes. All sequences shown belong to the American-African genotype. Other genotypes were not included to increase resolution of the Central American lineage. Black tapered lines indicate condensation of a monophyletic lineage with a single common ancestor. Scale bar indicates nucleotide substitutions per site. MX, Mexico.

infection was found by MAC ELISA for 7 (14%) of the 51 household members; all were older than those without recent infection (Table). Five (71%) of the 7 household members with recent DENV infection reported neither recent travel outside of Texas nor febrile illness, suggesting locally acquired but asymptomatic DENV infection. One (14%) of the 7 household members with recent DENV infection, a 79-year-old woman, reported having had fever, headache, body and eye pain, nausea and vomiting, and anorexia that began 2 weeks after her husband, the case-patient, became ill. She sought medical care for her illness in Mexico, where she was hospitalized for persistent vomiting and abdominal pain. After a 3-day hospitalization, she was discharged home in good condition.

The 7 household members with evidence of recent DENV infection resided in 6 households that had more household members and more adults than those without evidence of intrahousehold DENV transmission (Table). Other household characteristics were similar between households with and without evidence of intrahousehold DENV transmission, including frequency of having a visitor from Mexico and having air-conditioning and window screens.

Discussion

Consistent with previously reported outbreaks (*6*–*8*), the 2013 dengue outbreak in southern Texas occurred concurrently with an epidemic in Tamaulipas, Mexico. Enhanced surveillance enabled identification of 53 dengue cases in southern Texas, of which nearly one fifth initially had negative results from private laboratories and about half of the infections were locally acquired. This finding represents the largest number of locally acquired dengue cases in a single outbreak since dengue first reemerged in Texas in 1980. Molecular phylogenetic analysis of isolated DENVs determined that the viruses circulating in northern Mexico and southern Texas in 2013 were closely related to viruses that had recently circulated in Mexico and Central America. Investigations of households of dengue patients enabled identification of 7 additional persons who had recently been infected with DENV; 6 of these infections probably resulted from intrahousehold DENV transmission, and 1 was associated with an illness that was consistent with dengue.

Fewer than half of the dengue cases identified in this investigation were reported to the Texas Department of State Health Services, partly because nearly 1 in 5 case-patients received a false-negative serologic diagnostic test result from commercial laboratories. The high proportion of false-positives were probably the result of serologic testing being ordered for a specimen collected during the first 5 days of illness. Clinicians should be aware that anti-DENV IgM is typically not detectable until 3–5 days after illness onset (*10*), whereas DENV nucleic acid is typically detectable by rRT-PCR for the first 5 days of illness (*9*). Clinicians should therefore be encouraged to request both molecular and serologic diagnostic testing for dengue patients.

Of 5 DENV-1 isolates that were sequenced, 2 were from patients with locally acquired DENV infection and 3 were from patients who had traveled to Mexico. One DENV-1 isolate was determined to be closely related to a virus isolated from Nuevo Leon, Mexico, in 2012. The remaining DENV-1 isolates grouped together and were probably descendants of a common ancestor from northern Central America and Mexico. This grouping of isolates from Mexico and Texas demonstrates that multiple DENV-1 strains were co-circulating in northern Mexico and southern Texas in 2013. Moreover, the phylogenetic, geographic, and temporal data suggest that transmission of this lineage of DENV-1 is moving northward from Central America. More virus isolates will be required to further

Table. Characteristics of households and household members with evidence of intrahousehold transmission of DENV, southern Texas, 2013*

Characteristics	No evidence of intrahousehold transmission, n = 9†	Evidence of intrahousehold transmission, n = 6†
Households		
No. residents, mean (range)	2 (2–6)	4 (3–5)
Ratio of adults:children, mean (range)	0.8 (0.2–1)	2.8 (1–4)
Recent visitors from Mexico, no. (%)‡	4 (44)	1 (17)
Window screens, no. (%)	8 (89)	4 (67)
Air-conditioning, no. (%)	7 (78)	6 (100)
Mosquitoes recently seen in house, no. (%)	7 (78)	2 (33)
Household members	No evidence of recent DENV infection, n = 44	Evidence of recent DENV infection, n = 7
Age, y, median (range)	26 (1.4–71)	45 (3–79)
Portion of life lived in Mexico, mean % (range)	42 (0–96)	33 (0–71)
Recent travel to Mexico, no. (%)‡	25 (57)	1 (14)
Recently used mosquito repellant, no. (%)‡	27 (61)	4 (57)
Recent febrile illness, no. (%)‡	0 (0)	1 (14)

*DENV, dengue virus.
†7 households were excluded from analysis because not all household members provided a serum specimen for diagnostic testing and therefore could not be confidently defined as not having evidence of intrahousehold transmission.
‡Within past 3 mo.

ascertain the relationships of DENV-1 and DENV-3 circulating along the United States/Mexico border region.

Consistent with previously reported data (*13*), half of dengue case-patients in southern Texas were hospitalized during the 2013 outbreak. This hospitalization rate (55%) is higher than rates in areas where dengue is endemic (10%–20%) (*14*). Potential explanations for this rate of patient hospitalization include detection bias based on severity of dengue disease, overhospitalization of dengue patients, and appropriate hospitalization of patients who were older than those in dengue-endemic areas and who had concurrent conditions or warning signs for severe dengue.

A strength of this investigation included identification of cases by further testing specimens by rRT-PCR after initial testing by IgM ELISA. However, a limitation of this investigation was that not all specimens initially tested at commercial laboratories were available for retesting by rRT-PCR. Thus, the true number of cases that occurred in southern Texas in 2013 was probably underestimated. Also, because most DENV infections in dengue-endemic areas occur in and around the home of infected persons (*15*), the finding of other household members with evidence of recent DENV infection in one quarter of case-patients' households was not unexpected. Although only 1 person with recent DENV infection had an illness consistent with dengue, such infections are still relevant to identify, because asymptomatically infected persons have recently been shown to play a role in DENV dissemination (*15–17*). Nonetheless, these and other observations of the frequency and characteristics of infected persons cannot be applied to the population of southern Texas because they were derived from a convenience sample of households where known dengue case-patients resided. Last, because we were unable to definitively determine where infection of household members occurred, some households defined as having evidence of intrahousehold transmission may have been misclassified.

In 2013, >27 million travelers crossed the Mexico border into southern Texas (*18*), where *Ae. aegypti* mosquito populations are established (*19*). Therefore, future dengue epidemics in northern Mexico are likely to result in local DENV transmission in southern Texas. Residents of southern Texas should therefore empty, cover, or dispose of mosquito breeding sites (e.g., discarded tires, rain barrels, buckets) and use mosquito repellent to avoid mosquito bites. Clinicians should order both molecular and serologic diagnostic testing for suspected dengue patients, and positive results should be reported to public health authorities. Additional information on recommended diagnostic algorithms and dengue patient clinical management is available from CDC (http://www.cdc.gov/dengue/training/cme.html).

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Transmission of *Mycobacterium chimaera* from Heater–Cooler Units during Cardiac Surgery despite an Ultraclean Air Ventilation System

Rami Sommerstein, Christian Rüegg, Philipp Kohler, Guido Bloemberg, Stefan P. Kuster, Hugo Sax

Heater–cooler units (HCUs) were recently identified as a source of *Mycobacterium chimaera* causing surgical site infections. We investigated transmission of this bacterium from HCUs to the surgical field by using a thermic anemometer and particle counter, videotape of an operating room equipped with an ultraclean laminar airflow ventilation system, and bacterial culture sedimentation plates in a nonventilated room. Smoke from the HCU reached the surgical field in 23 s by merging with ultraclean air. The HCU produced on average 5.2, 139, and 14.8 particles/min in the surgical field at positions Off, On/oriented toward, and On/oriented away, respectively. Culture plates were positive for *M. chimaera* ≤5 m from the HCU in the test room. These experiments confirm airborne transmission of *M. chimaera* aerosols from a contaminated HCU to an open surgical field despite ultraclean air ventilation. Efforts to mitigate infectious risks during surgery should consider contamination from water sources and airflow-generating devices.

Several independent studies have reported postoperative prosthetic-valve endocarditis caused by waterborne bacteria, such as *Legionella* spp. and *Mycobacterium* spp. (*1*–*5*). The mode of transmission was not fully established until airborne transmission of *Mycobacterium chimaera* from heater–cooler units was reported (*1*,*6*). These units are widely used in open-chest heart surgery as an essential part of extracorporeal circulation but have been suggested as being a risk for infection (*7*).

Ultraclean air ventilation systems produce a vertically directed flow of filtered ultraclean air from the ceiling to the sterile operating area, also known as laminar airflow. The protective advantage of these systems compared with whole-room, turbulent, clean air ventilation has been questioned. Although Bosanquet et al. suggested that laminar airflow is superior in reducing surgical site infections in vascular patients (*8*), Brandt et al. reported an increase in surgical site infections in orthopedic surgery after use of laminar airflow systems (*9*). The hazard of horizontal air flow disrupting laminar airflow has been studied for forced-air warming systems that prevent hypothermia (*10*–*12*). Although these devices contaminate ultra-clean air ventilation systems, no definite link to an increased risk for surgical site infection has been established (*10*–*12*).

The considerable horizontal airflow generated by heater–cooler units might disrupt vertical ultraclean airflow, which could be a potential mechanism for transmission of pathogens from a contaminated heater–cooler unit to a surgical site. Thus, we conducted a series of technical and microbiological experiments to investigate the potential airborne transmission pathway of pathogens, such as *M. chimaera*, from a contaminated heater–cooler unit to the surgical field.

Methods

Setting

Experiments were performed at the University Hospital of Zurich (Zurich, Switzerland) in 2014. This hospital is an 800-bed tertiary-care center.

Heater–Cooler Units

A single model of heater–cooler unit (Model 3T; Sorin, Milan, Italy) was used as a test unit for all experiments in this study. Heater–cooler units are stand-alone devices that contain a tank that holds filtered tap water, which serves as transfer fluid to control the temperature of patient blood and cardioplegia solution (Figure 1). The tank has 2 main compartments, 1 for warming water and 1 for cooling water. Water from the 2 tanks is continuously pumped through 2 circuits that consist of tubes and temperature exchange elements attached to a heart–lung machine. The warm water circuit controls blood temperature of the patients, and the cold water circuit cools the

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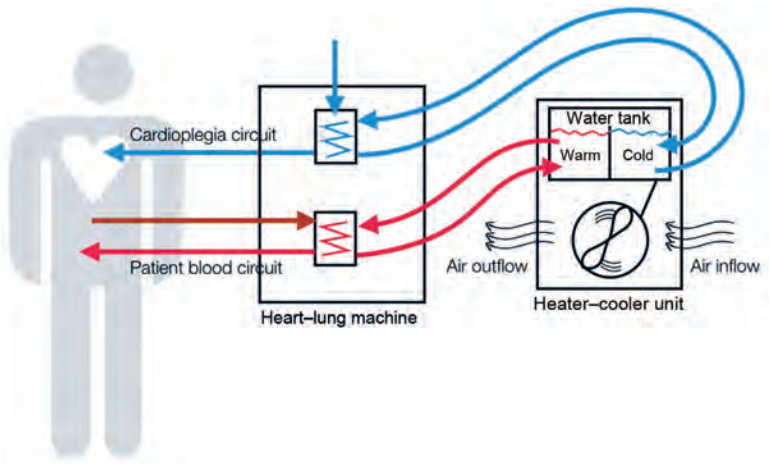

Figure 1. Schematic representation of heater–cooler circuits tested for transmission of *Mycobacterium chimaera* during cardiac surgery despite an ultraclean air ventilation system. Blue arrows indicate cold water flow, and red arrows indicate hot water flow and patient blood flow.

cardioplegia solution. Thus, water temperature in the tank might range between 2°C and 41°C during operation; water returns to room temperature on standby. The tank features filling and overflow tubes, heating and cooling coils, probes, and stirring devices. It is not airtight. The space beneath the tank holds a radiator to dissipate superfluous heat produced through water cooling. The efficacy of this radiator is increased by a fan that ensures a constant airflow through ventilation grid openings on either side of the heater–cooler unit housing.

For all experiments, the heater–cooler unit was preset to cooling of the cardioplegia circuit water to 4°C and the patient blood circuit water to 37°C to ensure maximal performance and ventilation when turned on.

Cardiac Surgery Operating Room and Ventilation System

The cardiac surgery operating room used in this study is fully functional: floor surface area is 6 m × 7 m and height 3 m. The ultraclean air ventilation system in the operating room consists of a ceiling outlet (2 m × 2.4 m) equipped with a high-efficiency particle air filter (HEPA H14). At the annual quality control check on December 14, 2014, the airflow was 0.24 m/s at 0.2 m beneath the ceiling in the center of the outlet (check was performed according to standard 14644–3:2005 by the International Organization for Standardization) (*13*).

Smoke Dispersal Experiments in a Functional Operating Room

On September 26, 2014, smoke dispersal experiments were performed in the surgical operating room in the presence of 3 researchers but no clinical staff or patient. The smoke source (Ventilax/Lüftax; AB Björnax, Strassa, Sweden) was placed at the vertical and horizontal center 0.2 m from the ventilation grid openings of the heater–cooler unit facing the operating table. Two test runs with opposite heater–cooler unit orientation were conducted, the first with ventilation airflow directed away from the operating table, and the second with ventilation airflow directed toward the operating table. The experiments were recorded by using 2 video cameras at eye level at a horizontal angle of 90°.

Particle Counts in a Functional Operating Room

On December 11, 2014, a laser counter (Handilaz Mini; Particle Measuring Systems Inc., Boulder, CO, USA) was placed at the usual location of the open chest surgical field 2 m below the ultraclean air ventilation system ceiling outlet with the sampling opening pointing upwards. The heater–cooler unit was positioned at its usual location 2 m from the operating table and turned off to obtain background measurements. During a break in obtaining measurements, the heater–cooler unit was turned on and the airflow was oriented toward the operating table. After another short break in obtaining measurements, the airflow was oriented away from the operating table. The particle counter sampled air with a rate of 1.0 ft^3/min for at least 4 min/configuration. Particle counts were reported overall and according to the 6 gates of the device that yield counts per particle size range.

M. chimaera Sedimentation in a Test Room

On September 26, 2014, a heater–cooler unit with proven contamination of the water tank by *M. chimaera* (*6*) was used. The unit was positioned in the middle of a nonventilated office (area 4 m × 7 m, height 2.8 m). Sampling was performed by using passive air sedimentation on Middlebrook 7H11 agar culture plates (diameter 10 cm). Three plates were placed on the ground and positioned in a line 3 m, 4 m, and 5 m from the operating room heater–cooler unit, and the main ventilation airstream was directed toward the plates. A room of identical configuration 2 floors below was used as a control setting without a heater–cooler unit. Identical culture plates were placed at the same locations. Plates

were left open for 4 hours and then sealed with parafilm, wrapped in a plastic bag to prevent desiccation, and immediately transported to a laboratory where they were incubated for 7 weeks at 37°C. Numbers of mycobacterial CFUs units were obtained, and species identification was performed by using 16S rRNA gene sequencing as described (*14*).

Results

Smoke Dispersal Experiments in a Functional Operating Room

In the first scenario, in which the heater–cooler unit ventilation airflow was directed away from the operating field, the test smoke was aspirated by the heater–cooler unit through the ventilation grid, expelled through the ventilation grid on the opposite side, and evacuated by the ventilation exhaust opening in the wall of the operating room (Figure 2; Video, http://wwwnc.cdc.gov/EID/article/22/6/16-0045-V1.htm). In the second scenario, in which the heater–cooler unit ventilation airflow was directed toward the operating field, smoke was blown away from the heater–cooler unit in a climbing airflow at an angle of 30°–60°. The smoke reached the ultraclean airflow 0.5 m beneath the ceiling outlet after 13 s, which resulted in a calculated mean airflow velocity of 0.23 m/s. The smoke then reached the surgical field 10 s later, which resulted in a calculated mean velocity of 0.15 m/s.

Particle Counts in a Functional Operating Room

The laser particle counter captured 5.2 particles/min (range 1–12 particles/min) when the heater–cooler unit was turned off for 5 min (background measurement), 139 particles/min (range 62–212 particles/min) when the heater–cooler unit was turned on and its airflow oriented toward the operating table for 5 min, and 14.8 particles/min (range 5–24 particles/min) when the heater–cooler unit was turned on and its airflow was oriented away from the operating table for 4 min. Results are shown in Figure 3.

M. chimaera Sedimentation in a Test Room

With the heater–cooler unit operating for the full 4 hours during which the plates were left open, the plate 3 m from the heater–cooler unit grew 2 CFUs of *M. chimaera* and the plate 5 m from the heater–cooler unit grew 1 CFU. The plate 4 m from the unit and all plates in the control test office without a unit remained sterile.

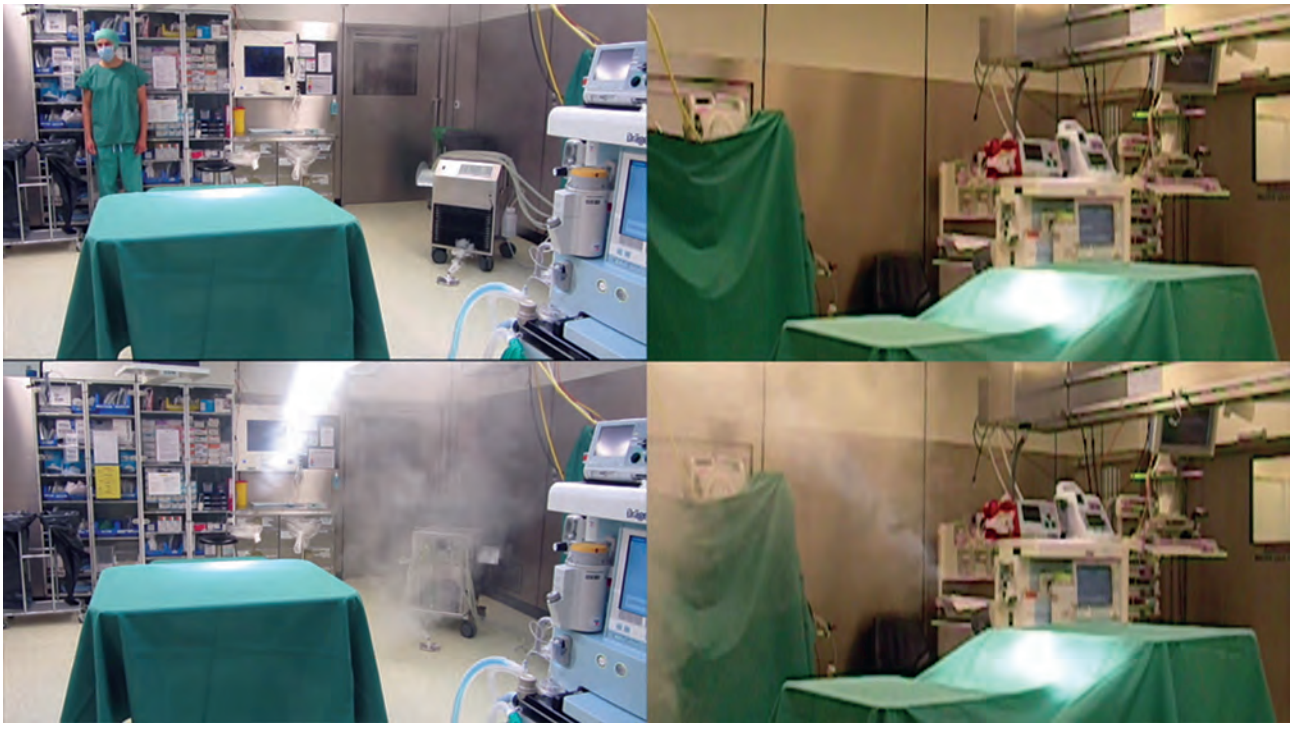

Figure 2. Video image captures showing effect of heater–cooler unit orientation on smoke dispersal in a cardiac surgery room and transmission of *Mycobacterium chimaera* during cardiac surgery despite an ultraclean air ventilation system (Video, http://wwwnc.cdc.gov/EID/article/22/6/16-0045-V1.htm). The device was switched on and began to ventilate 10 s after the start of the video. Frames on the left show an overview including unit placement. Frames on the right provide a lateral view of the operating field under the laminar airflow. Simultaneously recorded videos in the upper 2 frames show the first scenario, in which the main ventilation exhaust was directed *away* from the operating field. Simultaneously recorded videos in the lower 2 frames show the second scenario, in which the main ventilation exhaust was directed toward the operating field.

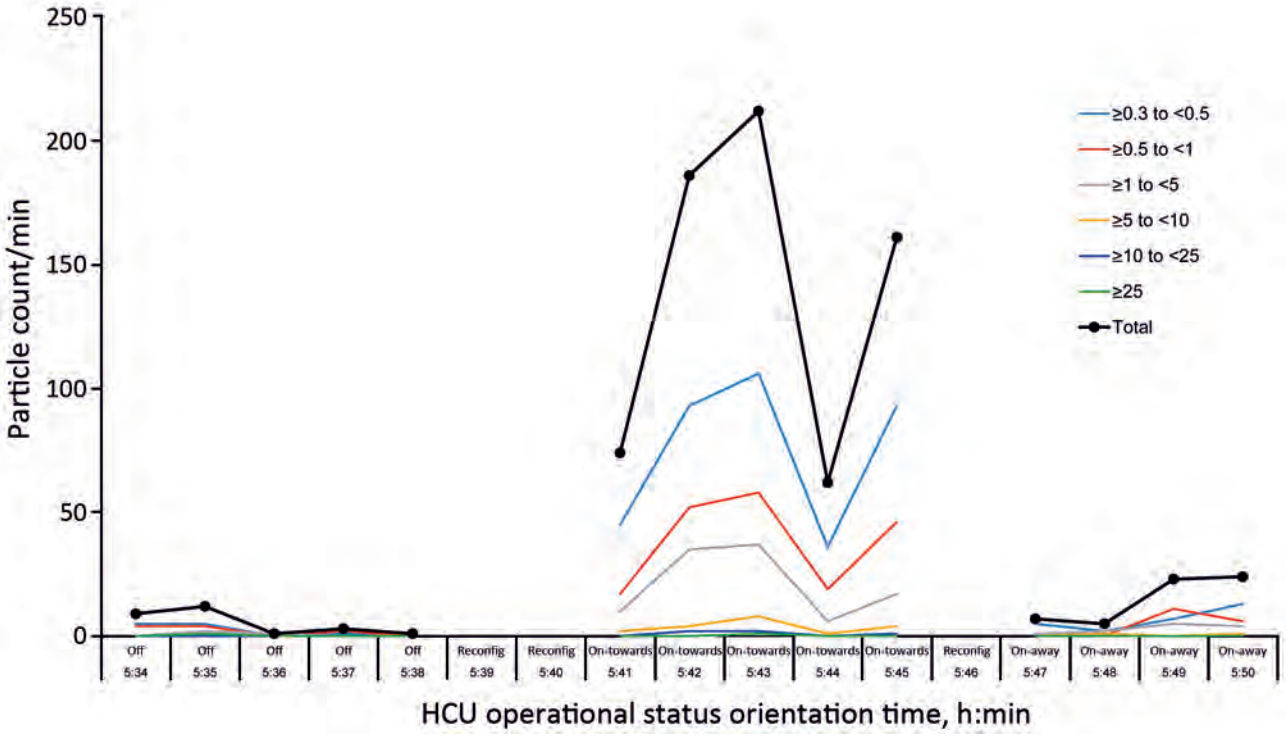

Figure 3. Laser particle measurements in cardiac operating room tested for transmission of *Mycobacterium chimaera* during surgery despite an ultraclean air ventilation system. Shown are measurements over time regarding heater–cooler unit (HCU) operational status (Off/On) and orientation (toward/away) with respect to the operating table. Lines indicate particle size ranges (in micrograms) captured by 6 gates and total particle count of the laser particle counter. Reconfig, time to reconfigure HCU status.

Discussion

Our goal was to substantiate the recently hypothesized transmission pathway of potential pathogens (*1*,*6*) from a heater–cooler unit to the surgical field by an airborne route in a cardiac surgical operating room equipped with an ultraclean air ventilation system. Smoke dispersal experiments demonstrated that air originating from the heater–cooler unit was propelled by the ventilator of the unit to merge with the ultraclean airflow near the ceiling and ultimately reached the surgical field. This finding was corroborated by particle measurements in the same setup and showed increased particle counts in the surgical field when the heater–cooler unit airflow was oriented toward the operating table than at baseline and when the opposite heater–cooler unit orientation was used. Furthermore, we demonstrated that viable *M. chimaera* can be recovered on sedimentation plates placed in the air outflow path at a distance of up to 5 m from a contaminated heater–cooler unit. These results corroborate and extend previous observations (*6*). Previously, bacteria-positive air cultures had been demonstrated only when an active air sampling device was used.

These findings have major and novel implications in 3 areas. First, they substantiate the airborne transmission pathway for outbreaks of *M. chimaera* cardiac implant–related infections (*1*,*6*). Second, they provide prototypical evidence for the failure of ultraclean airflow ventilation systems in preventing surgical site infection. Third, they exemplify the potential infectious risks related to add-on airflow producing devices in the operating room.

A recent outbreak investigation of 6 case-patients with postoperative *M. chimaera* infections showed that *M. chimaera* contaminate heater–cooler unit water and are dispersed into the air (*6*). In that study, when heater–cooler units contaminated with *M. chimaera* were turned on, air cultures associated with the unit became contaminated with the same mycobacteria, which was proven in 1 instance to display the same random amplified polymorphic DNA PCR pattern. Conversely, air cultures remained bacteria negative when heater–cooler units were switched off or were running but not contaminated. Given the bacteria-positive air cultures and the absence of another common source of infection among the 6 patients, contamination of cardiac implants or the surgical field during surgical intervention had to be assumed. Similar to results of that report, contaminated heater–cooler units and associated infections have been reported in other hospitals in Europe and the United States (*1*,*6*,*15*,*16*).

Smoke dispersal experiments visualize the airflow pathway by which bacterial aerosols can access the

Extended Human-to-Human Transmission during a Monkeypox Outbreak in the Democratic Republic of the Congo

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A >600% increase in monkeypox cases occurred in the Bokungu Health Zone of the Democratic Republic of the Congo during the second half of 2013; this increase prompted an outbreak investigation. A total of 104 possible cases were reported from this health zone; among 60 suspected cases that were tested, 50 (48.1%) cases were confirmed by laboratory testing, and 10 (9.6%) tested negative for monkeypox virus (MPXV) infection. The household attack rate (i.e., rate of persons living with an infected person that develop symptoms of MPXV infection) was 50%. Nine families showed ≥1 transmission event, and ≥6 transmission events occurred within this health zone. Mean incubation period was 8 days (range 4–14 days). The high attack rate and transmission observed in this study reinforce the importance of surveillance and rapid identification of monkeypox cases. Community education and training are needed to prevent transmission of MPXV infection during outbreaks.

Monkeypox virus (MPXV), which belongs to the genus *Orthopoxvirus*, is zoonotic and endemic to western and central Africa. MPXV is a close relative of the variola virus, and monkeypox illness resembles a smallpox-like infection but is less severe than smallpox. Most patients initially develop a fever, followed by rash a few days later. Lymphadenopathy is a common sign and occurs just before or concomitant with the rash (*1*,*2*). Up to 11% of unvaccinated affected persons die (*3*). No targeted medications are licensed to treat this infection. Although smallpox vaccination can provide some protection against infection, this vaccination is not used in MPXV-endemic areas because of cost considerations and safety concerns about using a vaccine that contains live vaccinia virus.

MPXV transmission among close contacts within households is well documented; previous reports have shown up to 6 intrafamily transmission events (*4*). Transmission is thought to occur by means of salivary or respiratory droplets or contact with lesion exudate (*5*,*6*); however, evidence suggests that infection can occur by direct inoculation (*7*). Previous household attack rates (i.e., rates of persons living with an infected person and developing symptoms of MPXV infection) of 3%–11% have been reported (*6*,*8*). Although some reports show a high incidence of households with single isolated cases (*8*), other reports have documented frequent transmission events within households (*6*,*9*). Attack rates have been found to be much higher among persons living in households with an MPXV patient and among persons with no evidence of prior smallpox vaccination (*6*,*8*).

Monkeypox is a reportable disease in the Democratic Republic of the Congo (DRC), and cases are reported from 26 health districts (containing 512 health zones). During 2013, a substantial increase in the number of suspected human monkeypox cases was noted in the Bokungu Health Zone within Tshuapa District of DRC's Equateur province (Figure 1). In December 2013, we conducted an

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cooler unit handling, and Lauren Clack for providing support with video editing.

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Extended Human-to-Human Transmission during a Monkeypox Outbreak in the Democratic Republic of the Congo

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A 600-fold increase in monkeypox cases occurred in the Bokungu Health Zone of the Democratic Republic of the Congo during the second half of 2013; this increase prompted an outbreak investigation. A total of 104 possible cases were reported from this health zone; among 60 suspected cases that were tested, 50 (48.1%) cases were confirmed by laboratory testing, and 10 (9.6%) tested negative for monkeypox virus (MPXV) infection. The household attack rate (i.e., rate of persons living with an infected person that develop symptoms of MPXV infection) was 50%. Nine families showed ≥1 transmission event, and ≥6 transmission events occurred within this health zone. Mean incubation period was 8 days (range 4–14 days). The high attack rate and transmission observed in this study reinforce the importance of surveillance and rapid identification of monkeypox cases. Community education and training are needed to prevent transmission of MPXV infection during outbreaks.

Monkeypox virus (MPXV), which belongs to the genus *Orthopoxvirus*, is zoonotic and endemic to western and central Africa. MPXV is a close relative of the variola virus, and monkeypox illness resembles a smallpox-like infection but is less severe than smallpox. Most patients initially develop a fever, followed by rash a few days later. Lymphadenopathy is a common sign and occurs just before or concomitant with the rash (*1*,*2*). Up to 11% of unvaccinated affected persons die (*3*). No targeted medications are licensed to treat this infection. Although smallpox vaccination can provide some protection against infection, this vaccination is not used in MPXV-endemic areas because of cost considerations and safety concerns about using a vaccine that contains live vaccinia virus.

MPXV transmission among close contacts within households is well documented; previous reports have shown up to 6 intrafamily transmission events (*4*). Transmission is thought to occur by means of salivary or respiratory droplets or contact with lesion exudate (*5*,*6*); however, evidence suggests that infection can occur by direct inoculation (*7*). Previous household attack rates (i.e., rates of persons living with an infected person and developing symptoms of MPXV infection) of 3%–11% have been reported (*6*,*8*). Although some reports show a high incidence of households with single isolated cases (*8*), other reports have documented frequent transmission events within households (*6*,*9*). Attack rates have been found to be much higher among persons living in households with an MPXV patient and among persons with no evidence of prior smallpox vaccination (*6*,*8*).

Monkeypox is a reportable disease in the Democratic Republic of the Congo (DRC), and cases are reported from 26 health districts (containing 512 health zones). During 2013, a substantial increase in the number of suspected human monkeypox cases was noted in the Bokungu Health Zone within Tshuapa District of DRC's Equateur province (Figure 1). In December 2013, we conducted an

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investigation of monkeypox for this health zone and focused on cases reported during July 1–December 8, 2013.

Methods

DRC has a regional surveillance system that collects reports of all suspected monkeypox cases. When possible, cases are investigated, a monkeypox-specific case report form is completed, and replicate diagnostic specimens (derived from lesions) are collected. During this investigation, additional retrospective cases within affected villages and households were identified on the basis of physical symptoms that were reported by patients and family members but that had not previously reported to the surveillance system.

Case Definitions

Cases in our investigation must have occurred during July 1–December 8, 2013. We used the following case definitions as part of the enhanced surveillance system in Tshuapa District. A confirmed case occurred in a person with a history of high fever, a vesicular-pustular rash, and ≥1 of the following 3 characteristics: 1) rash on the palms and soles, 2) lymphadenopathy, 3) fever preceding rash. In addition, this person has a PCR-tested diagnostic specimen that yielded a positive result for *Orthopoxvirus* or had MPXV DNA signatures. A probable case occurred in a person with a history of high fever, a vesicular-pustular rash, and ≥1 of the following 3 characteristics: 1) rash on the

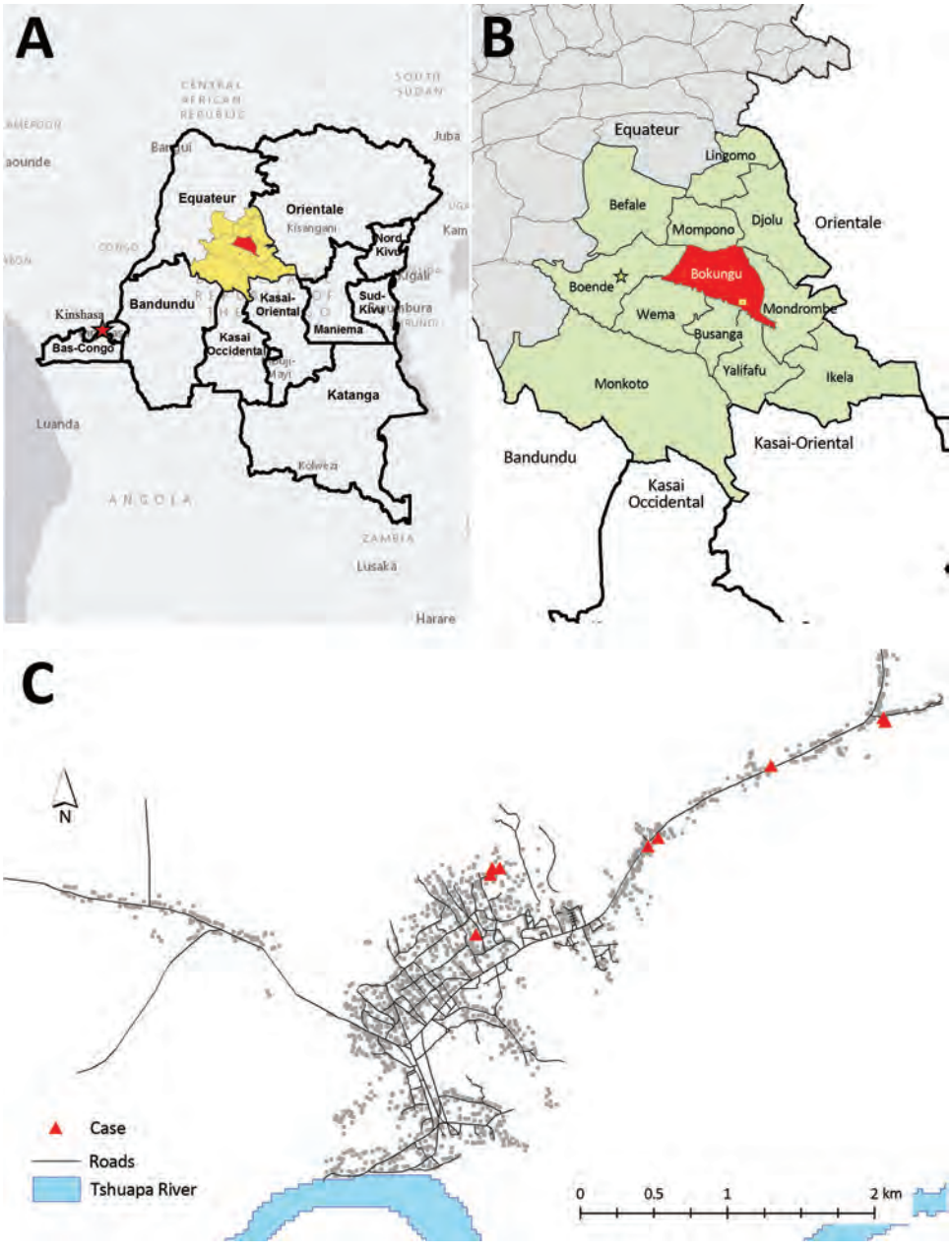

Figure 1. Region affected by monkeypox illness. A) The Democratic Republic of the Congo is outlined; Tshuapa District is highlighted in yellow and Bokungu Health Zone in red. B) Health zones within Tshuapa District; Bokungu Health Zone is highlighted in red. The village with the largest cluster of cases is indicated by a yellow square. C) Distribution of cases (shown by red triangles) in the village with the most cases during this outbreak.

palms and soles, 2) lymphadenopathy, 3) fever preceding rash. In addition, this person has a history of contact with a person or animal with confirmed monkeypox during the 14 days before illness onset. A suspected case occurred in a person with a history of high fever, a vesicular-pustular rash, and ≥1 of the following three characteristics: 1) rash on the palms and soles, 2) lymphadenopathy, 3) fever preceding rash.

Clinical symptoms were documented by a trained healthcare provider or investigator who used a standardized case-reporting form. Smallpox vaccination status was documented on the basis of patient recall and presence of a vaccination scar on the left upper arm. Analysis of vaccination status was performed with χ^2 test by using STATA 14.0 (https://www.stata.com/). Age was considered dichotomous because vaccination was available only to those >33 years of age, as a result of the discontinuation of smallpox vaccination in DRC (and other countries) 33 years earlier (1980) after the announcement of smallpox eradication.

Diagnostic specimens (crusts, vesicular fluid, or ocular fluid) were collected and shipped to the Institut National de Recherche Biomedicale in Kinshasa, DRC, for analysis. DNA was extracted from each specimen, and an *Orthopoxvirus*-specific real-time PCR assay (*10*) was performed for diagnostic confirmation. If no *Orthopoxvirus* DNA was amplified, then a second real-time PCR assay was performed for varicella zoster virus (VZV)–specific DNA (US Army Medical Research Institute of Infectious Diseases, unpub. data).

Symptom Intervals

Incubation period was defined as the number of days between contact with a symptomatic monkeypox patient and development of rash. Rash was chosen as the benchmark of infection for estimating incubation periods because families were better able to recall the day of rash onset than to recall the day of fever onset. To obtain the best estimate of the MPXV incubation period, we identified patients who reported clear dates of exposure and rash onset in our investigation and in the published literature (*4*,*9*,*11*). We determined a mathematical distribution of incubation times and calculated the mean, median, and range for the central 75% of the cases (that is, we excluded data for patients at either end of the distribution).

We conducted a larger analysis that combined the data from those persons with clearly identified dates of exposure with data containing household transmission intervals. The household data was calculated by determining the time between onset of rash for the first and second cases in a household. Cases were eliminated from this analysis if the first 2 cases in a household were separated by ≤3 days because we assumed that these case-patients were infected by the same source. We determined a mathematical distribution for the incubation times of this larger group. A secondary analysis of the dataset containing only persons with clearly defined dates of exposure and the dataset which included household transmission was performed by using an alternative formula that was developed to model serial case intervals for respiratory infections (*11*).

Transmission Chains

We estimated transmission chains (i.e., a series of persons who sequentially pass the infection to the next person) within families and villages on the basis of the calculated incubation periods for household transmission. Cases were considered independent when the interval between the onset of rash for a case-patient in the household or village was >8 days from the onset of symptoms for the previous case-patient. This value was chosen because we assumed that the first case occurred after the shortest possible incubation period (5 days) and that the last possible case occurred after the longest possible incubation period (13 days). Any cases occurring after this window of 5–13 days are considered to result from an independent infection either inside or outside the household. Coordinates for case households were recorded with handheld global positioning system units (eTrex 10; Garmin, Olathe, KS, USA) and compiled with the locations of residential structures digitized from satellite images (DigitalGlobe, Westminster, CO, USA). Household counts were aggregated into a 50-square-meter grid covering the entire populated area. Tests for spatial autocorrelation were performed by using the Global Moran's I tool in ArcGIS 10.2 (ESRI, Redlands, CA, USA) at distances of 50–1,000 meters in 50-meter increments.

Results

Monkeypox in Bokungu Health Zone

During 2013, a total of 104 suspected case-patients with human MPXV infection and 10 deaths (9.6%) were reported from the Bokungu Health Zone to the national surveillance system, with October showing a dramatic increase in number of cases (Table 1). Of the 104 suspected case-patients, 60 (57.7%) had active lesions, and specimens

Table 1. Reported monkeypox cases and deaths by month, Bokungu Health Zone, 2013*

Cases and deaths	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Cases, no.	3	0	2	3	0	10	6	0	1	61	2	16	104
Deaths, no.	0	0	0	0	0	1	0	0	0	8	0	1	10

*All cases, not yet characterized as confirmed, probable, or suspected.

Table 2. Characteristics of patients with monkeypox infections, Bokungu Health Zone, July–December 2013

Characteristic	Total cases, N = 63	Confirmed cases, n = 20	Probable cases, n = 19	Suspected cases, n = 24	Unaffected household members,* n = 53
Median age, y (mean)	10 (15.5)	14 (20.4)	7 (6.7)	10 (16.4)	20 (23)
Age range	4 mo–68 y	8 mo–68 y	4 mo–21 y	6 mo–65 y	2 mo–72 y
Male sex, no. (%)†	36 (57)	12 (60)	9/18 (50)	15/22 (68)	19/50 (38)
Vaccinated, no. (%)†	9/59 (15)	5/18 (28)	0/18 (0)	4/23 (17)	14/53 (26)

*Persons in households without symptoms and not tested.
†Denominators indicate no. patients with data available in that category.

were collected from these persons for testing. Of tested specimens, 50 (83.3%) were confirmed MPXV infections. Because MPXV infection and VZV infection have clinical similarities, testing for VZV was also performed. Five (8.3%) of the 60 patients had specimens that tested positive for VZV, and specimens for 5 (8.3%) failed to yield a positive result for either virus.

During the focused investigation period (July–December 2013), we identified and interviewed 63 case-patients in 16 households (Table 2). Of these case-patients, 26 had previously been identified, investigated, and reported by local health authorities; our investigation identified an additional 37 case-patients, including 4 with acute illness. Of the total 63 case-patients, 20 were confirmed, 19 were probable, and 24 were suspected cases. Median age of case-patients was 10 years (range 4 months–68 years); 17.7% were <5 years of age (Table 2). Of the 63 case-patients, 36 (57.1%) were male. Most cases occurred within a 74-day period between the first week of September and the last week of October (Figure 2). All 63 cases included in the 6-month investigation occurred within a 144-day window.

In the 16 investigated households, 9 (15%) of affected household members had evidence of a prior smallpox vaccination, compared with 30% of unaffected household members; χ^2 analysis showed that that this difference was not significant ($p>0.05$). However, vaccination status and age >33 years were nearly perfectly correlated ($p< 0.001$).

The median number of persons affected within each household was 3 (mean 3.9; range 1–8). The median attack rate within households was 50%; mean was 52.1% (range 50%–100%). For 1 of the 16 families investigated, all 6 household members were affected. For all households, the median interval between the time that rash developed in the first person in the household to time that rash developed in the last person was 10 days (range 2–41 days).

Incubation Period

Four case-patients were able to identify a specific date of monkeypox exposure and rash onset. These persons reported that rash developed 5–8 days after contact with an earlier case-patient. A PubMed (http://www.ncbi.nlm.nih.gov/pubmed) search identified 12 additional persons who had confirmed or probable infection and well-defined incubations periods; these case-patients had an incubation period of 9–14 days (*4*,*9*,*11*). When the 4 case-patients in our investigation and the 12 historical case-patients were considered together, mean incubation period for all was 9.6 days and median was 9 days. The central 75% of these case-patients had an incubation period of 6–13 days (Figure 3).

A second analysis was conducted with additional incubation periods that were calculated by using the difference in time of onset between the first and second cases within 12 households. These additional data were added to the 16 data points from the first analysis. For the total 28 data points available for this second analysis, the mean incubation period was 8.3 days and median was 8 days. From the second analysis, the central 75% of case-patients had an incubation period of 5–12 days (Figure 3). For subsequent

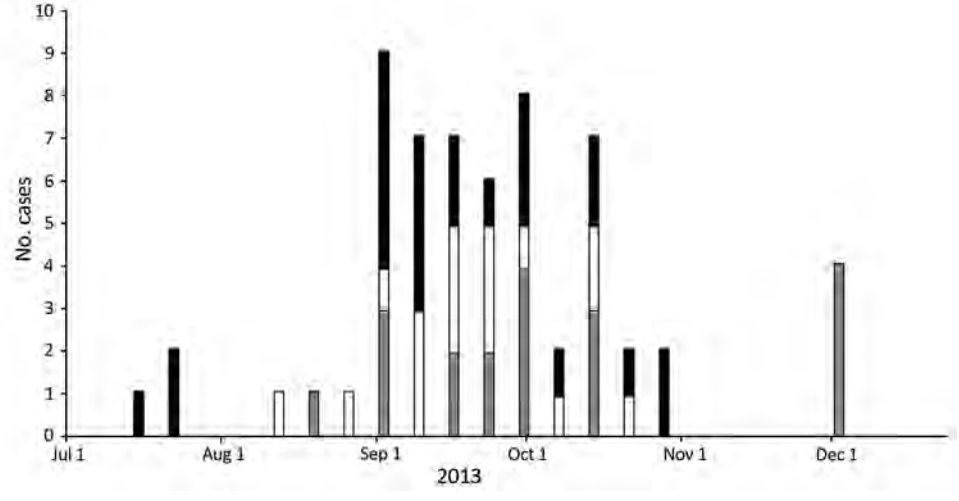

Figure 2. Epicurve of cases included in investigation and monkeypox cases during investigation period (July 1–December 8, 2013). Black represents suspected cases, white represents probable cases, and gray represents confirmed cases.

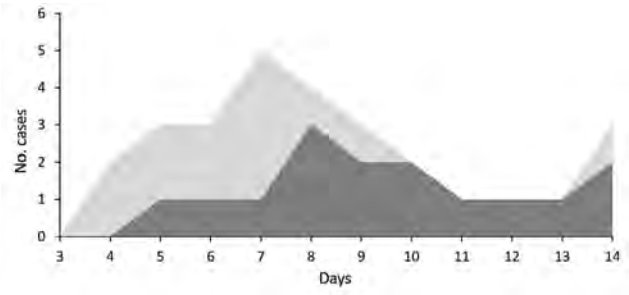

Figure 3. Distribution of incubation periods from 2 separate analyses. Dark gray shows the distribution of incubation periods on the basis of case-patients with well-defined dates of exposure identified in our investigation and in the published literature (n = 16). Light gray shows the distribution of incubation periods from the literature and incubation periods calculated by using the first 2 case-patients in each family (n = 28).

calculations in this investigation, we used the extremes in the 2 analyses as the incubation range (5–13 days, a range indicating the least and most number of days between exposure and onset of rash).

A third analysis of this data was performed by using the model described by Jezek et al. (*11*). In this analysis, which generates a model of serial intervals from the observed data, the transmission interval of the 16 well-described cases was 9.7 (95% CI 8.35–10.95) days; the interval for all 28 cases was 7.4 (95% CI 6.76–7.99) days.

Transmission Chains

Using the range of 5–13 days as the incubation period, we reconstructed transmission events within families and villages. When the longest incubation period (13 days) was used, 9 of 16 households showed ≥1 transmission event. When the shortest time of incubation was used (5 days), an additional 4 households showed patterns consistent with transmission within the household (Figure 4, panel A). Two households had cases separated by a considerable period, suggesting the occurrence of either an unknown transmission event within the household or an exposure outside of the household (Figure 4, panel B). When community-wide transmission was considered within the health zone, longer transmission chains were observed, with the longest being in the village of Bokungu, where ≥7 suspected transmission events resulted in 42 apparent cases (Figure 4, panel C). Tests for spatial autocorrelation showed that case households for were more spatially clustered (z-scores >2.0) than would be expected randomly at all distances of 50–1,000 meters.

Discussion

Human MPXV infection is endemic to DRC, with cases occurring throughout the Congo Basin. Many of these cases occur in isolation or in small clusters, but large outbreaks occasionally occur that involve many persons over a large geographic area. During 2013, a total of 104 cases of human monkeypox illness were reported in the Bokungu Health Zone. In contrast, only 17 cases were reported in 2011 and 13 in 2012. The surveillance system did not change substantively during this period; consequently, the rate in 2013 represents an increase of >600%, compared with rates for previous years. Our investigation focused on cases that occurred during the height of the 2013 outbreak.

Within the investigation period, 57% of affected persons were male, and median age was 10 years; 18% were <5 years of age. According to the United Nations World Population Prospects (http://esa.un.org/unpd/wpp/), 17.8% of the population in DRC is <5 years of age. Consequently, the age demographics of patients during the monkeypox outbreak correspond to those of the general population, suggesting that young children are not more prone to MPXV infection than others. A parallel study performed during this outbreak found no association between monkeypox illness and hunting or consumption of specific animals (*12*).

Previous publications have reported attack rates of 3%–11% (*6*,*8*); our investigation found a median attack rate of 50%, and 1 family had 100% of persons affected. The previously published attack rates are considerably lower than those for other viruses with similar routes of transmission; for example, smallpox has attack rates of 35%–88% (*13*–*15*), and variola virus had an attack rate of 90% (*16*). The difference between the findings reported here and those reported previously may result from several different causes. First, the high attack rate reported here possibly results from changing individual- and population-level immunity caused by elimination of routine childhood smallpox vaccination (*17*). Earlier investigations may have found lower attack rates because more persons had vaccine-derived immunity; however, some persons in the outbreak that we investigated had MPXV infection and prior smallpox vaccination, which suggest possible waning immunity over time, a factor that should be considered in future investigations. Second, a viral strain different from that found in previous investigations could have circulated in this outbreak and resulted in the high attack rate. However, we have no evidence for accepting or rejecting this possibility. Third, the high attack rate possibly reflects a high rate of case-patient identification in this investigation. We found that many persons are often affected in a household but that only 1 household member usually seeks medical attention, causing only 1 case to be recorded or investigated for surveillance. Case reporting on the basis of persons seeking healthcare may have caused the surveillance system to underestimate of the number of human monkeypox cases. Because we used in-home interviews, many previously unidentified cases were uncovered, enabling the calculation of a more accurate attack rate.

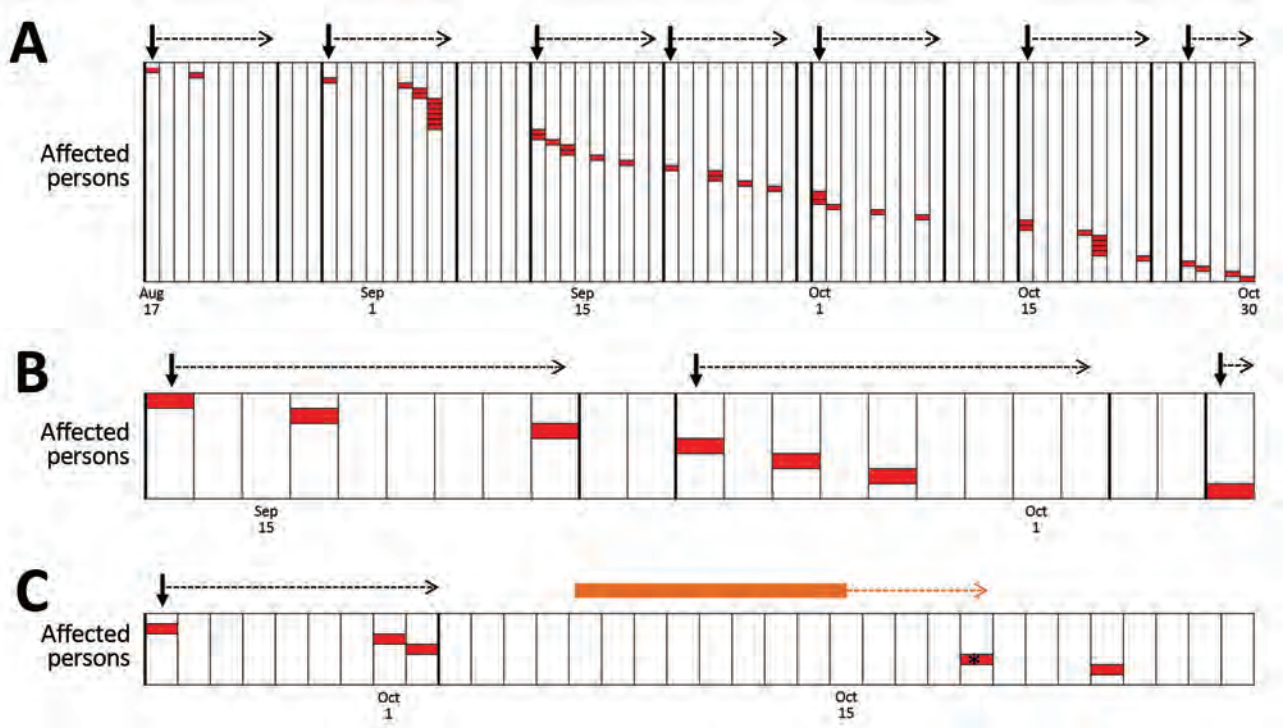

Figure 4. Reconstruction of monkeypox virus transmission events in the Democratic Republic of the Congo by using an estimated incubation period. Each column represents a calendar day. Red boxes represent a single case of monkeypox infection. A cluster is defined as a set of case-patients that could have resulted from a single exposure and are delimitated with dark vertical lines. Dark arrows indicate the first case within a cluster, and the dotted arrow indicates the time during which a potential single exposure could have produced symptoms in the first person in that group to the last (i.e., 5–13 days). A) Transmission events in the village of Bokungu in the Democratic Republic of the Congo. B) A household with evidence of 3 known transmission events. C) A household with evidence of 1 known and 1 unknown transmission event. The orange bar represents the days when the case-patient, represented by an asterisk (*), would be expected to have been exposed.

Previous investigations have shown a limited transmission capacity of MPXV within the human population. The highest number of suspected serial transmission events previously recorded is 6 (*4*,*9*). The ability to identify transmission events is limited by our lack of knowledge of the dynamics of infection. Often, whether 2 persons were coinfected by the same person and have different incubation periods or whether the persons were sequentially infected is difficult to determine. Understanding the incubation period of MPXV is vital for creating accurate transmission chains and determining if multiple introductions (human or zoonotic) occurred.

Sixteen patients with well-defined incubation periods were identified in our investigation and in the literature. Although these defined incubation periods are the best information available, they are also limited in number. Consequently, we also included apparent incubation periods within households. The time between onset of rash in 1 case and onset of rash in a subsequent case within a household provide an approximate incubation period. The longer that MPXV is present in a household, the more difficult identifying a clear infection chain is; therefore, only the transmission between the first and second case was used for the analysis. However, these 2 persons could have been infected by an outside source instead of by human-to-human transmission. The 3 analyses that we developed and presented here yielded similar results; 75% of the incubation periods were 5–13 days. Analysis of the same data by using the model proposed by Jezek et al. (*11*) yielded transmission intervals that matched data from our mathematical distribution model when we analyzed the 16 well-described cases. When all 28 cases were analyzed, the Vink model produced an interval 1 day shorter than that for the mathematical distribution. The difference between these numbers likely results from the weighting that is included in the Vink model. Further work is needed to evaluate which model best fits the biology of MPXV.

Our investigation suggests a shorter incubation period for MPXV than that observed in many animal models (*7*,*18*,*19*). Differences in organism and exposure may account for this difference. Experimental animals are often exposed to a virus for a brief time, and the interval between that exposure and development of symptoms is recorded as the incubation period. In contrast, the incubation period

in this study was defined as the time between onset of rash for the first person infected and onset of rash in the second. Although high levels of viral shedding begin with the onset of rash, virus may be transmitted before the onset of rash. Research in prairie dogs has shown that oral shedding of virus begins before the development of dermal rash (*5*); this finding indicates that spread of MPXV is possible before the appearance of external skin lesions. Therefore, the time from first exposure to development of symptoms calculated in laboratory settings may be longer than the time calculated in this analysis.

Little information is available regarding the incubation period of MPXV in humans. A monkeypox outbreak in the United States revealed that the incubation period varied (range 12–14 days), and this period was dependent on the route and nature of exposure (*7*). All US cases resulted from exposure to infected pets. In contrast, the infections described in this article are likely caused by exposure to wild animals or an infected human. The type of exposure and route of virus transmission may result in incubation periods during the US outbreak that differ substantially from those observed in the outbreak that was the focus of our investigation. In addition, previous outbreaks were caused by viruses from a different genetic clade than that which caused the outbreak reported here. Transmission times may differ because of the specific virus involved.

Altogether, the Bokungu Health Zone had 42 cases in ≥7 infection clusters (i.e., a group of cases that could have resulted from a single infectious exposure). These clusters could have been created in 3 different ways. First, the clusters could be linked sequentially, whereby the infection could be externally introduced into a cluster and then passed by 1 person from that group to cluster 2 and so on. Second, transmission may not have occurred in a clear linear fashion, but persons may have had multiple human exposures. Third, MPXV could have been reintroduced into the community from an external source (zoonotic or human) during the course of the outbreak. Although we cannot determine which of these possibilities is most likely, we favor the second model because community interactions would make a strictly linear pattern of spread unlikely to occur. Further, the limited number of cases in the population as a whole makes it less likely that an external source was causing frequent reintroductions. We can conclude that ≥6 transmissions or introductions occurred in this health zone after the initial infection.

This report has limitations that should be considered. First, MPXV infection was laboratory confirmed in 48% of the cases by using PCR; the remaining cases were identified by patients' symptoms. Laboratory confirmation was not possible for many cases because patients were interviewed after symptoms had resolved. Local resources for performing specimen collection were unavailable during all phases of the outbreak, so confirmation of MPXV infection for many cases was not possible. The lack of specimen collection has been noted as a limitation of the current surveillance program, and we are actively addressing this issue. Second, modeling of the incubation period was limited by the inability of most patients to identify a specific source of infection or a date of exposure. We assumed a minimum incubation period of 3 days when we created the incubation period model. This assumption was necessary to prevent bias toward very short incubation periods and is appropriate because of the longer incubation periods observed in animal models (*18*,*20*). Third, calculations were performed in our investigation with the assumption that transmission occurs once a person is symptomatic. Because data regarding transmission of MPXV are limited, this assumption was necessary; however, this assumption should be considered for evaluating incubation times and transmission.

This analysis provides insight into the dynamics of MPXV infection. We observed an average household attack rate of 50%, a much higher rate than reported in previous studies. Measures to decrease this attack rate should be implemented, including family-based education related to hygiene and isolation of patients. The transmission patterns observed in this outbreak also suggest transmission at the community level; therefore, community-wide education should begin as soon as the first monkeypox case is identified in an area. The calculated incubation period of 5–13 days further refines our understanding of the longest period of MPXV transmission risk after exposure in a natural setting. Knowledge of transmission risk is helpful for considering the appropriate monitoring period for exposed persons. This investigation and future work will improve our understanding of MPXV infection and our ability to limit its spread.

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Use of Population Genetics to Assess the Ecology, Evolution, and Population Structure of *Coccidioides*

Marcus M. Teixeira, Bridget M. Barker

During the past 20 years, a general picture of the genetic diversity and population structure of *Coccidioides*, the causal agent of coccidioidomycosis (Valley fever), has emerged. The genus consists of 2 genetically diverse species, *C. immitis* and *C. posadasii*, each of which contains 1 or more distinct populations with limited gene flow. Genotypic data indicate that *C. immitis* is divided into 2 subpopulations (central and southern California populations) and *C. posadasii* is divided into 3 subpopulations (Arizona, Mexico, and Texas/South America populations). However, admixture within and among these populations and the current paucity of environmental isolates limit our understanding of the population genetics of *Coccidioides*. We assessed population structure of *Coccidioides* in Arizona by analyzing 495 clinical and environmental isolates. Our findings confirm the population structure as previously described and indicate a finer scale population structure in Arizona. Environmental isolates appear to have higher genetic diversity than isolates from human patients.

Coccidioides immitis and *C. posadasii* are the only 2 species recognized within the genus *Coccidioides* (*1*). These fungi are endemic to arid or semi-arid regions of the Americas. Both species cause the disease coccidioidomycosis (Valley fever), which is contracted by dogs, humans, and other mammals living in or visiting *Coccidioides*-endemic areas (*2*,*3*). Infection is acquired through inhalation of air-dispersed arthroconidia (asexual single-cell fungal propagules). When a mammalian host inhales these conidia, a switch from polar to isotropic growth is initiated, resulting in the development of a specialized infectious structure called a spherule (*4*). Within 4 to 5 days, the mature spherules disrupt, releasing potentially hundreds of endospores, each of which are capable of developing into a new spherule (*5*). This cycle continues until the host's immune system represses fungal propagation or the fungus goes quiescent (*4*). If infection is not controlled, it can disseminate to other organs and tissues and is capable of crossing the blood–brain barrier and causing meningitis, which is fatal if untreated (*6*). Approximately 40% of infections are symptomatic (*4*).

The geographic distribution of *C. immitis* was thought to be restricted to central and southern California (*7*). However, the range extends south into Baja California and east into Arizona, and recent work shows this species was also found in eastern Washington (*8*,*9*), at Dinosaur National Monument in Utah (*10*), and in a patient in Colombia with no travel history (*11*). The species *C. posadasii* is present in Arizona, with its range extending into Utah, Texas, and Mexico and dispersed populations in Central and South America (*12*–*15*). *C. immitis* and *C. posadasii* probably co-occur in nature, given that both species have been isolated from patients in San Diego and Mexico and hybrid strains have been identified (*1*,*16*). Environmental sampling and recovery of isolates would be more helpful in confirming this hypothesis than using isolates derived from patients.

One approach to assessing genetic diversity in fungal populations is to develop microsatellite markers (*17*,*18*). Microsatellites are short (1–6 bp) tandem repeats, which are found throughout eukaryotic genomes and are thought to be evolving under neutrality in fungi (*19*). These markers have been useful in population genetics studies that compare genotypes among closely related fungal species or populations (*17*,*20*–*23*). Here we focus on the genotyping of *Coccidioides* strains from various origins by combining multiple studies in a meta-analysis and by using population genetics to clarify the causative agents of coccidioidomycosis.

Because coccidioidomycosis is increasing and disease severity is highly variable, defining genotypic distribution is important for monitoring outbreaks and determining whether increased pathogenicity is an emerging trait (*24*). Previous analysis showed that a single clone did not cause the rise in infection rates in Arizona; rather, each isolate recovered from a patient was unique (*25*). Thus, the question remains: why is coccidioidomycosis on the rise? It has

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been hypothesized that climate change, changes in human susceptibility, changes in reporting, or a result of the interaction of these factors, overlaid with high genetic variation and the possibility that *Coccidioides* can colonize new hosts and new environments, are some of the factors responsible (*3*,*26*,*27*). We aimed to answer 4 main questions: 1) if the subpopulation structure previously proposed has support when a larger dataset is analyzed by using multiple methods; 2) if there is evidence for population structure within Arizona; 3) if environmental isolates from Arizona are distinct from Arizona human host isolates; and 4) if patient data confound population structure because of incorrect identification of the point source of infection.

Methods

Strains

In total, we compiled data from 66 soil-derived isolates retrieved by mouse passage in Tucson, Arizona (*28*); 141 isolates from Arizona patients with Valley fever (*25*); 106 *C. posadasii* and 62 *C. immitis* isolates from a broad geographic range (*1*); and 266 clinical *C. posadasii* isolates (human and veterinary) newly analyzed for this study (online Technical Appendix 1 Table, http://wwwnc.cdc.gov/eid/article/22/6/15-1565-Techapp1.xlsx). Of these 641 isolates, 22 were removed from final analysis for failure to amplify >2 of the 9 loci.

DNA Extraction

To extract DNA, we placed ≈0.2 g of mycelia in a 2-mL screw-cap tube containing 0.5-mm–diameter sterile glass beads (BioSpec, Bartlesville, OK, USA) and 1 ml of lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 100 mmol/L EDTA [pH 8.0], 100 mmol/L NaCl, 0.5% sodium dodecyl sulfate, and 100 mmol/L β-mercaptoethanol) and subjected it to mechanical disruption by vortexing on a flat 12-tube holder (MoBio, Carlsbad, CA, USA) at 3,700 rpm for 10 minutes. Samples were incubated at 65°C for 60 minutes and centrifuged at 8,000 rpm for 5 minutes. We extracted nucleic acids from the supernatant with buffered phenol:chloroform:isoamyl alcohol pH 8.0 (25:24:1) and again with cholorform:isoamyl (24:1) and precipitated from the aqueous layer with 0.6 volumes of isopropyl alcohol. We washed the pellets twice with ethanol and resuspended them in 150 μL of double-distilled H_20. DNA concentration was determined on NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and was diluted to 20 ng/μL.

Multilocus Microsatellite Typing Markers and PCR

To genotype isolates, we used 9 microsatellite primers developed for phylogenetic analysis and tested for concordance in *Coccidioides* (*17*,*18*,*25*). All microsatellite fragments were first denatured for 2 min at 96°C, followed by 30 amplification cycles (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) and 1 extension cycle of 5 min at 72°C with 2.5× Hotmaster mix (Eppendorf, New York, NY, USA). One primer from each set was end-labeled with a fluorescent tag (either NED dye [ABI, Shirley, NY, USA] or FAM or HEX [Eurogentec, Seraing, Belgium). Primer concentrations were 200 nmol/L each per reaction, and 100 ng of DNA was used for each reaction.

Fragment Analysis

We grouped microsatellite fragments from each isolate into 3 sets of 3 fragments and labeled 1 primer set in each grouping with HEX, FAM, or NED. Pooled PCR products were separated on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Arizona Genomic Analysis and Technology Core sequencing facility, using a ROX-labeled ladder (Invitrogen, Carlsbad, CA, USA) for sizing. Chromatographs were read in Genotyper (ABI, Shirley, NY, USA), and a single peak was scored (*Coccidioides* is haploid). No evidence of multiple peaks was detected. Microsatellites were amplified and analyzed at least twice to verify their size. To compare our isolates to those described in published data, we analyzed the microsatellite sizes from a subset of previously analyzed isolates on our ABI 3730 system (online Technical Appendix 1 Table, duplicates tab). Calibration was necessary to compare the published microsatellite sizes to our data (online Technical Appendix 1 Table, correction tab).

Population Analyses

We tabulate data from the Genotyper program maintained them in a spreadsheet (online Technical Appendix 1 Table). Files were checked for duplicates and clone–correction checked using GenAlEx 6.501 (*29*). We found identical isolates from multiple isolations from the same patient and from isolates collected from the same soil site. Any samples that were missing ≥3 loci were eliminated from the final dataset. Locations were incorporated into a nexus file containing 619 isolates. We assigned locations based on the isolation/hospital origin as follows: Phoenix, Yuma, and Tucson (Arizona); San Diego and San Joaquin Valley (California); Texas; Mexico; and South America (Brazil, Argentina, and Venezuela).

We analyzed microsatellite matrices by using STRUCTURE 2.3.4 (Pritchard Laboratory, Stanford University, Stanford, CA, USA) to determine population structure within *Coccidioides* (*30*). The running length of burn-in period was 100,000 repetitions with 1,000,000 Markov chain Monte Carlo repetitions. Default settings in STRUCTURE 2.3.4 were as follows: the admixture model

was used to infer α along with the previous sampling location information model (LOCPRIOR) (*30*). We used CLUMPP, a cluster matching and permutation program (https://web.stanford.edu/group/rosenberglab/clumpp.html), to define populations within the STRUCTURE algorithm. K is the number of significant populations in each main group. Allele frequencies were assumed to be correlated among populations, assuming that there are different Fst values for different subpopulations, the previous mean of Fst for populations is 0.01, and λ is constant at 1.0. Ten runs for each *k* from 1 to 10 were performed, and results were analyzed using Evanno's method implemented in StructureHARVESTER (*31*). We generated a consensual STRUCTURE plot from the admixture values using the Clustering Markov Packager Across K (CLUMPAK) (http://www.clumpak.tau.ac.il) and built final plots with STRUCTURE PLOT (*32*,*33*).

We also inferred *Coccidioides* population splits and mixtures trees using a statistical model related to common ancestors through a graph of ancestral populations via TreeMix software (Pritchard Laboratory) (*34*). In brief, we inferred a population tree on the basis of microsatellite data for each of the identified populations in STRUCTURE (online Technical Appendix 2 Table, http://wwwnc.cdc.gov/eid/article/22/6/15-1565-Techapp2.xlsx). Migration events were placed on admixed edges, which are correlated with the degree of ancestry for each population and represents unidirectional gene flow between populations. Horizontal branch lengths are proportional to the accumulated genetic drift (drift parameter) from each population that was placed in a given branch. The drift parameter measures the variance in allele frequency that changes along each population of the tree. We also analyzed the same data were by using Nei's unbiased genetic distance estimate (Table 1), to complete a principal coordinate analysis (PCoA) (Table 2) in GENALEX 6.501 (http://www.biology-assets.anu.edu.au/GenAlEx/Welcome.html) (*29*). We documented allele frequencies, private alleles, and haploid diversity calculations (Table 3) for Arizona samples (online Technical Appendix 2 Table).

Results

Combining Data from Multiple Sources

We documented microsatellite frequencies (online Technical Appendix 3 Figure 1, http://wwwnc.cdc.gov/eid/article/22/6/15-1565-Techapp3.pdf; online Technical Appendix 1 Table). Three loci (GAC2, 621.1, and ACJ) had low diversity in *C. posadasii*, and these same loci were variable in *C. immitis*. Three loci showed the opposite pattern (K01, K03, and K07) and had low diversity for *C. immitis* and are variable in *C. posadasii*. Three loci (K09, GA1, and GA37) were diverse for both species. These results were similar to those of earlier reports (*1*). We merged datasets were merged for analysis (online Technical Appendix 1 Table) and analyzed isolates from both published datasets (online Technical Appendix 1, duplicates tab). Manual corrections of 1 or 2 bp were needed because of slight variations among machines and ladders (online Technical Appendix 1, corrections tab).

Population Structure of *Coccidioides* Subspecies

STRUCTURE analysis based on 619 isolates revealed 3 *Coccidioides* populations for *C. immitis* (n = 61), *C. posadasii* Mexico/Texas/South America (n = 63), and *C. posadasii* Arizona (n = 495) (online Technical Appendix 3 Figure 2). We detected low gene flow between the 3 major populations as observed by unique bar plots for each of these populations and observed gene flow between *C. posadasii* Mexico/Texas/South America and *C. posadasii* Arizona and between *C. immitis* and *C. posadasii* Mexico/Texas/South America (online Technical Appendix 3 Figure 2). The population tree displays the 3 main populations and population assignments for each isolate along the bar plots (online Technical Appendix 3 Figure 2). PCoA analysis using Nei's unbiased genetic distance estimates revealed 3 main groupings when considering all data (Figure 1, panel A). Principal component (PC) 1 explains 93.92% of the variation, mainly attributable to variation between the species (Eigen value 1.202). PC2 explains 3.95% of the variation, reflecting the subpopulation structure in both species

Table 1. Pairwise population matrix of Nei's unbiased genetic distance for principal coordinates analysis of *Coccidioides* populations, Arizona, USA*

Population	PHOENIX	AZSOIL	TUCSON	SJV	SDMX	MEXICO	TXSA
PHOENIX	0.000						
AZSOIL	0.128	0.000					
TUCSON	0.158	0.277	0.000				
SJV	2.582	2.571	1.675	0.000			
SDMX	2.519	2.570	1.737	0.143	0.000		
MEXICO	0.354	0.324	0.477	1.480	1.546	0.000	
TXSA	0.602	0.638	0.526	1.580	1.734	0.373	0.000

*The larger the genetic distance value, the greater the genetic difference between populations. PHOENIX represents primarily *Coccidioides posadasii* human clinical isolates from Yuma and Phoenix, Arizona. AZSOIL represents primarily environmental and veterinary clinical *C. posadasii* isolates from Arizona. TUCSON represents primarily human clinical *C. posadasii* isolates from Tucson, Arizona. SJV represents primarily *C. immitis* human clinical isolates from Bakersfield, California. SDMX represents primarily *C. immitis* human clinical isolates from San Diego, California, and Mexico. MEXICO represents primarily human clinical *C. posadasii* isolates from Mexico. TXSA represents primarily human clinical *C. posadasii* isolates from Texas, Brazil, Argentina, and Venezuela.

Table 2. Principal coordinates analysis results indicating percentage of variation among *Coccidioides* populations, Arizona, USA*

	Axis		
Value	1	2	3
% Variation	93.92	3.95	1.44
Total eigenvalue	1.202	0.051	0.018
PHOENIX	0.491	−0.061	0.009
AZSOIL	0.498	−0.018	0.009
TUCSON	0.213	−0.012	−0.103
SJV	−0.552	0.089	−0.023
SDMX	−0.552	−0.098	0.022
MEXICO	0.162	0.048	0.081
TXSA	0.179	0.163	−0.001

*PHOENIX represents primarily *Coccidioides* posadasii human clinical isolates from Yuma and Phoenix, Arizona. AZSOIL represents primarily environmental and veterinary clinical *C. posadasii* isolates from Arizona. TUCSON represents primarily human clinical *C. posadasii* isolates from Tucson, Arizona. SJV represents primarily *C. immitis* human clinical isolates from Bakersfield, California. SDMX represents primarily *C. immitis* human clinical isolates from San Diego, California, and Mexico. MEXICO represents primarily human clinical *C. posadasii* isolates from Mexico. TXSA represents primarily human clinical *C. posadasii* isolates from Texas, Brazil, Argentina, and Venezuela. Axis 1 explains 93.92% of the genetic variation among the populations, which is due mainly to separation of *C. immitis* (SJV and SDMX) from *C. posadasii.* Axis 2 suggests gene flow between SJV, MEXICO, and TXSA. Axis 3 indicates that the TUCSON population contains unique genetic signatures.

(Eigen value 0.051). PC3 explains 1.44% of variation and further separates Mexico from Arizona (Table 2).

Population Structure within *C. immitis* Population

Results of PCoA analysis strongly indicated population structure within *C. immitis*, separating San Joaquin Valley (SJV) from San Diego and Mexico (SDMX) isolates (Figure 1, panel A). STRUCTURE analysis also indicates a strong population subdivision within *C. immitis* (Figure 1, panel B). According to the optimal number of clusters determined by using StructureHARVESTER, the SJV and SDMX isolates are clustered into 2 different populations ($k = 2$) (Figure 1, panel B; online Technical Appendix 3 Figure 3). Bar plots show that limited gene flow was observed between these subpopulations; however, the bar plots also indicated that the *C. immitis* isolates 17TX, 4SD, 8SD, 4M3, and 8M3 share alleles from both populations (online Technical Appendix 3 Figure 3). The population tree indicates that the *C. immitis* SJV population has a migration event from the *C. immitis* SDMX population (Figure 2). The isolate population distribution frequency for *C. immitis* reveals differences between SDMX and SJV populations (Figure 3).

Population Structure within *C. posadasii* Mexico/Texas/South America Population

For *C. posadasii* Mexico/Texas/South America population, we detected 2 optimal clusters (online Technical Appendix 3 Figure 3), 1 including Texas/South America isolates and 1 constituting isolates from Mexico (Figure 1). The Mexico isolates display a high level of hybridization between 2 different populations as well as within *C. immitis* (online Technical Appendix 3 Figures 1, 3). The migration event from *C. immitis* SDMX to *C. posadasii* Mexico and the more basal *C. immitis* to *C. posadasii* Tucson migration event implicate the Sonoran desert as a convergent source of multiple *Coccidioides* genotypes and possible center of origin of the genus (Figures 2, 3).

Population Structure within *C. posadasii* Arizona Population

Population structure analysis of 495 separate fungal isolates suggests at least 3 different *C. posadasii* subpopulations in Arizona, in agreement with PCoA data (Figure 1). Clinical samples from Yuma and Phoenix (designated PHOENIX) and Tucson patients (designated TUCSON) fall in 2 different populations according to STRUCTURE (Figure 1,

Table 3. Summary of diversity indices for the *Coccidioides posadasii* population, Arizona, USA*

Source of isolate	No.	Different alleles	Effective alleles	Shannon's informative index	Diversity	Unbiased diversity	Private alleles
Tucson clinic							
Mean	251.444	9.444	3.139	1.288	0.581	0.584	20
SE	3.158	1.676	0.533	0.220	0.085	0.085	
Yuma clinic							
Mean	9.000	3.333	2.568	0.905	0.480	0.540	0
SE	0.000	0.577	0.473	0.204	0.101	0.114	
Phoenix clinic							
Mean	128.333	6.889	2.952	1.169	0.554	0.558	5
SE	1.546	1.296	0.560	0.207	0.083	0.084	
Soil							
Mean	64.778	6.667	3.155	1.257	0.598	0.607	4
SE	1.103	1.118	0.529	0.192	0.077	0.078	
Veterinary							
Mean	13.556	4.222	2.748	1.044	0.528	0.571	0
SE	0.338	0.703	0.437	0.199	0.091	0.099	

*Tucson clinic isolates are all human clinical isolates from Tucson, Arizona. Yuma clinic isolates (previously included in the PHOENIX population) are all human clinical isolates from Yuma, Arizona. Phoenix clinic isolates are all human clinical isolates from Maricopa County, Arizona. Soil isolates are the 66 environmental isolates from Tucson, Arizona, previously grouped in AZSOIL. Veterinary isolates are from various host animals, previously grouped in the AZSOIL population.

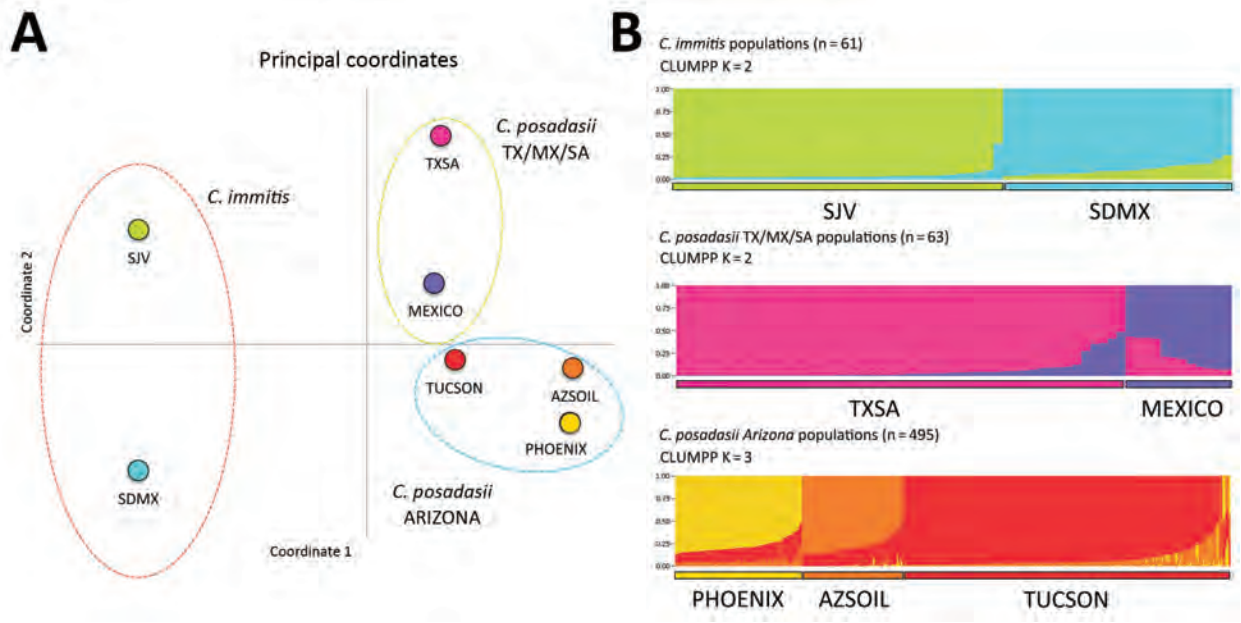

Figure 1. Results of principal coordinate analysis and STRUCTURE analyses of *Coccidioides spp.* populations. A) Principal coordinate analysis using Nei's unbiased genetic distance estimates supports 3 main groupings: *C. immitis, C. posadasii* TX/SA/MX, and *C. posadasii* Arizona (see also online Technical Appendix 3 Figure 2). The greatest separation occurs between species and is reflected in principal coordinate 1 (93.92% of variance). Color-coding for populations: lime green, San Joaquin Valley (SJV); aqua, San Diego/Mexico (SDMX); pink, Texas/South America (TXSA); purple, Mexico (MEXICO); red, Tucson (TUCSON); yellow, Phoenix/Yuma (PHOENIX); orange, soil (AZSOIL). B) STRUCTURE analysis. Microsatellite matrices were analyzed with STRUCTURE 2.3.4 to determine population structure within *Coccidioides* populations (*30*). The running length of burn-in period was 100,000 repetitions with 1 million Markov chain Monte Carlo repetitions. Default settings in STRUCTURE 2.3.4 were as follows: the admixture model was used to infer α along with the previous sampling location information model (LOCPRIOR) (*30*). We used CLUMPP, a cluster matching and permutation program (https://web.stanford.edu/group/rosenberglab/clumpp.html), to define populations within the STRUCTURE algorithm. K is the number of significant populations in each main group. A consensual STRUCTURE plot was generated from the admixture values by using the Clustering Markov Packager Across K (CLUMPAK) server, and final plots were built with STRUCTURE PLOT (*32*,*33*).

panel B). All environmental samples and some veterinary/clinical samples from Tucson, Phoenix, and Yuma regions constitute a third population (designated AZSOIL) apart from the TUCSON and PHOENIX populations (Figure 1; online Technical Appendix 3 Figure 3). We detected high level of admixture in the Arizona population, suggesting gene flow between 3 populations. However, the presence of private alleles for different loci within each of the 3 Arizona populations supports genetic isolation (online Technical Appendix 2 Table, AZ_PAL tab). Structure plots of AZSOIL, PHOENIX, and TUCSON populations contain isolates with genotypes from all 3 populations (online Technical Appendix 3 Figure 3). AZSOIL and TUCSON populations arose from the same geographic origin (Figure 3). The population tree (Figure 2) supports a migration event from AZSOIL to TUCSON. The AZSOIL is placed nearer to the ancestral branch for Arizona subpopulations. In addition, a low number of clinical isolates clustered with AZSOIL, leading us to consider variable pathogenicity or host specificity (Figure 2). We propose that a mammalian host or its close microenvironment (e.g., mammal burrows) could contribute to increased fitness of a virulent phenotype. Thus, the environmental reservoir could play a role in the emergence of pathogenic strains.

Clinical Isolates

Data obtained from genotyping human patient isolates might lead to incorrect estimates of population structure. Two *C. immitis* were found in patients in Phoenix hospitals, and both patients had confirmed travel to California; however, we analyzed only 1 because the other did not meet our cutoff criteria (*25*). A Texas patient isolate was determined to be *C. immitis* (*1*). Patients from China, Switzerland, and Colorado (1 patient from each) and 7 California patients were infected with *C. posadasii* (*1*). One of the widely used laboratory *C. posadasii* strains (Silveira) was isolated from a patient with coccidioidomycosis diagnosed in California. In northern Mexico (including Baja California) and southern Mexico (Michoacán state), many strains are genotyped as *C. immitis* but have evidence of hybridization with *C. posadasii* and signatures of introgression (*16*). Less is known about the prevalence of introgression found in the *C. posadasii* Mexico population. For Arizona isolates newly analyzed for this study, no *C. immitis* were identified (online Technical Appendix 1 Table).

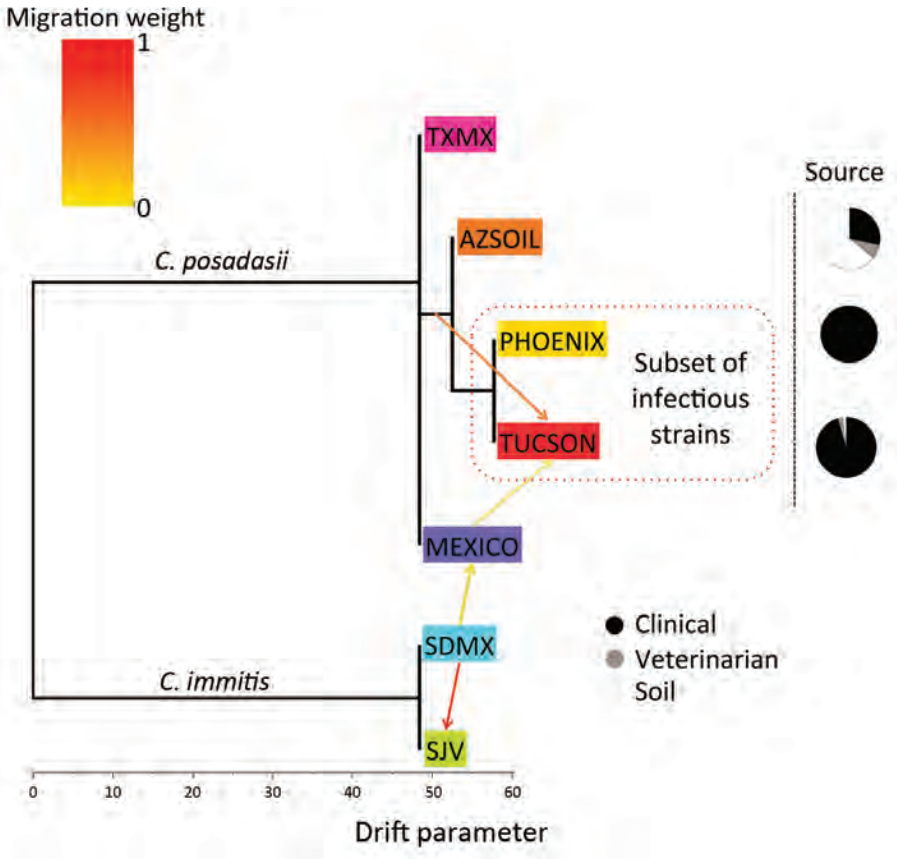

Figure 2. Population tree of *Coccidioides* subspecies population splits and mixtures. Maximum-likelihood population tree and presence of gene flow between diverged *Coccidioides* populations were inferred by using TreeMix software and microsatellites data (*34*). Direction of arrow indicates migration or gene flow based on admixture models; migration weights are shaded according their importance, supporting gene flow from a soil-derived population (AZSOIL) recovered from animal passage to a clinical-associated population (TUCSON). Color-coding for populations: lime green, San Joaquin Valley (SJV); aqua, San Diego/Mexico (SDMX); pink, Texas/South America (TXSA); purple, Mexico (MEXICO); red, Tucson (TUCSON); yellow, Phoenix/Yuma (PHOENIX); orange, soil (AZSOIL). The drift parameter, represented by horizontal scale, measures the variance in allele frequency change along each branch of the tree. The actual source of each evaluated isolate (clinical, veterinary, or soil) is represented proportionally in the pie chart.

Discussion

Multiple methods and previous reports show that there are 2 species within *Coccidioides* defined as *C. posadasii* and *C. immitis* (*1*,*7*). Within species, *C. posadasii* contains the 2 main populations of Texas/South America/Mexico and Arizona, and within *C. immitis*, 2 populations are suggested, SJV and SDMX, supported by our data and previous reports (Figure 1) (*7*). Gene flow between *C. immitis* populations is not abolished, as exemplified by the admixture isolates 17TX and 22SD (online Technical Appendix 3 Figure 3). STRUCTURE analysis suggests that *C. posadasii* Arizona and Texas/Mexico/South America populations are highly differentiated, with few isolates sharing genotypes among them (online Technical Appendix 3 Figure 3). Additionally, divergence between Mexico and South America/Texas is evident, such that they are evolving independently (Figure 1).

Within the Arizona population, we observed 3 clusters: PHOENIX, TUCSON, and AZSOIL (Figure 1). PHOENIX consistently groups separately from TUCSON and AZSOIL, which might reflect differences in ecology between Arizona upland (Tucson) and the Lower Colorado Valley (Phoenix and Yuma) or variation in pathogenicity among hosts. Variation in mean soil temperature, precipitation, natural hosts, and vegetation could exert differential selection pressure on the fungus in the environment (*35*,*36*). In addition, according to the population tree, the AZSOIL subpopulation appears to be basal within Arizona. The migration event from AZSOIL to TUCSON might reflect selection of more pathogenic genotypes because only ≈40% of infections are symptomatic (*4*), and even fewer of these would result in severe disease where the isolate would be collected from the patient (Figure 2). This leads us to propose that the AZSOIL subpopulation reflects greater diversity than the TUCSON and PHOENIX subpopulations and that this greater diversity might be driven by selection of certain pathogenic strains in humans.

Moreover, our soil sampling reflects diversity at only 7 locations in and around Tucson, and all samples were collected with a single year, whereas the patient isolates from Tucson were collected over a period of 30 years. These soil isolates were obtained using a highly sensitive murine model of coccidioidomycosis. Not all mice had evidence of illness, and infection was only realized upon necropsy. Thus, we might have selected for infectious strains, but we believe we captured diversity in pathogenesis. This assumption would suggest that we have underestimated diversity in the environment. Diversity at some soil locations was high (i.e., multiple genotypes were recovered), whereas other sites were clonal, or we only recovered a

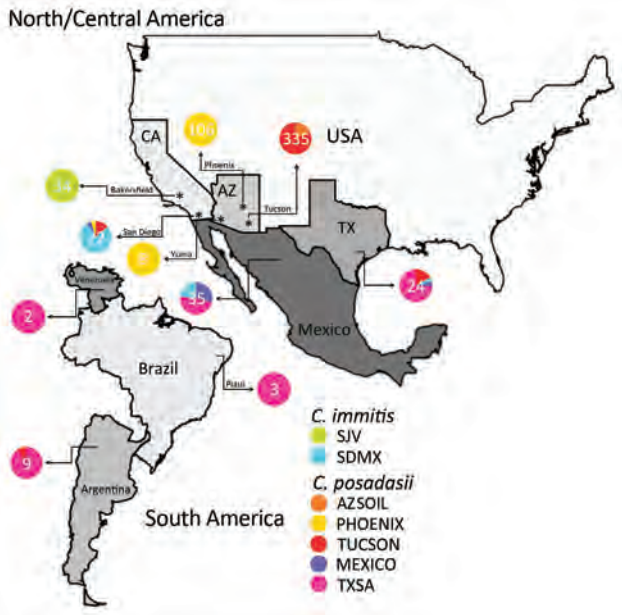

Figure 3. *Coccidioides* subspecies distribution for North, Central, and South America. The frequency of assignment for each *Coccidioides* population was plotted in a pie chart for each location, and numbers of isolates from each location are displayed. For example, patients from Mexico were infected with isolates from Texas, San Diego, and Mexico populations, as determined by analysis with STRUCTURE. Because each of the patients' location is the hospital, no fine-scale population is assessed.

single colony. Thus, it was surprising to find higher unbiased genetic diversity in AZSOIL (0.607, ±0.078 SE) than in TUCSON (0.584, ±0.085 SE) (Table 3). Patient isolates can provide information on a coarse level, but finer-scale mapping of geographic and population boundaries will require environmental sampling and analysis of genotypes. Our data suggest that environmental isolates reflect a broad diversity of genotypes and only a subset may be capable of causing severe disease in humans. A primary concern with our analysis is the precise location of the isolate origin. Few environmental isolates of Coccidioides exist, and methods to obtain them for genotypic analysis are currently inadequate (*9*,*10*,*28*,*36*).

Admixtures were found in the Arizona population, and gene flow was observed between the three defined subpopulations (online Technical Appendix 3 Figure 3); however, the presence of private alleles within each of those subpopulations and high genetic distance supports genetic isolation (Tables 1–3). The same was observed for the Mexico isolates nested in the Texas/Mexico/South America population. Additionally, the presence of private alleles and high diversity within C. immitis suggests that our results are not affected by oversampling in the Arizona subpopulation (Table 3). Because a sexual life cycle has not been observed, questions related to frequency, timing, and directionality of genetic exchange remain to be explored experimentally. Additional multilocus microsatellite types might be needed to support populations or could be resolved by using whole genome sequence comparison. Our data support previous work identifying the same main populations (1,7) and can be further tested with additional single nucleotide polymorphisms identified using whole genome sequence comparison.

Questions remain about the population biology of *Coccidioides*. The spatial and temporal distribution of individual genotypes, the amount of spatial overlap between the 2 species, and population boundaries within each species are still unclear (*9*,*10*,*16*). Overlap between species is likely, because of the identification of both *C. immitis* and *C. posadasii* recovered among patients in San Diego and northern Mexico and the observation of hybridization and introgression (*1*,*7*,*16*). This work shows that analyzing a large number of patient isolates and assigning regional population information reveals the potential for population structure within Arizona, at a much finer scale than previously thought (Figure 1). Thus, genetic differences and population subdivision among isolates and populations are likely greater than has been shown to date. The question of which population is basal to the *C. posadasii* lineage remains unanswered, and greater efforts to explore genotypic variation in Texas, Mexico, and Central and South America are needed.

Understanding the ecology of *Coccidioides* has been a longstanding goal (*3*,*4*). We used multiple methods to understand population genetics and determine population structure (*29*,*30*). However, environmental isolates must be more deeply explored by using direct fungal isolation (not passaging in mice) or high-coverage metagenomic sequencing, so that a specific location can be assigned to each isolate and potential for greater genetic diversity in the environment could be specifically tested. Surveying human patient isolates will continue to be valuable to track new outbreaks, such as the current coccidioidomycosis cases in Washington State (*9*).

Investigating the ecology and distribution of genotypes within and among populations of a pathogen is important for monitoring outbreaks, determining variance in virulence, and predicting disease progression (*37*). Correlating disease severity with pathogen genotype by using genome-wide association studies might assist in identifying genetic-based differences in virulence (*38*). Monitoring disease progression and response to antifungal therapy in animal models of coccidioidomycosis with more than a few well-characterized laboratory strains might provide information that could assist with better treatment options (*39*). Finally, a better understanding of ecologic and environmental factors that influence the growth and reproduction of the organism will assist in predicting and preventing exposure to the pathogen (*28*).

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Infection, Replication, and Transmission of Middle East Respiratory Syndrome Coronavirus in Alpacas

Danielle R. Adney, Helle Bielefeldt-Ohmann, Airn E. Hartwig, Richard A. Bowen

Middle East respiratory syndrome coronavirus is a recently emerged pathogen associated with severe human disease. Zoonotic spillover from camels appears to play a major role in transmission. Because of logistic difficulties in working with dromedaries in containment, a more manageable animal model would be desirable. We report shedding and transmission of this virus in experimentally infected alpacas (n = 3) or those infected by contact (n = 3). Infectious virus was detected in all infected animals and in 2 of 3 in-contact animals. All alpacas seroconverted and were rechallenged 70 days after the original infection. Experimentally infected animals were protected against reinfection, and those infected by contact were partially protected. Necropsy specimens from immunologically naive animals (n = 3) obtained on day 5 postinfection showed virus in the upper respiratory tract. These data demonstrate efficient virus replication and animal-to-animal transmission and indicate that alpacas might be useful surrogates for camels in laboratory studies.

Middle East respiratory syndrome coronavirus (MERS-CoV) was first detected in samples from a man in Saudi Arabia who had severe respiratory disease in 2012 (*1*). Since its identification, >1,600 cases of infection have been documented, and the case-fatality rate is ≈36% (*2*). Although efficient human-to-human transmission has been documented, zoonotic spillover probably plays a major role in human infection (*3*–*7*).

Dromedary camels were identified early after recognition of the virus as a possible reservoir host for the disease, although not all patients report contact with camels. Numerous investigators have reported the presence of MERS-CoV RNA or infectious virus in nasal swab specimens of dromedary camels in Saudi Arabia (*3*,*4*,*8*–*10*), Qatar (*5*,*11*–*13*), Oman (*14*), the United Arab Emirates (*15*), Nigeria (*16*), and Egypt (*17*). In some areas of the Middle East and Africa, nearly 100% of animals tested were serologically positive for MERS-CoV, which suggested widespread circulation among camel populations (*9*,*18*,*19*).

Historical samples contained specific antibodies against MERS-CoV as long ago as 1992, which indicated that MERS-CoV has been circulating much longer than originally believed (*19*,*20*). Young animals appear to be at a greater risk for productive infection, and handling practices, such as weaning or shipping animals, might play a major role in animal-to-animal transmission. Many dromedary camels tested had high antibody titers. These results support field data suggesting that young animals become infected, and their immune responses probably are repeatedly boosted by subsequent exposure to the virus (*18*). However, it is currently unknown whether these repeated exposures result in productive infection or whether antibodies generated from a previous infection are protective.

We have previously demonstrated that dromedary camels can be experimentally infected with MERS-CoV and found that mild upper respiratory tract disease associated with shedding copious amounts of virus by nasal secretions develops during the first week after infection (*21*). However, because of the cost of dromedaries, their size, and the requirement for specialized facilities to conduct such studies, it would be useful to identify alternative animal models that respond similarly to infection with MERS-CoV.

We report characterization of an alpaca model of MERS-CoV infection in which we evaluated virus shedding and pathology, transmission by contact, and protective immunity 10 weeks after initial infection. Results indicate that alpacas might be a useful substitute for dromedary camels in certain types of MERS-CoV experiments.

Materials and Methods

Ethics

Animal experiments were approved by the Animal Care and Use Committee of Colorado State University. Every effort was made to minimize stress and pain of the animals.

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DOI: http://dx.doi.org/10.3201/eid2206.160192

Virus and Cells

Animals were infected with a low-passage human isolate of MERS-CoV (strain HCoV-EMC/2012). This strain was propagated in Vero E6 cells cultured in Dulbecco modified Eagle medium as described (*21*).

Animal Study

Nine locally bred alpacas were obtained by private sale for use in this study. Animals were allowed to acclimate to the facility for 1 week before infection and were fed hay ad libitum. One day before infection, animals were subcutaneously injected with an identification and temperature-sensing transponder (Lifechip; Destron Fearing, Dallas/Fort Worth Airport, TX, USA), and their body temperatures were monitored throughout the study. Alpacas A1–A3 were housed together and experimentally infected by intranasal instillation of 10^7 PFU of MERS-CoV diluted in sterile phosphate-buffered saline (3 mL/nare). Two days later, alpacas A4–A6 were introduced into the same room as alpacas A1–A3 and housed together for the duration of the study.

Nasal swab specimens were collected by inserting and rotating sterile swabs into both nares, immediately placed in virus transport medium, and frozen until assay. Blood was collected weekly into serum-separating tubes for detection of neutralizing antibodies. Animals A1–A6 were held in the facility for 70 days postinfection, and all 6 animals were then reinfected intranasally with 10^7 PFU of MERS-CoV. Three additional alpacas (A7–A9) were also infected to serve as infection controls and evaluate tissue distribution of virus replication.

Nasal swab specimens were collected daily from all animals for 5 days, at which time animals A7–A9 were humanely euthanized. Tissues collected at necropsy for detection of infectious virus from these 3 animals included nasal turbinates, trachea, larynx, and all 4 lung lobes. These samples plus additional samples, including brain, kidney, liver, skeletal muscle, heart, spleen, bladder, mesenteric lymph node, submandibular lymph node, and mediastinal lymph node, were fixed in formalin for histopathologic and immunohistochemical analysis. Nasal swab specimens and serum samples collected from alpacas A1–A6 were sampled for 2 weeks after the second infection, and then these animals were then humanely euthanized.

Histopathologic and Immunohistochemical Analysis

Tissues were fixed in 10% neutral-buffered formalin for >7 days and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and evaluated by a veterinary pathologist (H.B.-O.). Immunohistochemical analysis was preformed to detect MERS-CoV antigen by using a rabbit polyclonal antiserum against HCoV-EMC/2012 antigen (diluted 1:1,000) as a primary antibody as described (*22*).

Virus Isolation and Plaque Reduction Neutralization Test

MERS-CoV was titrated from nasal swab specimens in virus transport medium and homogenized tissue by plaque assay as described for camels (*21*). A 1-mL volume of virus transport medium was considered a 10^{-1} dilution, and 10-fold serial dilutions were prepared in BA1 medium. Neutralizing antibodies were detected by plaque reduction neutralization test (PRNT) as described, and seropositive animals were identified by using a 90% neutralization cutoff (*23*).

Results

Clinical Signs of MERS-CoV in Infected Alpacas

Field studies and experimental infections suggest that mild respiratory disease associated with nasal discharge develops in MERS-CoV–infected camels (*21*,*24*,*25*). Similar to dromedaries, none of the alpacas had any appreciable increase in body temperature during challenge or rechallenge (Figure 1). Unlike dromedary camels, none of the alpacas

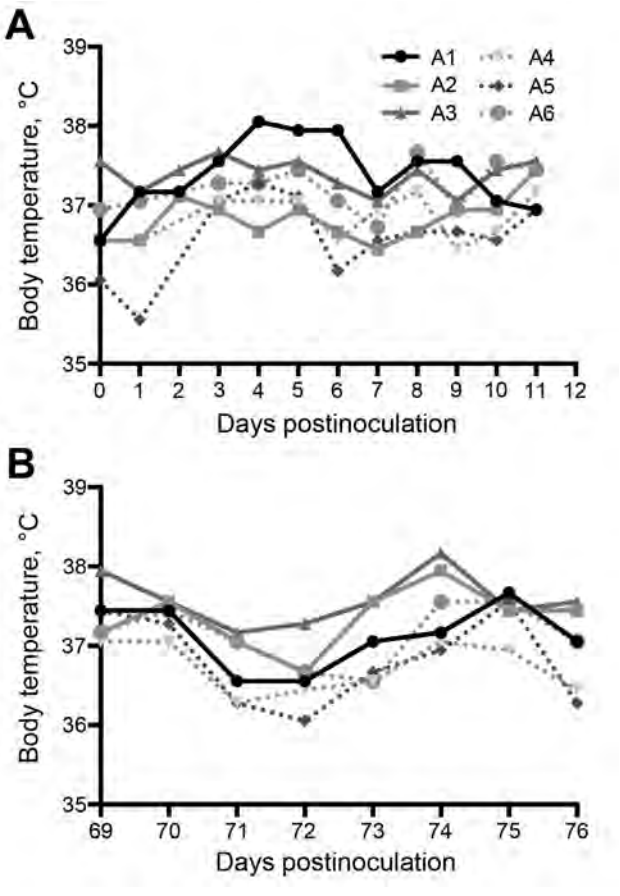

Figure 1. Body temperatures of 6 alpacas (A1–A6) experimentally infected with Middle East respiratory syndrome coronavirus after A) initial challenge and B) after rechallenge on day 70 postinfection.

had any observable nasal discharge over the course of infection. All alpacas maintained consistent activity level, temperament, and food intake throughout the study.

Virus Shedding

Nasal swab specimens were collected from infected animals immediately before challenge, on days 1–5 postinfection, and on day 10 postinfection. All 3 experimentally infected animals (A1–A3) had detectable infectious virus on day 15 postinfection but had stopped shedding virus by day 10 postinfection (Figure 2, panels A, B). The 3 co-housed animals (A4–A6) were placed in the room with the infected animals 2 days after initial virus infection. Nasal swab specimens were collected from co-housed animals on days 3–10 after infection of animals A1–A3, and then 3 times/week through day 19.

Infectious virus was detected from animal A6 during days 7–14 and from animal A4 only on day 14. We did not isolate infectious virus from animal A5 (Figure 2, panel A). Although infectious virus was detected in animal A6 on day 7, infectious virus was not detected in animal A4 until day 14 (Figure 2, panel A). We speculate that animal A4 became infected by contact with animal A6 after animals A1–A3 had cleared their infections, which suggested that transmission is linked to intimate animal contact, rather than to aerosol transmission.

To test whether previous infection was protective against subsequent virus challenge, all 6 original study animals (A1–A6) were allowed to clear their infections and rechallenged by intranasal infection on day 70 postinfection. Challenge was also performed with 3 immunologically naïve alpacas (A7–A9) (infection controls). The 3 immunologically naive animals became infected and shed virus during days 1–5 postinfection, at which time they were euthanized. The 3 animals that became infected through contact (A4–A6) shed minimal virus between days 1–2 after rechallenge, but not on days 3–5. In contrast, animals that had been experimentally infected were completely protected against rechallenge and did not shed detectable quantities of virus (Figure 2, panels C, D).

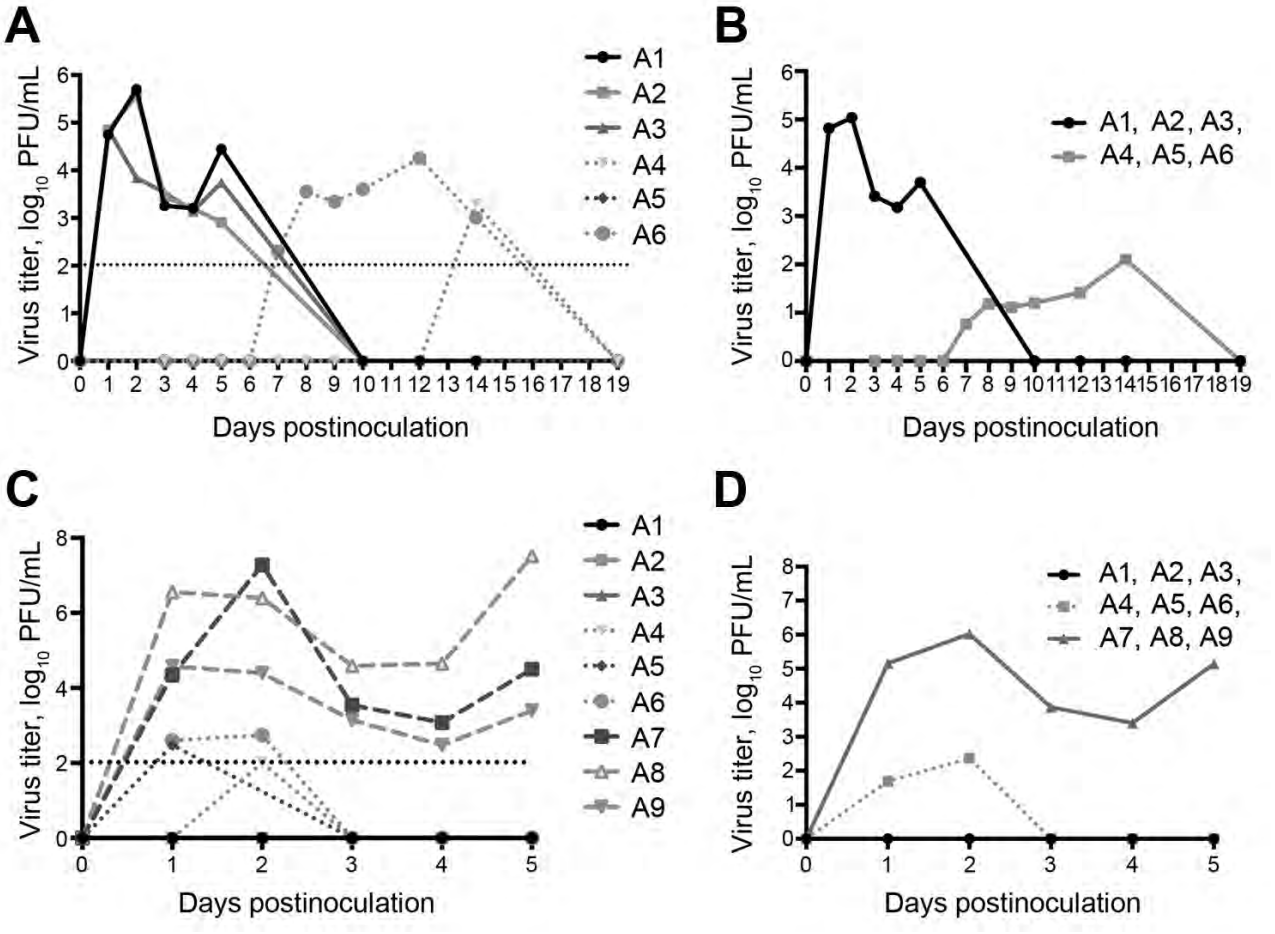

Figure 2. Virus shedding (nasal swab specimens) in 6 alpacas experimentally infected with Middle East respiratory syndrome coronavirus (A1–A3) and co-housed with infected animals (A4–A6). A, B) initial challenge; C, D) rechallenge with addition of 3 immunologically naive alpacas (A7–A9). Individual animal results (A, C) and group means (B, D) are shown. Dotted vertical lines indicate detection limit of the assay.

Table. Neutralizing antibody titers in 6 alpacas after experimental infection with Middle East respiratory syndrome coronavirus and after rechallenge on day 70 postinfection*

Day	Alpaca, antibody titer					
	A1	A2	A3	A4	A5	A6
0	<10	<10	<10	<10	<10	<10
14	40	40	40	<10	<10	<10
21	40	40	40	<10	10	20
28	40	80	80	10	160	20
35	80	160	160	20	80	40
42	160	320	160	20	40	20
49	80	320	80	20	80	80
56	80	640	160	20	80	80
63	80	640	160	40	80	80
70	160	640	80	20	40	80
77	320	640	80	160	320	80
84	320	640	160	320	320	80

*Alpacas A1–A3 were experimentally infected, and alpacas A4–A6 were co-housed with infected alpacas. Titers were determined by using a 90% cutoff.

Humoral Response in Infected Alpacas

Serum was collected weekly and tested for neutralizing antibodies against MERS-CoV. All 3 experimentally infected animals (A1–A3) had detectable levels of antibodies beginning on day 14 (Table). Although infectious virus was isolated only from 2 of the 3 co-housed animals, these 3 animals had neutralizing antibodies detected first on day 21 (animals A5 and A6) or day 28 (animal A4) (Table).

Virus in Organs

Nasal turbinate, upper trachea, lower trachea, larynx, and all 4 lung lobes were sampled at necropsy from alpacas A7, A8, and A9 and tested for infectious virus by using a plaque assay. Virus was detected in the nasal turbinates, larynx, and trachea of the 3 alpacas but not in any of the lung lobes tested (Figure 3).

Pathologic and Immunohistochemical Analysis

Gross lesions were not observed at necropsy in any of the alpacas. However, microscopic analysis of formaldehyde-fixed tissue sections from animals A7–A9 showed mild squamous metaplasia of the epithelium of the turbinates in animal A8 (Figure 4, panel A) and rare foci of mucosal erosion accompanied by minimal-to-mild subepithelial infiltration of neutrophils and macrophages and fewer lymphocytes (Figure 4, panel C). All 3 animals also had follicular hypertrophy and hyperplasia of the draining lymph nodes, which suggested immune activation.

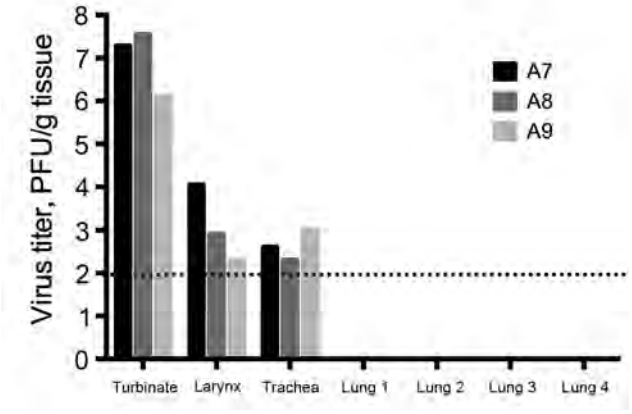

Figure 3. Virus titers from tissues collected from 3 immunologically naive alpacas (A7–A9) challenged with Middle East respiratory syndrome coronavirus and sampled at necropsy on day 5 postinfection. Dotted line indicates detection limit of the assay.

Immunohistochemical analysis detected rare, scattered, virus antigen–positive cells in respiratory epithelium of turbinates (Figure 4, panel B) and in rare cells interpreted to be intraepithelial leukocytes. Virus antigen was not detected in any of the other tissues examined. Animals A7 and A9 had histopathologic evidence of mild encephalitis with perivascular infiltrates of lymphocytes and monocytes and mild gliosis (Figure 4, panel D). We did not assay brain tissue for virus, either by isolation or PCR, because of the high potential of contamination from the nasal cavity during extraction. Brain tissue was negative for virus by immunohistochemical analysis, but the etiology of the encephalitis observed remains unknown and might have been unrelated to MERS-CoV infection.

Discussion

Many difficulties are associated with high containment experiments involving dromedary camels. Thus, additional animal models are necessary for MERS-CoV research. Because of their greater availability in the United States and smaller size, we tested an alpaca model.

We report an alpaca model of MERS-CoV infection in camelids and analysis of animal-to-animal transmission and reinfection dynamics. Infected alpacas shed considerable quantities of infectious virus nasally, although at lower concentrations than those reported for dromedary camels (*21*,*24*). In addition, none of the infected alpacas had a noticeable nasal discharge, which is distinctly different from what has been observed in camels and might explain the relatively low efficiency of contact transmission we observed with alpacas.

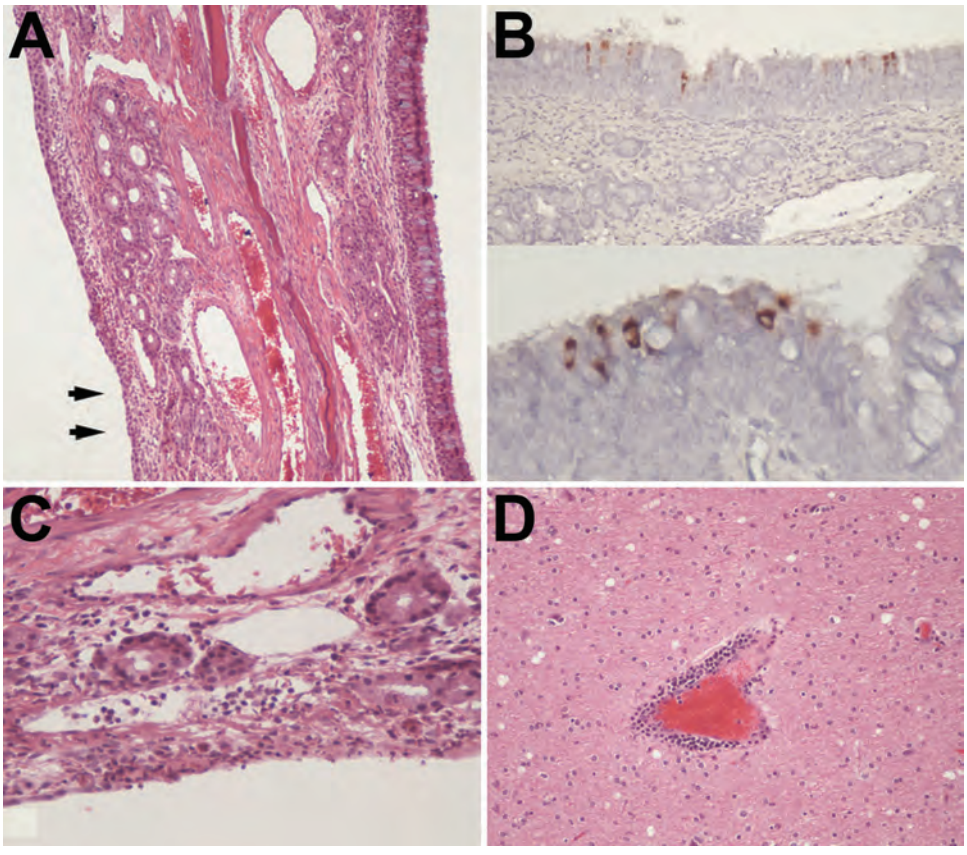

Figure 4. Signs of mild upper respiratory inflammation, encephalitis, and virus antigen detection in respiratory epithelium of alpacas experimentally infected with Middle East respiratory syndrome coronavirus. A) Turbinate from alpaca A8 showing normal respiratory epithelium on the right with goblet cells (blue cells). Epithelium on the left has undergone squamous metaplasia (arrows) and is focally eroded with mild subepithelial inflammation (original magnification ×100). B) Virus antigen in apparently intact respiratory epithelium of alpaca A8 detected by immunohistochemical analysis and lack of subepithelial inflammation (original magnification ×200 [top] and ×400 [bottom]). C) Erosion in turbinate epithelium from alpaca A8 showing leukocytosis in underlying blood vessels (original magnification ×400). D) Perivascular infiltration of lymphocytes and monocytes in the brain of alpaca A9 (original magnification ×200). Hematoxylin and eosin stain in panels A, B, and D; immunohistochemical stain in panel C.

Infectious virus was detected in nasal swab specimens from 2 of 3 alpacas co-housed with experimentally infected animals, and each of the 3 co-housed animals had neutralizing antibodies against MERS-CoV, which indicated virus transmission. The antibody titers observed approximate those seen for infected dromedaries with the exception of A4, whose antibodies titers remained low until after rechallenge (*21*). Finally, experimentally infected alpacas were completely protected against subsequent virus rechallenge, and contact-infected alpaca were only partially protected.

These results suggest that infection can easily spread among closely grouped camelids infected with MERS-CoV. Camels are frequently moved within the Middle East for grazing, camel shows, and races. Such movement enables mixing and close mingling of animals and could play a major role in MERS-CoV transmission among animals and to handlers. Khalafalla et al. reported that animals bound for slaughter were held in a livestock market for several days, transferred to an abattoir, and kept for up to 24 hours before slaughter (*25*). Our data suggest that these handling practices could promote animal-to-animal virus transmission and that at the time of slaughter virus could potentially be transmitted to slaughterhouse workers.

A major question related to the pathogenesis of MERS-CoV infection in camels, and of great relevance to vaccination strategies, is whether animals that have been infected are resistant to reinfection and virus shedding and, if so, for how long. Our experimentally infected animals were completely protected against rechallenge 70 days later, which suggests that sterilizing immunity can be achieved. However, the animals that were infected through contact (animals A4–A6) shed infectious virus after reinfection, albeit at much lower levels than infected control animals (animals A7–A9).

Although not tested in the present study, it might be surmised that the 3 in-contact animals would have acquired sterilizing immunity from the second (booster) infection. These results support field data that suggest that young animals become infected and probably receive booster infections; most older animals have acquired immunity and are not susceptible to infection and virus shedding (*26*). This finding also highlights the possibility that widespread vaccination of dromedary camels could result in a major decrease in virus transmission to humans.

To date, neutralizing antibodies against MERS-CoV have not been detected in camelids outside Africa or the Middle East. However, if virus were to be introduced into immunologically naive camelid populations, it probably would be readily transmitted among animals. Many New World camelids are valued for their fiber, and such transmission might devastate fiber-related industries (*27*). Thus, as travel-associated cases of MERS-CoV infection continue to be documented, human-to-human virus transmission and possible human-to-animal virus transmission should be monitored.

This study had several limitations. Each of the 3 experimental groups had only 3 animals, which limited our ability to perform statistical analyses. In addition, we evaluated protective immunity 10 weeks after the original infection, which is a relatively short period and does not fully recapitulate seasonal exposures. Thus, further studies are necessary to better understand duration of immunity in camels and alpacas.

Note Added in Proof: Crameri et al. also report experimental infection and response to rechallenge of alpacas with Middle East respiratory syndrome coronavirus in this issue of Emerging Infectious Diseases (*28*).

Acknowledgments

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Ms. Adney is a graduate student at Colorado State University, Fort Collins, Colorado. Her primary research interest is the pathogenesis of emerging infectious diseases.

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Rapid Detection of Polymyxin Resistance in *Enterobacteriaceae*

Patrice Nordmann, Aurélie Jayol, Laurent Poirel

For identification of polymyxin resistance in *Enterobacteriaceae*, we developed a rapid test that detects glucose metabolization associated with bacterial growth in the presence of a defined concentration of colistin or polymyxin B. Formation of acid metabolites is evidenced by a color change (orange to yellow) of a pH indicator (red phenol). To evaluate the test, we used bacterial colonies of 135 isolates expressing various mechanisms of colistin resistance (intrinsic, chromosomally encoded, and plasmid-mediated MCR-1) and 65 colistin-susceptible isolates. Sensitivity and specificity were 99.3% and 95.4%, respectively, compared with the standard broth microdilution method. This new test is inexpensive, easy to perform, sensitive, specific, and can be completed in <2 hours. It could be useful in countries facing endemic spread of carbapenemase producers and for which polymyxins are last-resort drugs.

Among the most clinically significant multidrug-resistant bacteria are carbapenemase-producing *Enterobacteriaceae*. Because these bacteria usually remain susceptible to polymyxins, an old class of antimicrobial drugs almost abandoned in the 1970s because of their potential toxicity, interest in polymyxins (colistin and polymyxin B) has been renewed worldwide (*1*,*2*). However, the increasing use of colistin explains why acquired colistin resistance may now be added to the carbapenem resistance trait in *Enterobacteriaceae* (*3*).

The standard reference technique for determining susceptibility to polymyxins is broth microdilution, which requires fastidious attention and a long time (24 h) to perform (*4*). Other techniques for determining susceptibility to polymyxins (disk diffusion and Etest) have been proposed and also provide results in 18–24 h. Because of poor diffusion of polymyxin molecules in agar, rates of false susceptibility are high (up to 32%) (*4*,*5*).

Acquired resistance to colistin in *Enterobacteriaceae* results mostly from modification of lipopolysaccharide (*6*). Addition of phosphoethanolamine, 4-amino-L-arabinose cationic groups, or both to lipopolysaccharide decreases polymyxin binding to the bacterial outer membrane. Addition of these groups may be associated with chromosome-encoded mechanisms (mutations in PmrAB or PhoPQ two-component systems or alterations of the *mgrB* gene) (*6*). A recent report revealed that addition of phosphoethanolamine may also be plasmid mediated through the *mcr-1* gene, which confers the first known plasmid-mediated resistance to colistin in isolates from humans and animals (*7*). More recently, the *mcr-1* gene was identified in several plasmid backbones, mostly in *Escherichia coli* (*8*–*10*). There is therefore a need for a test that enables rapid detection of polymyxin resistance in *Enterobacteriaceae* and that may contribute to its containment.

We developed a test (the rapid polymyxin NP [Nordmann/Poirel] test) that detects bacterial growth in the presence of a defined concentration of a polymyxin. Bacterial growth detection (or absence) is based on carbohydrate metabolism (*11*). Acid formation associated with carbohydrate metabolism in *Enterobacteriaceae* can be observed through the color change of a pH indicator. This test is rapid (<2 h) and easy to perform.

Materials and Methods

Isolate Collection

To evaluate the performance of the rapid polymyxin NP test, we used 200 isolates collected from clinical samples worldwide. This collection included 135 *Enterobacteriaceae* isolates resistant to polymyxin: 5 isolates of intrinsically polymyxin-resistant species (*Morganella morganii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia stuartii*, and *Serratia marcescens*) and 130 isolates of various enterobacterial species (*Klebsiella* spp., *E. coli*, *Enterobacter* spp., and *Hafnia alvei*) with acquired resistance to polymyxins (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-1840-Techapp1.pdf), including a previously reported heteroresistant *Klebsiella pneumoniae* isolate for which MIC for colistin was high (*12*). The other 65 enterobacterial isolates belonged to various species and were susceptible to polymyxins (online Technical Appendix).

MIC Determination

To determine MICs for polymyxins, we used the broth microdilution method in cation-adjusted Mueller-Hinton broth (MHB-CA, reference 69444; Bio-Rad, Marnes-La-Coquette, France) as recommended by Clinical Laboratory

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Standard Institute (CLSI) guidelines (*13–15*). We considered this method to be the standard for comparison with the rapid polymyxin NP results. Polymyxin B and colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) were tested over a range of dilutions (0.12–128 μg/mL). All experiments were repeated in triplicate in separate experiments. As recommended by CLSI, microdilution was performed without addition of Tween 80 (*15*), and *E. coli* ATCC 25922 was used as a control strain.

Because no breakpoint is available for polymyxins for *Enterobacteriaceae* according to CLSI guidelines (*14*), we used the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for reference (*16*). Enterobacterial isolates with colistin or polymyxin B MICs ≤2 μg/mL were categorized as susceptible; those with MICs >2 μg/mL were categorized as resistant.

PCR Amplification and Sequencing

We recovered the chromosomal DNA of the isolates by using a commercially available kit (QIAquick; QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. We sequenced the *pmrA, pmrB, phoP, phoQ*, and *mgrB* genes possibly involved in colistin resistance in *K. pneumoniae* and *K. oxytoca*, as described previously (*12*,*17–19*). We performed PCR amplification for detection of the plasmid-mediated *mcr-1* gene as described (*7*).

Isolate Genotyping by Pulsed-Field Gel Electrophoresis

We assessed the genetic relatedness of the colistin-resistant isolates with identical molecular mechanisms of colistin resistance. We used pulsed-field gel electrophoresis with *Xba*I-digested genomic DNA as described previously (*20*).

Rapid Polymyxin NP Test

Reagents and Solutions

The rapid polymyxin NP test uses 2 reagents and solutions: stock solutions of polymyxins and rapid polymyxin NP solution. Each is described below.

For stock solutions of polymyxins, colistin sulfate and polymyxin B powders (Sigma Aldrich) were diluted into MHB-CA medium in glass tubes to obtain a concentration of 0.2 mg/mL. These powders can be stored at 4°C before use, and the diluted polymyxin solutions can be kept at –20°C for 1 year. Of note, polymyxin-containing batches from commercial origin can be used, but the colistimethate sulfate powder, a therapeutic prodrug of colistin, cannot be used.

To prepare 250 mL of the rapid polymyxin NP solution, we mixed the culture medium and the pH indicator in a glass bottle as follows: 6.25 g of MHB-CA powder, 0.0125 g of phenol red (Sigma Aldrich), and 225 mL of distilled water. The pH of the solution was adjusted to 6.7 by adding drops of 1 mol/L HCL. This solution was then autoclaved at 121°C for 15 min. After cooling the solution to room temperature, we added 25 mL of 10% anhydrous D(+)-glucose (Roth, Karlsruhe, Germany) sterilized by filtration. The final concentrations in the rapid polymyxin NP solution were consequently 2.5% of MHB-CA powder, 0.005% of phenol red indicator, and 1% of D(+)-glucose. This rapid polymyxin NP solution can be kept at 4°C for 1 week or at –20°C for 1 year. This solution must be prewarmed at 37°C before use to prevent growth delay and therefore a delayed color change.

Just before performing the experiment, we added colistin to the rapid polymyxin NP solution and mixed it into sterile glass tubes to obtain a rapid polymyxin NP solution containing a final colistin concentration of 5 μg/mL. For example, we added 25 mL of colistin stock solution at 0.2 mg/mL to 1 mL of rapid polymyxin NP test solution for the testing of 1 isolate and respective negative and positive controls.

Bacterial Inoculum Preparation

We prepared a standardized enterobacterial inoculum by using freshly obtained (overnight) bacterial colonies grown on Luria-Bertani or Mueller-Hinton plates. We resuspended the bacterial colonies into 10 mL of sterile NaCL (0.85%) to obtain a 3.0–3.5 McFarland optical density (≈10^9 CFU/mL), which corresponds to an ≈10-mL full loop of bacterial colonies diluted in 10 mL of NaCL. A bacterial suspension was prepared for each isolate to be tested and for the colistin-susceptible and -resistant isolates used as controls (isolates FR-01 and FR-136; respectively; online Technical Appendix). As recommended by the EUCAST guidelines for susceptibility testing, we used the bacterial suspensions within 15 min of preparation and for no longer than 60 min after preparation (*16*).

Tray Inoculation

We performed testing in a 96-well polystyrene microtest plate (round base, with lid, sterile, reference 82.1582.001; Sarstedt, Nümbrecht, Germany). For each isolate, bacterial suspension was inoculated in parallel into 2 wells, with and without colistin. The following steps of the rapid polymyxin NP test were then performed (Figure):

Step 1: 150 mL of colistin-free solution was transferred to wells A1–A4.
Step 2: 150 mL of the rapid polymyxin NP solution containing colistin was transferred to wells B1–B4.
Step 3: 50 mL of NaCl 0.85% was added to wells A1and B1.
Step 4: 50 mL of the colistin-susceptible isolate suspension used as negative control was added to wells A2 and B2.

Step 5: 50 mL of the colistin-resistant isolate suspension used as positive control was added to wells A3 and B3.
Step 6: 50 mL of the tested isolate suspension was added to wells A4 and B4.

We mixed the bacterial suspension with the reactive medium by pipetting up and down. The final concentration of bacteria was ≈10^8 CFU/mL in each well, and the final concentration of colistin sulfate was 3.75 μg/mL.

Tray Incubation

We incubated the inoculated tray for up to 4 h at 35 ± 2°C in ambient air, without being sealed and without agitation. We did not seal the tray because oxygen is required for carbohydrate metabolism.

Tray Reading

We visually inspected the tray (checked for no spontaneous color change) after 10 min and then every hour for 4 h. We considered the test result positive (polymyxin resistance) if the polymyxin-resistant isolate grew in presence of colistin and negative (polymyxin susceptibility) if the polymyxin-susceptible isolate did not grow in presence of colistin. We considered the test result interpretable if the following 4 conditions were met: 1) both wells with 0.85% NaCl without bacterial suspension (wells A1 and B1) remained orange (absence of medium contamination); 2) the wells with bacterial suspension and colistin-free (wells A2–A4) turned from orange to yellow, confirming the metabolism of glucose by the isolates; 3) the wells with the colistin-susceptible reference bacterial suspension (negative control) gave negative results (wells A2 and B2); and 4) the wells with the colistin-resistant reference bacterial suspension (positive control) gave positive results (wells A3 and B3). The test result was positive when the well containing colistin (well B3) and the isolate to be tested turned from orange to yellow, giving exactly the same color as the well without colistin (well A3), indicating glucose metabolism and growth in presence of colistin (i.e., colistin resistance) (Figure). The test result was negative when the well containing colistin (well B2) with the isolate to be tested remained orange (unchanged color) (Figure) or was more clear than the wells with 0.85% NaCl but not exactly the same color as the well without colistin (not shown). Results were interpreted by 2 technicians who did not know which isolates were colistin resistant and colistin susceptible.

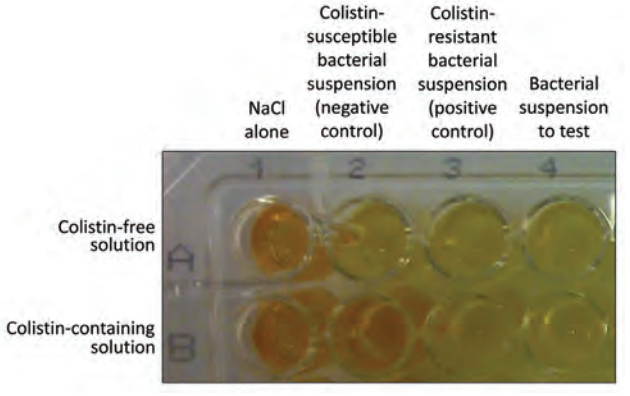

Figure. Representative results of the rapid polymyxin NP [Nordmann/Poirel] test. Noninoculated wells are shown as controls (first column). The rapid polymyxin NP test was performed with a reference colistin-susceptible isolate (second column) and with a reference colistin-resistant isolate (third column) in a reaction medium without (upper row) and with (lower row) colistin. The tested isolate grew in the presence (and absence) of colistin (wells B4 and A4, respectively) and was therefore reported to be colistin-resistant.

Other Experimental Conditions Tested

Polymyxin B Instead of Colistin

We evaluated the possibility of adapting the test to susceptibility testing of polymyxin B in countries where polymyxin B is prescribed. To do so, we performed the rapid polymyxin NP test with 20 colistin/polymyxin B–susceptible isolates and 20 colistin/polymyxin B–resistant isolates with polymyxin B at the same concentrations of colistin sulfate.

Incubation Conditions

To determine effects of the incubation atmosphere on time to result, we incubated the tray with 20 colistin-susceptible isolates and 20 colistin-resistant isolates, in parallel, under 2 conditions: ambient air and atmosphere with 5% CO_2. We also incubated the tray for 20 colistin-susceptible isolates and 20 colistin-resistant isolates in parallel with and without agitation.

Culture Media

To determine the potential effects of culture medium on the test results, we performed the test with 20 colistin-susceptible isolates and 20 colistin-resistant isolates cultured overnight on different agar plates. The following culture media were tested: 1) nonselective culture medium such as Columbia agar + 5% sheep blood (bioMérieux, La-Balme-Les-Grottes, France); 2) chocolate agar + PolyVitex (bioMérieux); 3) nonselective chromogenic medium UriSelect 4 (Bio-Rad); 4) Eosin methylene blue agar (Sigma Aldrich); 5) Drigalski agar (Bio-Rad); 6) MacConkey agar (VWR BDH Prolabo, Leuven, Belgium); and 7) bromocresol purple (bioMérieux).

Results

Of the 200 enterobacterial isolates tested to evaluate the performance of the rapid polymyxin NP test (online Technical Appendix), 5 isolates belonged to bacterial species that are

intrinsically resistant to colistin (*M. morganii*, *P. mirabilis*, *P. vulgaris*, *P. stuartii*, and *S. marcescens*) and 130 isolates of various species displayed an acquired mechanism of resistance to colistin. For 87 *Klebsiella* spp. isolates, resistance to colistin was associated with various chromosomal gene changes responsible for lipopolysaccharide modifications (online Technical Appendix): 10 isolates had mutations in the PmrAB two-component system (n = 3 in *pmrA* gene, n = 7 in *pmrB* gene); 2 isolates had structural modifications in the PhoPQ two-component system (n = 1 in *phoP* gene, n = 1 in *phoQ* gene); and 75 isolates had various alterations in *mgrB* gene, the negative regulator of the PhoPQ system (online Technical Appendix). Seven nonduplicate *E. coli* isolates harbored a plasmid-mediated *mcr-1* gene. Pulsed-field gel electrophoresis revealed that isolates with identical mechanisms of colistin resistance (chromosomal or plasmid-encoded) were not clonally related (data not shown). The mechanism(s) of colistin resistance remained unknown for the 43 remaining enterobacterial isolates (online Technical Appendix). With regard to performance of the rapid polymyxin NP test with colistin-susceptible strains, the 65 colistin-susceptible isolates tested (MICs of colistin 0.12–2 μg/mL) gave negative results, except for 3 isolates (isolates FR-180, 181, and 182) for which colistin MICs were 1–2 μg/mL (just below the EUCAST breakpoint) and which gave a positive (false-positive) result (online Technical Appendix).

As expected, isolates that were intrinsically resistant to colistin (n = 5), such as *Proteus* spp., *P. stuartii* and *S. marcescens*, gave a positive test result (online Technical Appendix). Colistin-resistant enterobacterial isolates (n = 130, MICs of colistin ranging from 4 to >128 μg/mL) also gave positive results, except for 1 colistin-resistant *E. coli* isolate (isolate FR-119) for which colistin MIC was 8 μg/mL and which gave a negative (false-negative) result (online Technical Appendix).

Correlation was high between colistin resistance and positive rapid polymyxin NP test results and, conversely, colistin susceptibility and negative test results (online Technical Appendix). Sensitivity (99.3%) and specificity (95.4%) of the test were also high, compared with the standard broth microdilution method.

By reading the color change of the wells every hour, we determined that final results were obtained 2 h after incubation when the tray was incubated at 35 ± 2°C under an ambient atmosphere. However, positive results (frank color change) were obtained as early as 1 h after incubation for *Klebsiella* spp. and *E. coli* isolates. Half of the *Enterobacter* spp. isolates gave positive results within 1 h of incubation and the other half within 2 h. In addition, all resistant isolates gave positive results after 1 h of incubation when trays were incubated at 35 ± 2°C under 5% CO_2. Agitating the tray did not improve the speed with which results were obtained.

The rapid polymyxin NP test results were the same whether performed with polymyxin B or with colistin (data not shown). Testing of several agar media revealed that 30% of the colistin-susceptible tested isolates gave false-positive results when bacterial colonies were recovered from acidifying media such as Drigalski, MacConkey, or bromocresol purple agar. Media that were adequate for culturing bacteria before performing the rapid polymyxin NP test were Luria Bertani agar, Mueller-Hinton agar, Columbia agar + 5% sheep blood, chocolate agar, UriSelect 4 agar, and eosin methylene blue agar.

Discussion

The rapid polymyxin NP test is easy-to-perform, rapid, sensitive, and specific. It detects polymyxin-resistant and -susceptible isolates from any enterobacterial species, regardless of the molecular mechanism of polymyxin resistance. This test offers the possibility of detecting polymyxin resistance from bacterial cultures from infected samples or from selective media before any antimicrobial drug susceptibility testing results are obtained. Results are obtained at least 16 h sooner with this test than with the reference broth microdilution method. This test is as reliable as the reference dilution technique but much less cumbersome and is not based on diffusion of large polymyxin molecules in agar (as are the Etest and the disk-diffusion techniques), which therefore prevents false susceptibility results (*15*). A commercial test for research-use only is available for determining MICs of polymyxins (TREK Diagnostic Systems, Inc., Cleveland, OH, USA) (*15*); however, this test is adapted to testing series of isolates rather than single isolates, and results are available in 16–20 h. Sensitivity and specificity of the rapid polymyxin NP test were high (99.3% and 95.4%, respectively), making it a potential useful clinical technique.

The rapid polymyxin NP test can be performed on cultured bacteria grown on media such as Luria Bertani, Mueller-Hinton, Uriselect-4, eosin-methylene blue, blood agar, and chocolate agar. Interference may be observed with colonies grown on acidifying culture media such as Drigalski, Mac Conkey, and bromocresol purple agars. The test has been optimized in its present form; testing under other conditions (e.g., changes in pH indicator, inoculum size, glucose and pH indicator concentrations; preparation of the solution with non-cation–adjusted culture medium; or use of other polystyrene-containing trays) gave less optimal results.

The rapid polymyxin NP test uses commercially available polymyxin B and colistin sulfate powders, which are unspecified mixtures of chemically related compounds that differ by single amino acid changes and fatty acyl moieties. The changes of the relative proportion of the polymyxin components of these mixtures is poorly defined and a potential source of variability (*21*).

We believe that the rapid polymyxin NP test may useful for first-step screening of polymyxin resistance because use of molecular-based techniques for identification of all polymyxin-resistance mechanisms cannot be foreseen. Indeed, polymyxin resistance in *Enterobacteriaceae* may be associated with many nonrelated mechanisms, some of which are known (defects in outer-membrane proteins, structural modification of lipopolysaccharide, efflux overexpression; *16*) and others still unknown.

This study was subject to at least 4 limitations. First, we did not assess the ability of the rapid polymyxin NP test to detect heteroresistant isolates with low MICs for polymyxin by broth microdilution; such isolates are problematic to detect. Second, the rapid polymyxin NP test involves an orange-to-yellow color change, which is readily apparent for resistant organisms, but interpretation may require more vigilance from laboratory technicians testing organisms with low-level resistance; thus, larger scale studies in different laboratories are needed to fully evaluate the reliability of rapid polymyxin NP test performance. Third, we could not determine the mechanism for colistin resistance in 43 isolates for which several mechanisms of colistin resistance may exist and may be expressed in various ways. Last, we did not evaluate the rapid polymyxin NP test in species of bacteria with metabolic pathways that differed from those of *Enterobacteriaceae*; further work is needed to adapt the rapid polymyxin NP test to detection of polymyxin-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which have different metabolic pathways.

The rapid polymyxin NP test can be used to determine susceptibility or resistance to polymyxins in countries facing endemic spread of carbapenemase producers and for which polymyxins are last-resort drugs (*22*). The test can also rapidly identify carriers of polymyxin-resistant isolates, leading to rapid implementation of adequate hygiene measures to control their spread. This test can also support the development of novel polymyxin-like molecules, facilitating patient enrollment in pivotal clinical trials.

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Human Adenovirus Associated with Severe Respiratory Infection, Oregon, USA, 2013–2014

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Several human adenoviruses (HAdVs) can cause respiratory infections, some severe. HAdV-B7, which can cause severe respiratory disease, has not been recently reported in the United States but is reemerging in Asia. During October 2013–July 2014, Oregon health authorities identified 198 persons with respiratory symptoms and an HAdV-positive respiratory tract specimen. Among 136 (69%) hospitalized persons, 31% were admitted to the intensive care unit and 18% required mechanical ventilation; 5 patients died. Molecular typing of 109 specimens showed that most (59%) were HAdV-B7, followed by HAdVs-C1, -C2, -C5 (26%); HAdVs-B3, -B21 (15%); and HAdV-E4 (1%). Molecular analysis of 7 HAdV-B7 isolates identified the virus as genome type d, a strain previously identified only among strains circulating in Asia. Patients with HAdV-B7 were significantly more likely than those without HAdV-B7 to be adults and to have longer hospital stays. HAdV-B7 might be reemerging in the United States, and clinicians should consider HAdV in persons with severe respiratory infection.

Human adenoviruses (HAdVs) are a common cause of respiratory infection in persons of all ages. Acute upper and lower respiratory tract diseases, including pneumonia and bronchitis, have been attributed to HAdVs. Although many infections are mild, some persons, such as very young children, elderly or immunocompromised persons, or persons who have underlying pulmonary or cardiac disease, might be at increased risk for severe disease (*1*–*4*). HAdV infection can occur sporadically, endemically, or epidemically and often is influenced by HAdV species and type (*4*). Common settings for infection include the community, military recruit training centers, hospitals, and chronic care facilities (*3*,*5*–*8*).

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HAdV comprises 7 species (A–G), and >51 types have been characterized by immunotypic and molecular methods. HAdVs that are most often associated with symptomatic respiratory infections include species B (types 3, 7, 14, and 21), species C (types 1, 2, and 5), and species E (type 4) (*9*). Infections with HAdV-C often are endemic, mild, and most commonly seen in young children (*2*). HAdVs can be shed from the respiratory and gastrointestinal tracts for weeks or longer, even in persons who are no longer symptomatic, especially young children and immunocompromised persons (*10*). In comparison, HAdV-B– and HAdV-E–associated respiratory infections are more commonly seen as part of an epidemic or as sporadic cases in adults, and infections with these viruses are often more severe (*11*).

Circulating HAdVs can vary temporally and geographically; emergent genomic variants are possibly associated with more severe illness (*8*,*12*,*13*). Recent reports have noted severe respiratory disease associated with the reemergence of HAdV-B7 and genomic variant 7d in China and other countries in Asia (*14*–*16*). However, HAdV-B7 was rarely reported in the United States during the past decade (*17*,*18*). Among a convenience sample of 291 specimens sent to the Centers for Disease Control and Prevention (CDC) from mid-2004 through 2013 for HAdV typing, only 7 (2.4%) were identified as HAdV-B7 (D.D. Erdman, pers. comm.).

In March 2014, clinicians in the metropolitan areas of Eugene and Portland, Oregon, USA, reported an increase in the number of HAdV detections among specimens from hospitalized patients with severe respiratory infections to the Oregon Public Health Division (OPHD). In this study, we describe the clinical, epidemiologic, and viral molecular features of this cluster of HAdV-positive cases identified during October 2013–July 2014.

Methods

OPHD asked 3 major hospital systems that perform HAdV diagnostic testing to participate in the investigation. The hospital systems comprised 14 facilities located primarily within the Eugene and Portland metropolitan areas.

Facilities averaged 210 beds (range 25–688) and 11,774 admissions annually (range 1,263–35,614). These hospital systems were asked to provide information on HAdV-positive specimens from patients with respiratory infections diagnosed after September 2013. Requested information comprised basic demographic and clinical data and whether specimens were available for HAdV typing. On April 19, 2014, OPHD released a statewide notice through the Health Alert Network requesting providers in Oregon to consider HAdV in the differential diagnosis for patients presenting with severe pneumonia or for unusual clusters of pneumonia.

We also obtained historical HAdV detection data from Oregon clinical laboratories that reported to the National Respiratory and Enteric Virus Surveillance System to review recent trends in HAdV detections. In addition, 2 large Oregon hospital systems with available HAdV detection data compared HAdV detections for November 2013–April 2014 with those for November–April from the respiratory disease seasons of the previous 3 years (i.e., 2010–11, 2011–12, 2012–13).

The study population comprised persons in whom HAdV was detected in respiratory specimens during October 2013–July 2014 by hospital laboratories that perform virus isolation, direct fluorescent antibody, or PCR for HAdV detection (retrospective and prospective) and were willing to submit data to OPHD and CDC. A case-patient was defined as a person with respiratory symptoms and a positive HAdV laboratory test result. Illness was defined as severe if the patient was hospitalized.

We reviewed available medical records and collected the following information: basic demographic information, symptom onset date, symptoms, hospital admission and discharge dates, intensive care unit (ICU) admission, use of mechanical ventilation, specimen type and collection date, and HAdV laboratory results. The medical record review was conducted under Oregon's special study statute for issues of public health significance (http://arcweb.sos.state.or.us/pages/rules/oars_300/oar_333/333_019.html).

Available clinical specimens were shipped to the Oregon State Public Health Laboratory and then submitted to the CDC for HAdV confirmation and molecular typing. HAdV-positive specimens were typed by either conventional PCR and sequencing of HAdV hexon gene hypervariable regions 1–6 or by HAdV type-specific real-time PCR assays, as previously described (*19*,*20*). For enhanced genetic comparisons, genomic sequencing was performed on 7 HAdV-B7–positive samples collected during January 2014–May 2014. Deep sequencing libraries were prepared by using the Nextera XT DNA Sample Prep Kit and sequenced (250-bp paired-end sequencing) on an Illumina MiSeq Desktop Sequencer (both from Illumina, San Diego, CA, USA) (protocol available on request). In silico genome restriction enzyme digestion profiles were generated by using NEBcutter V2.0 (*21*), and genome-type determinations were based on the classification system previously described (*22*). Sequences were compared with HAdV-B7 genome type d (HAdV-B7d) reference strains 0901HZ/Shx/CHN/2009 (GenBank accession no. JF800905.1) and human/CHN/DG01/2011/7[P7H7F7] (GenBank accession no. KC440171.1). We used sequence alignment and neighbor-joining phylogenetic tree construction to compare the phylogenetic relationships among a representative sample of HAdV-B7 genomic sequences using ClustalW implemented in BioEdit version 7.0.5 (*23*) and MEGA7, respectively (*24*). We excluded cases determined by CDC to be HAdV-negative.

We used Excel (Microsoft Corp., Redmond, WA, USA) for data entry and SAS version 9.3 (SAS Institute, Cary, NC, USA) for data analysis. Mantel-Haenszel χ^2 was used to assess associations. A p value <0.05 was considered significant.

Results

Comparison to historical data for the past 3 years demonstrated an increase in HAdV reports for November 2013–April 2014. We found 2-fold and 9-fold increases in HAdV detections for the 2 large Oregon hospital systems for which historical data were available (Figure 1). HAdV detections from laboratories in Oregon reporting to the National Respiratory and Enteric Virus Surveillance System (Figure 2) increased 11-fold during the same period.

For October 9, 2013–July 7, 2014, we identified 198 patients who had an HAdV-positive respiratory specimen. Most were reported from hospital systems in Portland and surrounding counties (56%), followed by Eugene and surrounding counties (39%), Medford (4%), and 2 other locations (1%). Most (97%) cases came from 3 major hospital systems in Oregon (hospital system A, 77 [39%] cases; hospital system B, 69 [35%]; hospital system C, 46 [23%]); the remaining 6 cases came from a variety of sources around the state. Most (91%) cases occurred in residents of Oregon; the remainder occurred in residents of Washington (7%), and California (2%). Case-patients ranged in age from 3 weeks to 80 years (median 8 years); 60% were male. For most (87%) HAdV detections, symptom onset occurred or specimens were collected during January–April 2014 (Figure 3).

Specimens from 109 (55%) HAdV patients were available for HAdV typing (Table 1). We identified 7 HAdV types; HAdV-B7 was most commonly detected (64 [59%] specimens), followed by HAdV-C2 (14 [13%]), HAdV-B21 (10 [9%]), HAdV-C1 (10 [9%]), HAdV-B3 (6 [6%]), HAdV-C5 (2 [2%]), and HAdV-E4 (1 [1%]). In 2 (2%) cases, HAdV-C1 and -C2 were co-detected.

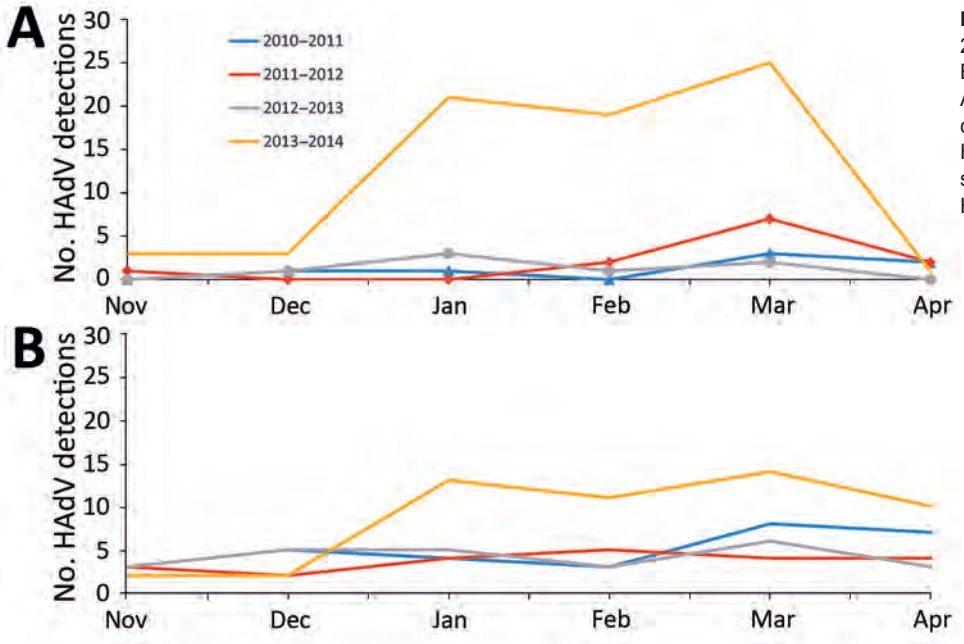

Figure 1. HAdV detections from 2 major hospital systems (A and B), Oregon, USA, November–April 2010–2014. Historical data collected by the Oregon Public Health Division. Data for hospital system C were not available. HAdV, human adenovirus.

The genome from 7 HAdV-B7–positive samples collected during January–May 2014 (GenBank accession no. KT963081) showed >99.9% sequence identity and gave identical in silico *BamH*I and *Bcl*I restriction profiles with a HAdV-B7d variant circulating in China in 2009 and 2011 (*22*) (Figure 4). Specimens from 89 (45%) HAdV-positive patients were not available for typing; 80% of these specimens came from hospital system A. HAdV type results were grouped by species (i.e., HAdV-C and HAdV-E), except for species B, which was grouped by type (i.e., HAdV-B7 and HAdVs-B3, -B21) to better highlight specific features of the rarely reported HAdV-B7. Most (74%) specimens tested were upper respiratory tract specimens (i.e., nasopharyngeal wash or nasopharyngeal swab samples). Case-patients with HAdV-C1, -C2, or -C5 detections were generally younger (median age 1.2 years) than those with HAdVs-B3 or -B21 (median age 24.0 years) and HAdV-B7 (median age 20.0 years) (Table 1). Case-patients with HAdV-B7 were significantly older than those with non–HAdV-B7 (Table 2).

For all case-patients, the most common symptoms were fever (75%), cough (61%), shortness of breath (26%), nausea or vomiting (24%), and rhinorrhea (22%) (Table 1). Pneumonia was reported for 32% of the 198 HAdV patients (41% of those with HAdV-B7, 31% of those with HAdVs-B3 or -B21, 21% of those with HAdVs-C1, -C2, or -C5). A

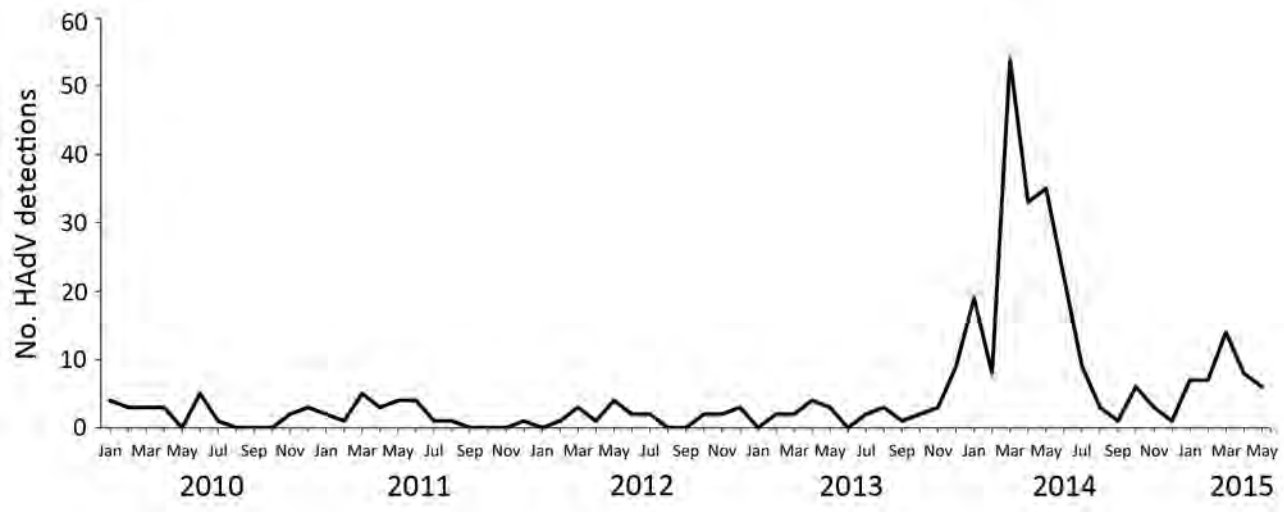

Figure 2. HAdV detections reported to the National Respiratory and Enteric Virus Surveillance System, Oregon, January 2010–May 2015. HAdV, human adenovirus.

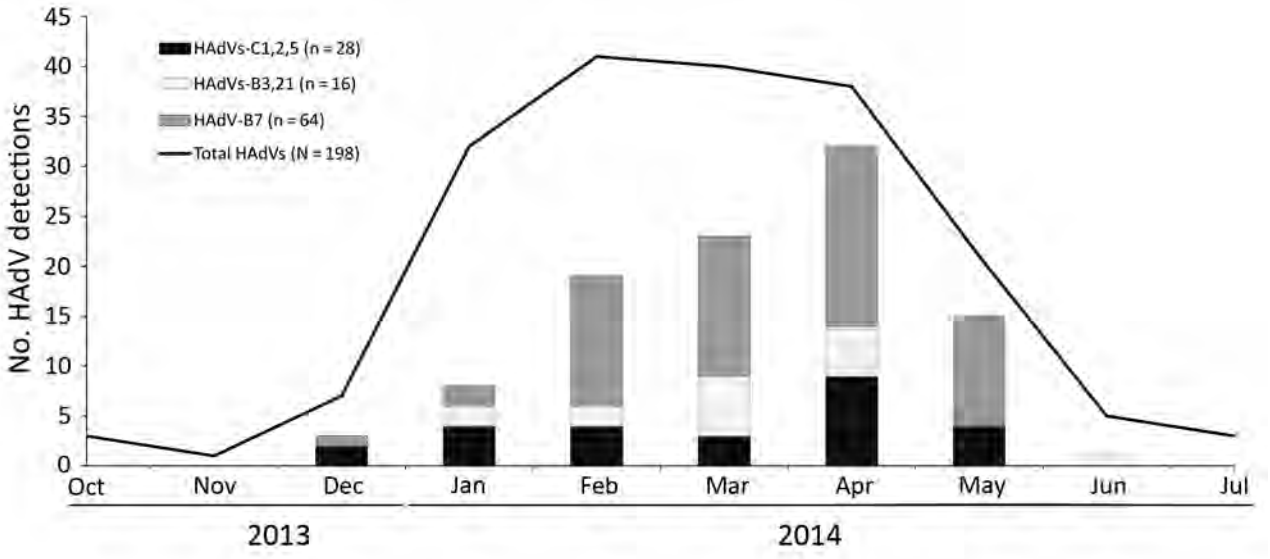

Figure 3. HAdV detections by type and by month of symptom onset, Oregon, USA, October 2013–July 2014. If month of symptom onset was not available, month of specimen collection was used. Total HAdVs include 109 HAdV-positive specimens that were typed (including 1 HAdV-E4 specimen) and 89 specimens that were not available for typing. HAdV, human adenovirus.

total of 136 (69%) persons were hospitalized (Table 1), and 62 (31%) were outpatients seen in the emergency department or in outpatient clinics. HAdV-B patients were more often hospitalized (HAdVs-B3 or -B21: 81%; HAdV-B7: 84%) than HAdVs-C1, -C2, or -C5 (64%) patients (Table 1). Among hospitalized case-patients, 32% were admitted to the ICU, and 18% required mechanical ventilation (Table 1); 46% of HAdV-B7 case-patients were admitted to the ICU (Table 1). Median length of hospital stay for HAdV-B7 case-patients was longer (6.5 days; interquartile range [IQR] 3–9.5 days) than for HAdVs-B3 or -B21 case-patients (5.0 days; IQR 3–5 days) and HAdVs-C1, -C2, or -C5 case-patients (2.5 days; IQR 2–4 days). However, when we compared HAdV-B7 with non–HAdV-B7 case-patients, only length of hospitalization remained statistically significant after adjustment for age; HAdV-B7 case-patients were significantly older (adults) and hospitalized longer than the median (4 days) than non–HAdV-B7 case-patients ($p<0.05$) (Table 2). Five (2.5%) case-patients died; for 2, specimens were available for typing, and both were HAdV-B7.

Discussion

We describe a community outbreak of HAdV in Oregon where HAdV-B7 was identified as the predominant HAdV type. HAdV-B7 was more often detected in adults than children and was associated with more severe disease than other HAdV types. In persons ≤18 years of age, 7 HAdV types were detected, whereas in most persons >18 years of age, HAdVs-B3, -B7, or -B21 were detected. These findings are consistent with other studies showing that HAdVs-C1, -C2, and -C5 are most frequently detected in young children (*17*,*25*). Many studies have demonstrated severe illness associated with HAdV-B7 in children or in military recruits, but few have documented such a widespread community outbreak in which many adults experience severe respiratory illness (*5*,*18*,*26*,*27*). However, a high percentage of adults with severe respiratory disease has been reported in other community outbreaks of related HAdV-B viruses (*6*,*13*,*28*,*29*).

Compared with non–HAdV-B7 case-patients, HAdV-B7 case-patients were more frequently admitted to the ICU and mechanically ventilated, were significantly older, and had significantly longer hospital stays. Our findings of severe infection with HAdV-B7 requiring ICU admission and extended hospitalization is consistent with other reports linking HAdV-B7 with more severe acute respiratory disease (*30*–*32*). HAdV also was detected in 5 fatal cases, 2 of which were identified as HAdV-B7. Although the contribution of HAdV to a patients' death is unclear, similar reports of severe outcomes associated with HAdV infection have been documented (*18*,*26*,*30*).

Further molecular analysis showed that genome sequences of 7 Oregon HAdV-B7 isolates were identical to each other and nearly identical to strains circulating in China in 2009 and 2011. Predicted restriction enzyme profiles have not previously identified the genome of this virus (HAdV-B7d) in the United States (A. Kajon, pers. comm.) until recently (*33*). HAdV-B7d was first isolated in China in 1980, where it became the predominant circulating genome type at least through 1990 (*22*) but then disappeared until reemerging 21 years later (*15*). Severe respiratory

Table 1. Demographic and clinical characteristics for HAdV-positive case-patients, Oregon, October 2013–July 2014*

Characteristic	Total	HAdVs-B3, -B21	HAdV-B7	HAdVs-C1, -2, -5	Not available†
Total	198 (100)	16 (8.1)	64 (32.3)	28 (14.1)	89 (45.0)
Age group, y					
Children, ≤18 y	118 (59.6)	8 (50.0)	28 (43.8)	27 (96.4)	54 (60.7)
<2	63 (31.8)	3 (18.8)	16 (25.0)	25 (89.3)	19 (21.4)
2–5	24 (12.1)	4 (25.0)	5 (7.8)	1 (3.6)	14 (15.7)
6–10	20 (10.1)	0	4 (6.3)	1 (3.6)	15 (16.9)
11–18	11 (5.6)	1 (6.3)	3 (4.7)	0	6 (6.7)
Adults, >18 y	80 (40.4)	8 (50.0)	36 (56.3)	1 (3.6)	35 (39.3)
19–25	16 (8.1)	0	6 (9.4)	0	10 (11.2)
26–45	19 (9.6)	2 (12.5)	10 (15.6)	0	7 (7.9)
46–65	35 (17.7)	6 (37.5)	18 (28.1)	1 (3.6)	10 (11.2)
>65	10 (5.1)	0	2 (3.1)	0	8 (9.0)
Common symptoms at presentation					
Fever	149 (75.3)	12 (75.0)	50 (78.2)	18 (64.3)	69 (77.5)
Cough	121 (61.1)	10 (62.5)	45 (70.3)	18 (64.3)	48 (53.9)
Shortness of breath	52 (26.3)	7 (43.8)	23 (35.9)	7 (25)	15 (16.9)
Nausea or vomiting	47 (23.7)	4 (25.0)	14 (21.9)	9 (32.1)	20 (22.5)
Rhinorrhea	43 (21.7)	5 (31.3)	6 (9.4)	10 (35.7)	22 (24.7)
Fatigue	27 (13.6)	2 (12.5)	15 (23.4)	3 (10.7)	7 (7.9)
Diarrhea	22 (11.1)	3 (18.8)	9 (14.1)	2 (7.1)	8 (9.0)
Sore throat	20 (10.1)	2 (12.5)	3 (4.7)	0	15 (16.9)
Myalgia	20 (10.1)	1 (6.3)	6 (9.4)	0	13 (14.6)
Wheezing	13 (6.6)	3 (18.8)	3 (4.7)	4 (14.3)	3 (3.4)
Illness severity					
Hospitalized	136 (68.7)	13 (81.3)	54 (84.4)	18 (64.3)	51 (57.3)
Admitted to ICU‡	43 (31.6)	4 (30.8)	25 (46.3)	4 (22.2)	10 (19.6)
Required mechanical ventilation‡	25 (18.4)	3 (23.1)	13 (24.1)	1 (3.6)	8 (15.7)
Died	5 (2.5)	0	2 (3.1)	0	3 (3.4)
Age, y, mean/median (IQR)§	20.6/8.0 (1.9–40)	26.8/24.0 (3–50.5)	26.8/20.0 (2.5–50.5)	3.5/1.2 (0.7–1.6)	20.6/9.0 (3–29)
Days from symptom onset to hospital admission, mean/median (IQR)‡§	4.2/3.0 (2–5)	3.6/3.0 (2–4)	4.6/3.0 (2–6)	3.9/3.0 (1–4)	4.2/ 4.0 (2–5)
Days hospitalized, mean/median (IQR)‡§	6.5/4.0 (2–8)	5.0/5.0 (3–5)	8.4/6.5 (3–9.5)	3.4/2.5 (2–4)	6.3/ 4.0 (2–6)

*Values are no. (%) except as indicated. Total HAdV also includes HAdV-E4 (1 case), which was in a nonhospitalized child (10–18 y). HAdV, human adenovirus; ICU, intensive care unit; IQR, interquartile range.
†Specimens from these HAdV-positive patients were not available for typing.
‡Among hospitalized HAdV patients.
§IQR = 25% and 75% quartiles.

disease and higher case-fatality rates have been associated with HAdV-B7, especially HAdV-B7d. Enhanced virus fitness, low herd immunity to HAdV-B7, or both might have contributed to the large community outbreak in Oregon.

The US military used an effective live oral vaccine for HAdV-E4 and HAdV-B7 from 1971 until 1999, and the vaccine was reintroduced in 2011 (*34*,*35*). During 1999–2011, when the vaccine was unavailable, vaccine-preventable HAdV infections increased substantially in US military personnel (*36*). Subsequent reintroduction of the vaccine in October 2011 resulted in a significant decrease in these HAdV infections, indicating HAdV vaccination is an effective prevention measure for HAdV infection in this setting (*35*). However, currently no HAdV vaccine is available for use in the general public.

Our study has several limitations. The HAdV cases described in this case series do not represent a population-based sample and might not represent all HAdV cases in Oregon during this period. Persons with milder illness were not sought out at participating facilities because case finding was primarily conducted in hospitals and because the Health Alert Network notice requested providers to consider HAdV in patients with severe pneumonia. Because of the timing of the investigation, almost half of the HAdV-positive specimens were not available for typing (many were cases diagnosed earlier in the study period), and 80% of these specimens came from 1 of the 3 major hospital systems that participated in the investigation. The molecular methods used for typing HAdVs in this study targeted the hexon hypervariable regions that have been shown to correlate closely with virus serotype. These methods do not provide genomic detail and might miss recombination events in other regions of the virus genome. Moreover, full-genome sequencing was performed on only 7 HAdV-B7 isolates, and although they were chosen from different time points in the outbreak, we cannot conclude that all HAdV-B7 detections were HAdV-B7d. Finally, detection of HAdV in a respiratory specimen, especially HAdV-C, does not necessarily imply causation because asymptomatic HAdV shedding can occur. HAdV-C detections in infants and young children with mild respiratory infections and the virus might persist in the adenoids and tonsils and be shed for prolonged periods

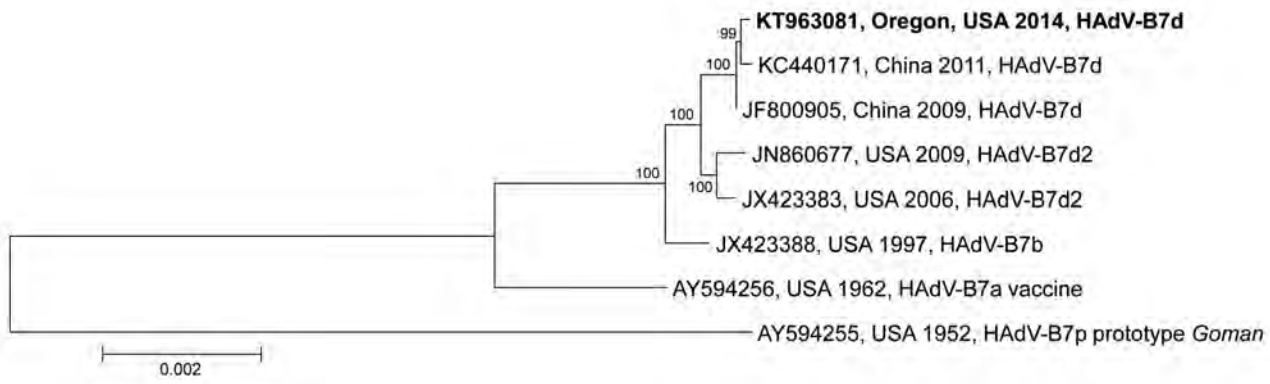

Figure 4. Phylogenetic analysis of human adenovirus 7 genome type d (HAdV-B7d), Oregon, USA, 2014. Genomic sequences were aligned by using ClustalW implemented in BioEdit version 7.2.5 and the neighbor-joining phylogenetic tree constructed by using MEGA7 software (*23,24*). Numbers at selected nodes indicate level of support using 1,000 bootstrap replicates. Sequences are identifoed by GenBank accession number, geographic location, year of sample collection, and virus genome type identified. Boldface indicates 7 identical genomic sequences identified during this study; sequences are from HAdV-B7 isolates derived from 7 different cases spanning the outbreak period. Scale bar indicates estimated number of nucleotide substitutions per site.

after symptoms resolve (*37*–*39*). The clinical significance of HAdV latency in tonsil and adenoid tissue is unclear.

HAdV type surveillance is an important tool for monitoring changes in predominant types and genome types. Shifts in HAdV types might be associated with more severe disease not only in vulnerable populations, such as children, elderly persons, and immunocompromised persons, but might also cause community outbreaks of severe respiratory disease in adults, as occurred in Oregon. Healthcare providers should consider HAdV in their differential diagnosis for patients with pneumonia and acute respiratory infection. Testing for HAdV using respiratory panel PCR assays and HAdV typing has been increasing nationwide. In response, CDC recently launched a voluntary and passive surveillance system to collect HAdV typing data from laboratories called the National Adenovirus Type Reporting System. The main objectives of this system are to better define circulation patterns of HAdV types and better monitor HAdV outbreaks in the United States.

HAdV-B7 might be reemerging in the United States and might be associated with increased numbers of severe respiratory infections. Tracking the emergence of HAdV types in the United States will lead to early identification of new types and potential variants of known types. Our results demonstrate how HAdV surveillance might help explain clusters and sporadic cases of severe illness possibly related to changes in HAdV species.

Acknowledgments

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Ms. Kendall Scott is the influenza epidemiologist for OPHD. Her research interests include influenza and other respiratory viruses.

Table 2. Comparison of HAdV-B7 cases with non-HAdV-B7 case-patients, Oregon, October 2013–July 2014*

Characteristic	HAdV-B7, no. (%)	Non–HAdV-B7, no. (%)†	p value
Total	64	45	
Age group, y			
Children, ≤18	28 (43.7)	36 (80.0)	<0.001
Adults, >18	36 (56.3)	9 (20.0)	
Illness severity			
Hospitalized	54 (84.4)	31 (68.9)	<0.05‡
Admitted to ICU§	25 (46.3)	8 (25.8)	0.06
Required mechanical ventilation§	13 (24.1)	4 (12.9)	0.26
Days hospitalized¶			
≤ Median, 4	14 (35.0)	19 (70.4)	<0.05
> Median, 4	26 (65.0)	8 (29.6)	

*HAdV, human adenovirus; ICU, intensive care unit
†Includes only the 109 specimens that were typed. Non-HAdV-B7: HAdVs-B3, -B21, HAdVs-C1, -C2, -C5, and HAdV-E4.
‡Not significant after adjustment for age.
§Among hospitalized case-patients.
¶A total of 40 HAdV-B7 case-patients and 27 non–HAdV-B7 case-patients had both hospital admission and discharge dates.

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Heterogeneous and Dynamic Prevalence of Asymptomatic Influenza Virus Infections

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Influenza infection manifests in a wide spectrum of severity, including symptomless pathogen carriers. We conducted a systematic review and meta-analysis of 55 studies to elucidate the proportional representation of these asymptomatic infected persons. We observed extensive heterogeneity among these studies. The prevalence of asymptomatic carriage (total absence of symptoms) ranged from 5.2% to 35.5% and subclinical cases (illness that did not meet the criteria for acute respiratory or influenza-like illness) from 25.4% to 61.8%. Statistical analysis showed that the heterogeneity could not be explained by the type of influenza, the laboratory tests used to detect the virus, the year of the study, or the location of the study. Projections of infection spread and strategies for disease control require that we identify the proportional representation of these insidious spreaders early on in the emergence of new influenza subtypes or strains and track how this rate evolves over time and space.

Infection of the respiratory tract with an influenza virus results in symptoms ranging from mild nonfebrile illness to severe disease and complications, including pneumonia, shock, renal failure, encephalopathy, and multiorgan dysfunction (*1*,*2*). Influenza viruses infect 5%–15% of the global population annually (*3*), accounting for ≈500,000 deaths (*4*) and 19 million disability-adjusted life years (*5*). The occurrence of asymptomatic influenza viruses infections has been recognized for some time (*6*), but determinations about their possible role in transmission are largely speculative (*7*,*8*). Clarifying the role of these infections in virus transmission requires a solid understanding of their rate of occurrence.

Interest in the contribution of asymptomatic infection to influenza virus transmission has risen in recent years after a series of outbreaks caused by newly emerging subtypes (*9*–*12*). Subclinical infection eludes symptomatic surveillance, and resulting illnesses thus manifest as sporadic disease. Social network analysis indicates that nearly one third of the attack rate for influenza A(H1N1)pdm09 virus in England was attributable to asymptomatic infection (*13*), a proportion mirrored by a recent review of volunteer challenge studies (*14*). Mathematical modeling studies designed to inform pandemic preparedness and vaccination thresholds and stockpiling strategies have typically had to resort to using these types of indirect metrics for parameterization (*15*–*17*). Current policy surrounding intervention planning for pandemic and interpandemic influenza is informed by estimates and simulations that arbitrarily assume a constant rate of asymptomatic infection in the range of 30%–50%.

However, mortality rates, clinical symptoms, and basic reproduction numbers (outbreak thresholds) vary greatly between influenza virus types, subtypes, and strains (*18*). Therefore, assigning an arbitrary value for asymptomatic infection rates that does not reflect this heterogeneity presents an important shortcoming in the current ability to accurately predict influenza outbreaks. Therefore, we conducted a systematic review and meta-analysis to determine the prevalence of asymptomatic influenza infection and to identify any factors associated with the heterogeneity reported across studies.

Methods

Search Strategy and Selection Criteria

A systematic review and meta-analysis was conducted in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (*19*). Literature searches were performed on the PubMed and Web of Science databases for the period from the inception of these databases to the beginning of 2015 to identify studies that reported laboratory-confirmed influenza infection (i.e., by culture, PCR, or serologic testing) and the proportion of symptomatic versus asymptomatic presentation. Search terms were chosen to ensure maximum coverage of possible literature and included the terms "influenza,"

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"carrier," "carriage," "shedding," "asymptomatic," "influenza AND prophylaxis NOT vaccine" (filtered for randomized control trials), "influenza AND (travel OR migration OR immigra*) AND (screening OR test OR testing OR detection)," "subclinical," "serosurvey OR seroprevalence OR seroepidemiology." Other keywords and connectors were also used (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/22/6/15-1080-Techapp1.pdf).

To be eligible for inclusion, studies needed to 1) be peer-reviewed and 2) report the prevalence of asymptomatic influenza virus infections in humans or present the appropriate data from which that prevalence could be calculated. Laboratory confirmation of influenza was a requirement, and it had to be possible to correlate these data to the number of symptomatic patients. We did not impose limitations in terms of study design, influenza virus type, or exposure type (community or experimental inoculation). According to current World Health Organization guidelines, laboratory confirmation consisted of 1) conventional PCR (referred to here as PCR) or real-time reverse transcription PCR (rRT-PCR); 2) virus antigen detection by immunofluorescence or enzyme immunoassay methods; 3) serologic detection of antibodies (hemagglutination inhibition); or 4) virus culture (*20*). Studies were excluded when the use of antiviral agents without a placebo group was reported. In cases in which a placebo group was used and an asymptomatic proportion could be determined, only this subset was used; otherwise, the study was excluded. Results were restricted to studies published in English; however, no restriction was placed on the publication date of studies that fit these criteria.

Study Selection and Data Extraction

Two authors (L.F-K. and M.C.) independently screened the publications for eligibility in a stepwise fashion. Search results were initially screened based on article titles and abstracts. Then, full-text analysis was performed to identify all studies which either reported asymptomatic prevalence or from which asymptomatic prevalence could be calculated. Any discrepancies that might have affected inclusion or exclusion of a study were resolved through discussion and consensus after independent evaluation by another author (L.Y.). The same 2 authors (L.F-K. and M.C.) assessed the risk for bias of the studies included by using a modified version of the tool developed by Hoy et al. (*21*) for prevalence studies (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/22/6/15-1080-Techapp2.pdf).

The definitions of asymptomatic influenza infection varied considerably between studies. Definitions ranged from a total absence of symptoms to a lack of influenza-like illness (ILI) or acute respiratory illness (ARI). For the sake of clarity, we used the term "asymptomatic" when there was a total absence of symptoms and "subclinical" when the patient did not meet the authors' criteria for ILI or ARI. Asymptomatic influenza prevalence was considered to be the proportion of all persons with laboratory-confirmed influenza who had no symptoms, whereas subclinical influenza prevalence was the proportion of persons with laboratory-confirmed influenza who failed to meet the study's definition of symptomatic infection. In addition to collecting data on asymptomatic and subclinical infection prevalence, we collected data on influenza virus type/subtype and study characteristics (e.g., study design, sample size, diagnostic test used to detect influenza virus infection, and the working definition of "symptomatic").

Statistical Analysis

We used prevalence of asymptomatic versus subclinical carriers among persons with laboratory-confirmed influenza as primary endpoints of interest. We pooled the prevalence estimates of asymptomatic and subclinical influenza across studies by using 2 meta-analytical models, the inverse variance heterogeneity model (*22*) and the random effects model.

We observed considerable heterogeneity across studies. This heterogeneity was unlikely to be attributable only to random or systematic errors, and actual clinical heterogeneity was deemed to exist. Therefore, we created subgroups by influenza virus type/subtype with the aim of generating more homogeneous groups within which we could anticipate that the differences indeed reflected variability caused by random or systematic error rather than actual clinical heterogeneity. In addition, we built a linear model to examine the variance explained by the influenza virus type/subtype, laboratory test used to detect the virus, year of the study, and geographic location of the study to gain insight into the considerable heterogeneity observed in the prevalence of asymptomatic and subclinical infections. We conducted the meta-analyses by using MetaXL version 2.0 (EpiGear Int Pty Ltd, Brisbane, QLD, Australia), which also included the inverse variance heterogeneity method, and the generalized linear model by using Stata version 12 (StataCorp LP, College Station, TX, USA). All tests were 2-tailed, and a p value <0.05 was deemed statistically significant.

Results

Yield of Search Strategy

A total of 13,219 records were identified from literature searches of the 2 databases. This number was reduced to 9,900 after removal of publications that were either duplicates or not original research papers (e.g., review papers). An additional 3,663 papers were removed based on the title and 5,652 papers more based on the abstract. The full texts of the remaining 585 studies were examined, and 55 articles met the inclusion criteria and were included in the final analysis (Figure; online Technical Appendix 1).

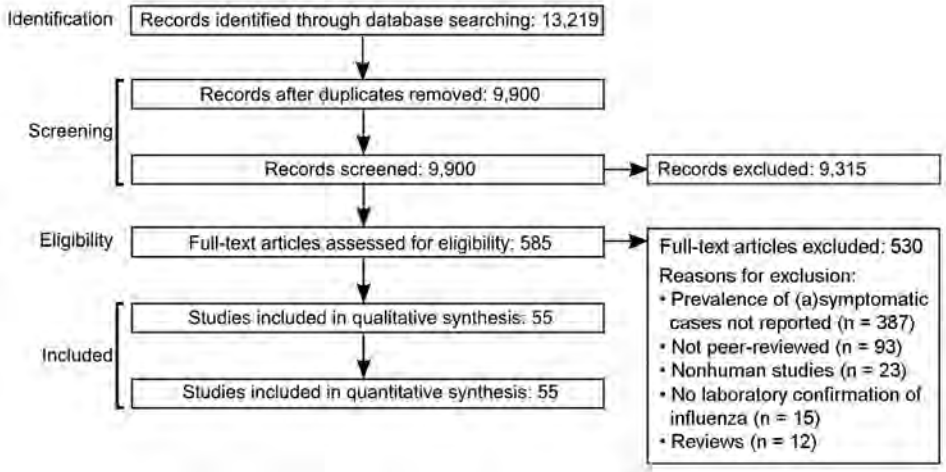

Figure. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) flowchart of literature search for systematic review and meta-analysis of asymptomatic and subclinical influenza infection prevalence.

Characteristics of the Studies Included

The 55 articles provided 59 data points because 4 papers reported the prevalence of asymptomatic and subclinical carriers for influenza A and B viruses separately. Overall, 19 studies (22 data points) defined asymptomatic infection as cases in persons lacking symptoms, and 44 studies (46 data points) reported subclinical influenza virus infections.

Infection was confirmed by serologic testing, rRT-PCR, or viral culture; 28 studies reported use of serologic testing alone to confirm infection, 18 used rRT-PCR alone, and the remaining 9 used a combination of methods (5 serologic testing and rRT-PCR, 3 serologic testing and culture, and 1 rRT-PCR and culture). Among the 55 studies, influenza A virus (predominantly H1N1) was the most common type of infection; 5 studies reported influenza B virus infections, and 1 study reported influenza C infections (online Technical Appendix 1 Table 2). Most studies reported on pandemic influenza virus types; 32 of these studies related to the 2009 pandemic influenza A/Mexico/4108/2009 strain. The risk for bias was moderate in 32% of the studies and low in the remaining 68%; no study was found to have a high risk for bias.

Quantitative Synthesis

The overall pooled prevalence for asymptomatic carriers was 19.1% (95% CI 5.2%–35.5%) for any type of influenza, 21.0% (95% CI 4.2%–41.0%) for influenza A, and 22.7% (95% CI 7.7%–39.8%) for influenza A(H1N1) (Table 1; online Technical Appendix 2 Figure 1). For subclinical carriers, the overall pooled prevalence was 43.4% (95% CI 25.4%–61.8%) for any type of influenza, 42.8% (95% CI 22.3%–63.9%) for influenza A, and 39.8% (95% CI 16.4%–64.5%) for influenza A(H1N1) (Table 1; online Technical Appendix 2 Figure 2). However, extensive heterogeneity was immediately evident for reported asymptomatic prevalence ($\tau^2 = 0.31$) and subclinical prevalence ($\tau^2 = 0.45$) that could not be explained by the influenza type/subtype alone. Similar results were obtained with the random effects model (online Technical Appendix 2 Figures 3, 4).

Investigation of Heterogeneity

The considerable heterogeneity observed within asymptomatic and subclinical influenza prevalence could not be explained by the type/subtype of influenza, the laboratory tests used to detect the virus, the location where the study was conducted, or the year of the study. The multivariate regression models could only explain 16.8% and 14.8% of the observed variance for the asymptomatic and subclinical prevalence, respectively. Influenza type/subtype as an independent predictor was found to account for almost the entire variance (16%) found for the prevalence of asymptomatic carriers (Table 2).

Table 1. Heterogeneity within asymptomatic and subclinical influenza infection cases, by virus type/subtype, as determined through a systematic review and meta-analysis of 55 studies

Type/subtype	Prevalence (95% CI)	Cochran's Q	p value (Cochran's Q)	I^2,* %
Asymptomatic				
All types of influenza	19.1 (5.2–35.5)	752.40	<0.001	97
Influenza A	21.0 (4.2–41.0)	692.94	<0.001	98
Influenza A(H1N1)	22.7 (7.7–39.8)	561.14	<0.001	97
Subclinical				
All types of influenza	43.4 (25.4–61.8)	1768.24	<0.001	97
Influenza A	42.8 (22.3–63.9)	1689.78	<0.001	98
Influenza A(H1N1)	39.8 (16.4–64.5)	1388.54	<0.001	98

*The I^2 statistic describes the percentage of variation across studies that is attributable to heterogeneity rather than chance.

Table 2. Variance attributable to predictors in univariate and multivariate regression models for asymptomatic and subclinical influenza infection prevalence, by study characteristics, as determined through a systematic review and meta-analysis of 55 studies

Model/characteristic	Asymptomatic	Subclinical
Univariate model		
Influenza type/subtype	0.1599	0.0345
Laboratory test used to detect influenza	0.0043	0.0546
Hemisphere where study was conducted	0.0001	0.0159
Continent where study was conducted	0.0045	0.0213
Decade when study was conducted	*	0.0064
Multivariate model	0.1676†	0.1478‡

*Variance not reported because all the studies were from the same decade.
†Model adjusted for influenza type/subtype, laboratory test, and location (continent) of the study.
‡Model adjusted for influenza type/subtype, laboratory test, location (continent) of the study, and decade when the study was conducted.

Publication Bias

For both asymptomatic and subclinical carrier prevalence, the funnel plots showed no indication of publication bias. This result was confirmed by Doi plots (data not shown).

Discussion

Studies of laboratory-confirmed influenza typically do not include details of the symptomatic versus asymptomatic rate of infection. Of the few that do include this information, ambiguity exists between definitions of asymptomatic versus subclinical infections. This has perpetuated the ubiquitous issue of absent denominators in documented influenza rates and has caused substantial aberrations in initial reports of newly emerging subtypes and strains (*23*). We propose that the term "asymptomatic" be used exclusively to describe the complete absence of symptoms associated with influenza virus infection in patients with laboratory-confirmed cases. Given that reporting of this rate in the clinical literature would require little to no additional effort for most study designs, we also propose that the asymptomatic rate of laboratory test–positive persons be declared explicitly by public health bodies and researchers.

We found no evidence to support a fixed asymptomatic rate (or even an informative range) between or even within influenza virus subtypes. For example, the prevalence of asymptomatic influenza A(H1N1) virus ranged from 0% to 65%, resulting in an overall failure to explain the extreme heterogeneity in this reported rate. Some alternative explanations for the extreme heterogeneity are plausible, one being that generally applicable biologic mechanisms underlie the asymptomatic rates of influenza virus infection and these have been missed (e.g., details of patient vaccination or infection history were not routinely described in the clinical studies and data on sex and age of patients were excluded). Alternatively, influenza viruses conferring asymptomatic infection mutate so rapidly that a meaningful single per–influenza type rate simply does not exist. Employing sensitive diagnostic testing and standardized reporting of the asymptomatic rate of influenza virus infection would elucidate any underlying mechanisms or demonstrate any temporal changes in this rate.

This lack of a convenient asymptomatic rate poses a considerable obstacle to public health planning. Disease surveillance and control strategy is contingent on reliable estimates for the asymptomatic rate and the contribution that asymptomatic persons have on transmissibility. For example, a low asymptomatic rate improves the utility of passive (i.e., symptom-based) surveillance, whereas a higher asymptomatic rate might prompt presumptive travel restrictions to curb the spread of newly emerging subtypes and strains, especially if a high mortality rate is evident early in the outbreak. Future analyses correlating asymptomatic rates with mortality rates are also required; although one could easily speculate that influenza subtypes and strains eliciting high asymptomatic rates probably incur correspondingly low mortality rates, no evidence supporting this assumption currently exists.

Our study clearly demonstrates the inappropriateness of a one-size-fits-all approach to mitigating the spread of human influenza viruses. As new subtypes and strains emerge, actively surveying infection status of local populations and tracking any changes in asymptomatic rates of infection should increasingly become a global health priority, possibly necessitating the provision of international resources and the deployment of dedicated rapid-response teams who are guided by standardized protocols.

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Mr. Furuya-Kanamori is an infectious disease epidemiologist. He is enrolled in a PhD program at the Australian National University where he uses modern quantitative methods to better understand risk factors associated with different infectious diseases.

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High MICs for Vancomycin and Daptomycin and Complicated Catheter-Related Bloodstream Infections with Methicillin-Sensitive *Staphylococcus aureus*

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We investigated the prognostic role of high MICs for antistaphylococcal agents in patients with methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection (MSSA CRBSI). We prospectively reviewed 83 episodes from 5 centers in Spain during April 2011–June 2014 that had optimized clinical management and analyzed the relationship between E-test MICs for vancomycin, daptomycin, oxacillin, and linezolid and development of complicated bacteremia by using multivariate analysis. Complicated MSSA CRBSI occurred in 26 (31.3%) patients; MICs for vancomycin and daptomycin were higher in these patients (optimal cutoff values for predictive accuracy = 1.5 μg/mL and 0.5 μg/mL). High MICs for vancomycin (hazard ratio 2.4, 95% CI 1.2–5.5) and daptomycin (hazard ratio 2.4, 95% CI 1.1–5.9) were independent risk factors for development of complicated MSSA CRBSI. Our data suggest that patients with MSSA CRBSI caused by strains that have high MICs for vancomycin or daptomycin are at increased risk for complications.

Staphylococcus aureus bacteremia is an issue of concern because of high rates of illness and death for this condition (*1*). Some studies have identified high MICs for vancomycin (defined as an MIC ≥1.5 μg/mL by E-test) as a simple laboratory-based marker, which has been shown to be associated with a worse prognosis in methicillin-resistant *S. aureus* (MRSA) bacteremia in terms of treatment failure (*2–4*) and increased mortality rates (*5*,*6*). Such increased mortality rates associated with a high MIC for vancomycin has also been reported in methicillin-sensitive *S. aureus* (MSSA) bacteremia (*7*,*8*). However, the use of death as a study outcome and the fact that these studies were performed with patients with different sources of bacteremia make it difficult to interpret study results. Such findings could not be confirmed in large cohorts of patients with MRSA (*9–11*) or MSSA (*12*) bacteremia from different sources.

To avoid these confounding factors, we focused on a more homogeneous population of patients with MSSA catheter-related bloodstream infection (MSSA CRBSI) for whom specific efforts were devoted to optimize clinical management. In a previous retrospective study, we observed that high MICs for vancomycin were associated with development of complicated MSSA CRBSI, regardless of the initial antimicrobial drug therapy used (vancomycin or β-lactams), which suggested that intrinsic characteristics of these strains might explain their pathogenic role in development of complicated bacteremia (*13*). We designed this multicenter prospective study to confirm the prognostic role of high MICs for vancomycin and to explore if increased MICs for other antistaphylococcal agents could also influence the risk for developing complicated MSSA CRBSI.

Materials and Methods

Study Design and Setting

We conducted a prospective, observational, multicenter study during April 2011–June 2014 in 5 hospitals in Spain in which we included patients given a diagnosis of MSSA

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CRBSI according to standard a definition (*14*) that fulfilled 5 criteria: 1) age ≥18 years and a life expectancy ≥7 days; 2) no use of daptomycin within the 3 months preceding the incident MSSA CRBSI episode; 3) removal of the intravascular catheter suspected to be the source of CRBSI within a maximum of 72 hours from sampling of the first blood cultures yielding MSSA; 4) a maximum of 72 hours of treatment with glycopeptides; and 5) at least the first blood culture isolate available for microbiological analysis. All included patients signed a study-specific informed consent form. The study was approved by the ethics research boards at all participating centers.

Management of MSSA CRBSI

Although the nature of the study was not interventional, the adherence to evidence-based quality-of-care measures was strongly encouraged among participating investigators to optimize the therapeutic management of MSSA CRBSI. These measures were extraction of follow-up blood cultures, ordering echocardiography or venous Doppler ultrasound examination for patients with clinical indication, early use of intravenous cloxacillin or other highly effective antimicrobial drugs for treatment of for MSSA as definitive therapy, and adjustment of treatment duration according to the complexity of the infection.

Definitions

Recorded clinical variables were age; site of acquisition of infection; concurrent conditions, as assessed by the Comorbidity Index of Charlson et al. (*15*); prognosis of the underlying disease (classified according to the McCabe and Jackson modified criteria as rapidly fatal [when death was expected within 3 months], ultimately fatal [when death was expected within a period of >3 months but <5 years] and nonfatal [when life expectancy was >5 years]) (*16*); type of intravenous catheter; timing of catheter removal; treatment administered; duration of fever; duration of bacteremia; Pitt bacteremia score (*17*); development of septic shock; endovascular infection or complicated bacteremia (defined below); and all-cause and MSSA CRBSI–attributable mortality rates at 30 days. MSSA CRBSI–attributable deaths at 30 days were identified as per the clinical judgment of the investigator.

Antimicrobial drugs were classified within 1 of the following mutually excluding categories: glycopeptides; daptomycin; antistaphylococcal β-lactams (parenteral cloxacillin, cefazolin or other active β-lactams [including parenteral amoxicillin/clavulanate, piperacillin/tazobactam, imipenem, or meropenem]); non−β-lactam agents with in vitro activity against MSSA (antistaphylococcal quinolones, tigecycline, or linezolid); and noneffective agents. Timing to initiation of antimicrobial drug therapy and duration of treatment were also recorded. Empiric therapy was defined as therapy administered before identification and antimicrobial susceptibility testing of the isolate. Complicated bacteremia (study outcome) was defined by 1 of 5 events occurring after the incident episode of MSSA CRBSI: 1) development of endocarditis (defined according to modified Duke criteria) (*18*); 2) septic thrombophlebitis (defined by persistent MSSA bacteremia for >72 hours after the initiation of active therapy associated to the documentation of a thrombus at the site of catheter insertion); 3) septic metastatic infection, including arthritis, spondylitis, infections involving vascular or osteoarticular prostheses (excluding intravascular catheter) or end-organ hematogenous seeding to other locations remote from the primary focus; 4) persistent bacteremia for >72 hours; or 5) persistent fever for >4 days after extraction of the first blood culture yielding MSSA, provided that alternative causes had been reasonably excluded (*19*).

Microbiological Methods

Blood cultures were processed and isolates were identified according to standard techniques at each participating center. The first MSSA isolate from each patient was stored at –80°C until analysis and sent to the central reference laboratory (Department of Microbiology, University Hospital 12 de Octubre, Madrid, Spain). We subsequently subcultured strains twice and performed antimicrobial susceptibility testing by using the E-test method according to manufacturer's recommendations (AB bioMérieux, Solna, Sweden). We tested vancomycin, daptomycin, oxacillin, and linezolid. In addition, the E-test macromethod was performed to screen for heteroresistant vancomycin-intermediate *S. aureus* (hVISA) (*20*). All the plates were visually read throughout the entire study period by 2 independent observers, who were blinded to each other and to the clinical outcomes. Interobserver discrepancies were resolved by reevaluation and a consensus decision.

Statistical Analysis

We used the Student unpaired *t*-test to compare normally distributed continuous variables, the Mann-Whitney U test to compare continuous variables with non-normal distribution, and the χ^2 and Fisher exact tests to compare proportions as appropriate. Areas under receiving operating characteristic curves for MICs for selected antistaphylococcal agents (on the basis of their discriminative value at the univariate comparisons) were plotted to select the optimal cutoff value in terms of sensitivity (S) and specificity (Sp) to distinguish between patients with or without complicated bacteremia by means of the Youden's J statistic (J = S + Sp −1). We calculated the Pearson correlation coefficient (r) to assess the strength of the linear relationship among E-test MICs for daptomycin and vancomycin.

To test the effect of high MICs for antistaphylococcal agents and other clinical variables on the occurrence of complicated MSSA CRBSI, we plotted event-free Kaplan-Meier survival curves and performed comparisons between groups by using the log-rank test. Statistically relevant variables ($p<0.10$) at the univariate level were entered into multivariate Cox proportional hazard models with a backward stepwise selection, and hazard ratios (HRs) with 95% CIs were estimated. Some variables considered clinically relevant were also forced into the models regardless of their univariate p values. All statistical tests were 2-tailed, and the threshold of statistical significance was set at $p<0.05$. We used statistical software SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA) to perform calculations of different analysis and generated graphics by using Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Variables with an unacceptable proportion of missing values (>30%) were excluded from the analysis.

Results

Study Population and Outcome

Of 108 patients initially recruited, 19 were excluded because they did not fulfill inclusion criteria after revision of the clinical charts (13 had received >72 hours of treatment with vancomycin and 6 had had a delay of >3 days in catheter removal). An additional 6 patients cases were excluded because of inability to recover the MSSA strain for microbiological analysis. Therefore, we included 83 patients in the final analysis; median follow-up was 479 days (range 5 days–1,014 days). No variables were excluded because of missing data. None of the included patients had received vancomycin, daptomycin, oxacillin, or linezolid within the 3 months preceding the incidence MSSA CRBSI episode.

We compared the main demographic and clinical characteristics of the 83 patients in the final cohort (Table 1). Catheter removal was performed for all patients within the first 72 hours from sampling of the first blood cultures and, in most (75 [90.6%]) patients, within the first 48 hours. Antistaphylococcal β-lactams were used as empiric therapy in 43 patients (51.8%), glycopeptides in 32 (38.5%), and daptomycin in 17 (20.4%). Combined therapy was used for 25 (31.1%) patients. Twelve (14.5%) patients received no empiric therapy or the regimen administered was based on antimicrobial drugs with no in vitro activity against MSSA. For all patients who received daptomycin, the dose used was ≥6 mg/kg/day (range 6–10 mg/kg/day). Venous Doppler ultrasound examination and echocardiogram were performed for 41 (49.5%) and 61 patients (73.5%), respectively.

Complicated MSSA CRBSI developed in 26 patients (31.3%) at a median of 4 days (interquartile range [IQR] 1–26 days) after sampling of blood cultures. No patients had late complications (i.e., >30 days). Septic thrombophlebitis was the most frequent complication (10 patients [12%]), followed by right-sided endocarditis (2 cases [2.4%]), hematogenous osteoarticular infection (2 cases [2.4%]), and pulmonary emboli (2 cases [2.4%]) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/6/15-1709-Techapp1.pdf). All-cause and MSSA CRBSI–attributable 30-day mortality rates were 12.0% and 2.4%, respectively.

Correlation between MICs and Outcome

All strains were sensitive to all antimicrobial drugs tested. None of the isolates had the hVISA phenotype. The 50% MIC and 90% MIC and distribution ranges values were 0.5 μg/mL and 0.75 μg/mL (range 0.5–0.75 μg/mL) for cloxacillin, 1.5 μg/mL and 2 μg/mL (range 0.5–2.5 μg/mL) for vancomycin, 0.38 μg/mL and 0.75 μg/mL (range 0.125–0.94 μg/mL) for daptomycin, and 1 μg/mL and 1.5 μg/mL (range 0.25–2 μg/mL) for linezolid.

We compiled a comparative description of geometric means of MICs determined by E-test for vancomycin, daptomycin, oxacillin, and linezolid among isolates from patients with or without complicated bacteremia (Table 2). We observed that the MICs for vancomycin and daptomycin were higher for isolates from patients in whom complicated bacteremia developed.

We subsequently performed an exploratory area under receiving operating characteristic curve analysis to establish the optimal cutoff point for the daptomycin MIC with the best discriminatory capacity to predict development of complicated bacteremia. An MIC for daptomycin of 0.5 μg/mL was selected (online Technical Appendix Figure 1). This approach also confirmed that 1.5 μg/mL was the optimal cutoff point for the vancomycin MIC (online Technical Appendix Figure 2). A total of 43 strains (41.8%) had a vancomycin MIC ≥1.5, μg/mL and 13 (15.7%) had a daptomycin MIC >0.5 μg/mL. On the basis of these thresholds, 11 (84.6%) of 13 isolates with high MICs for daptomycin also showed high MICs for vancomycin, and we found a moderate positive correlation between these variables ($r = 0.21$, $p = 0.05$) (online Technical Appendix Figure 3). The percentage of MSSA strains with high MICs for vancomycin or daptomycin was higher in the group of patients with complicated bacteremia (Table 2). Strains isolated from all the patients in whom septic thrombophlebitis developed had high MICs for vancomycin or daptomycin.

We compared clinical characteristics of patients according to MICs for vancomycin or daptomycin (Table 3). Rates of complicated MSSA CRBSI were significantly higher among patients with episodes caused by strains with MICs for vancomycin (42% vs. 20%; $p = 0.05$) or daptomycin (69.2% vs. 24.3%; $p = 0.004$) than for those with

Table 1. Demographics and clinical characteristics of 83 patients with methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection*

Variable	Value
Age, y	60 ± 1.9
Male sex	49 (59.0)
Recruiting center	
1	32 (38.6)
2	19 (22.9)
3	2 (2.4)
4	12 (14.5)
5	18 (21.7)
Prognosis of underlying disease	
Not fatal	31 (37.3)
Fatal	44 (53.0)
Rapidly fatal	8 (9.6)
Charlson comorbidity index	3.7 ± 2.3
Previous conditions	
Diabetes	32 (38.6)
Malignancy	45 (54.2)
Valvular prosthesis	1 (1.2)
Ostheoarticular prosthesis	3 (3.6)
Renal failure requiring hemodialysis	10 (12)
Type of intravascular catheter	
Peripheral venous	32 (38.6)
Nontunneled (temporary) central venous	25 (30.1)
Peripherally inserted central	10 (12)
Permanent central venous	16 (19.3)
Pitt score at bacteremia onset	1.6 ± 1.4
Severe sepsis or septic shock	17 (20.5)
Empiric treatment including†	
Glycopeptides	32 (38.5)
Antistaphylococcal β-lactams‡	43 (51.8)
Other antistaphylococcal antimicrobial drugs	4 (4.8)
Daptomycin	17 (20.4)
None or noneffective antimicrobial drugs	12 (14.5)
Antimicrobial regimen	
Glycopeptides followed by antistaphylococcal β-lactam	25 (30.1)
Only antistaphylococcal β-lactams	30 (36.1)
Daptomycin followed by antistaphylococcal β-lactam	12 (14.5)
Only daptomycin	1 (1.2)
Glycopeptides followed by daptomycin plus antistaphylococcal β-lactam	7 (8.4)
Daptomycin plus antistaphylococcal β-lactam	7 (8.4)
Other	1 (1.2)
Timing of catheter removal	
Same day or before sampling first blood cultures	47 (56.6)
1 day after sampling	12 (14.5)
2 days after sampling	17 (20.5)
3 days after sampling	7 (8.4)
Venous Doppler ultrasound examination	41 (49.5)
Echocardiogram	61 (73.5)
Complicated MSSA CRBSI	26 (31.3)
Persistent fever ≥4 d§	11 (13.3)
Persistent bacteremia ≥72 h¶	6 (7.2)
Septic thrombophlebitis	10 (12)
Endocarditis	2 (2.4)
Hematogenous osteoarticular infection	2 (2.4)
Pulmonary emboli	2 (2.4)
All-cause deaths at 30 days	10 (12)
MSSA CRBSI–attributable deaths at 30 days	2 (2.4)

*Values are mean ± SD or no. (%). MSSA CRBSI: methicillin-sensitive *S. aureus* catheter-related bloodstream infection.
†Some patients could be included in >1 category.
‡Parenteral cloxacillin, cefazolin, amoxicillin/clavulanate, piperacillin/tazobactam, imipenem, or meropenem.
§Coincident with other forms of complicated bacteremia for 5 cases.
¶Coincident with other forms of complicated bacteremia in 5 cases.

episodes caused by strains with lower MICs. Strains with high MICs for vancomycin were not associated with higher rates of severe sepsis or septic shock at bacteremia onset, or with increased all-cause or attributable mortality rates. Conversely, we found that high MICs for daptomycin were more common among hemodynamically unstable episodes

Table 2. Antimicrobial susceptibility testing (E-test) of isolates from patients with methicillin-sensitive Staphylococcus aureus catheter-related bloodstream infection with or without complicated bacteremia*

Variable	Complicated bacteremia	
	No, n = 57	Yes, n = 26
MIC, μg/mL		
Vancomycin	1.2 ± 0.4†	1.5 ± 0.48†
Oxacillin	0.49 ± 0.27	0.51 ± 0.2
Daptomycin	0.4 ± 0.16†	0.5 ± 0.2†
Linezolid	1.08 ± 0.13	1.05 ± 0.08
Frequency		
Vancomycin MIC ≥1.5 μg/mL	25 (43.9)†	18 (69.2)†
Daptomycin MIC >0.5 μg/mL	4 (7.0)†	9 (34.6)†
Vancomycin MIC ≥1.5 μg/mL or daptomycin MIC >0.5 μg/mL	26 (45.6)†	19 (73.1)†
Vancomycin MIC ≥1.5 μg/mL and daptomycin MIC >0.5 μg/mL	3 (5.3)†	8 (30.8)†

*Values are geometric mean ± SD or no. (%).
†p<0.05.

(i.e., severe sepsis or septic shock) than among remaining episodes (46.2% vs. 15.7%, p = 0.02). However, no effect on mortality rates was observed.

Risk Factors for Development of Complicated Bacteremia

We compared clinical characteristics of patients with or without complicated bacteremia (Table 4). Most (65.4%) patients with complicated cases were given a diagnosis at 1 recruiting center. Such an imbalance might be partially explained by the fact that such a center was the study promoter and the largest center at which the greatest diagnostic efforts were made. The probability of remaining free of complicated bacteremia at day 30 was significantly lower for patients with MSSA CRBSI caused by isolates with daptomycin MICs >0.5 μg/mL (33. 3% vs. 75%; log-rank test p = 0.002) and isolates with vancomycin MICs ≥1.5 μg/mL (59.2% vs. 79.6%; log-rank test p = 0.02) (Figure).

Because of low number of patients with complicated bacteremia (26 episodes), we could not assess the potential effect of MICs by performing a single logistic regression model adjusted for all the covariates found to be significant by univariate analysis. We alternatively attempted an exploratory approach based on different models. We used Cox regression models that incorporated a maximum of 3 variables at a time, always including the recruiting center as a potential confounder (Table 5; online Technical Appendix Table 2). The presence of high MICs for vancomycin (minimum adjusted HR 2.4, 95% CI 1.2–5.5) and daptomycin (minimum adjusted HR 2.4, 95% CI 1.1–5.9) were kept as independent risk factors for development of complicated MSSA CRBSI in all models.

We performed a sensitivity analysis by restricting the study outcome to the occurrence of septic thrombophlebitis. These associations remained essentially unchanged, and we observed even higher HRs (minimum adjusted HR for vancomycin MIC 6.0 [95% CI 0.7–52.2]; minimum adjusted HR for daptomycin MIC 5.5 [95% CI 1.3–22.5]) (online Technical Appendix Tables 2, 3).

Discussion

The aim of our study was to investigate the potential role of high MICs for vancomycin and other antistaphylococcal agents in predicting the risk for complications in a selected cohort of patients with MSSA CRBSI. We investigated this role after controlling for other well-defined prognostic factors, such as timing of catheter removal or appropriateness of empiric therapy.

We found a correlation between high MICs for vancomycin (≥1.5 μg/mL) or daptomycin (>0.5 μg/mL) and an increased incidence of complicated bacteremia, particularly in terms of local endovascular complications represented by septic thrombophlebitis. Venous Doppler ultrasound examination was performed for only 50% of patients. Notwithstanding this potential drawback, we included in the definition of the study outcome the presence of persistent bacteremia in the absence of proven hematogenous spread to distant locations. This criterion might act as an appropriate clinical surrogate for identifying potential cases of septic thrombophlebitis that could have eventually remained undiagnosed.

Results confirm those obtained in our previous single-center retrospective study (*13*), in which we demonstrated that MSSA strains with vancomycin MICs ≥1.5 μg/mL were associated with a >2-fold increase in risk for development of complicated bacteremia and, specifically, a >6-fold increase in the risk for development of septic thrombophlebitis. In both studies, and in contrast to other authors who evaluated MSSA bacteremia from different sources (*7*), including patients with endocarditis (*8*), we did not find any apparent effect of increased MICs on sepsis severity or risk for death. The relatively low 30-day mortality rate for our selected cohort could partially account for this discrepancy. However, López-Cortés et al. (*12*) did not demonstrate any apparent effect of MICs for vancomycin on mortality rates in a recent study of a nonselected cohort of patients with MSSA bacteremia. These authors also reported an increased incidence of septic thrombophlebitis among patients with MSSA bacteremia caused by strains

Table 3. Demographic and clinical characteristics of patients with methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection by MICs for vancomycin and daptomycin measured by E-test*

Variable	Vancomycin MIC, μg/mL			Daptomycin MIC, μg/mL		
	<1.5, n = 40	≥1.5, n = 43	p value	≤0.5, n = 70	>0.5, n = 13	p value
Age, y	61.9 ± 16.0	59.9 ± 19.0	0.42	60.2 ± 17.3	63.8 ± 21.0	0.5
Male sex	23 (57.5)	26 (60.5)	0.42	41 (58.6)	8 (61.5)	0.4
Prognosis of underlying disease						
Not fatal	18 (45.0)	13 (30.2)	0.3	24 (34.3)	7 (53.8)	0.3
Fatal	17(42.5)	27 (62.8)	0.1	39 (55.7)	5 (38.5)	0.4
Rapidly fatal	5 (12.5)	3 (6.9)	0.6	7 (10.0)	1 (7.7)	0.8
Charlson comorbidity index	3.5 ± 2.6	4.0 ± 2.1	0.31	3.7 ± 2.3	3.5 ± 2.1	0.9
Previous conditions						
Diabetes	20 (50.0)	12 (27.9)	0.06	28 (40)	4 (30.8)	0.6
Malignancy	17 (42.5)	28 (65.1)	0.12	37 (52.9)	8 (61.5)	0.5
Valvular prosthesis	0 (0.0)	1 (2.3)	0.9	1 (1.4)	0 (0.0)	0.9
Ostheoarticular prosthesis	1 (2.5)	2 (4.7)	0.79	2 (2.9)	1 (7.7)	0.6
Renal failure requiring hemodialysis	7 (17.5)	3 (6.9)	0.2	9 (12.9)	1 (7.7)	0.5
Type of intravascular catheter						
Peripheral venous	17 (42.5)	15 (34.9)	0.71	28 (40)	4 (30.8)	0.7
Nontunneled (temporary) central venous	13 (32.5)	12 (27.9)	0.82	19 (27.1)	6 (46.2)	0.3
Peripherally inserted central	5 (12.5)	5 (11.6)	0.76	10 (14.3)	0 (0.0)	0.3
Permanent central venous	5 (12.5)	11 (25.6)	0.2	13 (18.6)	3 (23.1)	0.6
Pitt score at bacteremia onset	1.5 ± 1.7	1.1 ± 1.4	0.9	1.3 ± 1.7	1.2 ± 1.0	0.9
Severe sepsis or septic shock	11 (27.5)	6 (13.9)	0.2	11 (15.7)	6 (46.2)	0.02
Empiric treatment including†						
Glycopeptides	16 (40.0)	16 (37.2)	0.7	27 (38.5)	5 (38.4)	0.7
Antistaphylococcal β-lactams‡	21 (52.5)	22 (51.1)	0.9	37 (52.8)	6 (46.1)	0.7
Other antistaphylococcal antimicrobial drugs	1 (2.5)	3 (6.9)	0.7	4 (5.7)	0 (0.0)	0.8
Daptomycin	7 (17.5)	10 (23.2)	0.8	13 (18.5)	4 (30.7)	0.5
None or noneffective antimicrobial drugs	7 (17.5)	5 (11.6)	0.8	10 (14.3)	2 (15.4)	0.8
Antimicrobial regimen						
Glycopeptides followed by antistaphylococcal β-lactam	11 (27.5)	14 (32.6)	0.7	21 (30.0)	4 (30.8)	0.9
Only antistaphylococcal β-lactams	14 (35)	16 (37.2)	0.8	26 (37.1)	4 (30.8)	0.8
Daptomycin followed by antistaphylococcal β-lactam	3 (7.5)	9 (20.9)	0.16	8 (11.4)	4 (30.8)	0.2
Only daptomycin	1 (2.5)	0 (0.0)	0.9	1 (1.5)	0 (0.0)	0.9
Glycopeptides followed by daptomycin plus antistaphylococcal β-lactam	6 (15.0)	1 (2.3)	0.09	6 (8.6)	1 (7.7)	0.9
Daptomycin plus antistaphylococcal β-lactam	4 (10.0)	3 (6.9)	0.8	7 (10)	0 (0.0)	0.5
Other	1 (1.25)	0 (0.0)	0.6	1 (2.5)	0 (0.0)	0.7
Timing of catheter removal						
Same day or before sampling first blood cultures	28 (70.0)	19 (44.2)	0.03	41 (58.6)	6 (46.2)	0.7
1 day after sampling	6 (15.0)	6 (13.9)	0.9	10 (14.3)	2 (15.4)	0.9
2 days after sampling	5 (12.5)	12 (27.9)	0.1	12 (17.1)	5 (38.5)	0.2
3 days after sampling	1 (2.5)	6 (13.9)	0.1	7 (10.0)	0 (0.0)	0.6
Complicated MSSA CRBSI	8 (20.0)	18 (41.9)	0.05	17 (24.3)	9 (69.2)	0.004
Persistent fever for ≥4 d	3 (7.5)	8 (18.6)	0.2	9 (12.9)	2 (15.4)	0.8
Persistent bacteremia for ≥72 h	2 (5.0)	4 (9.3)	0.7	5 (7.1)	1 (7.7)	0.9
Septic thrombophlebitis	1 (2.5)	9 (20.9)	0.02	4 (5.7)	6 (46.2)	<0.0001
Endocarditis	1 (2.5)	1 (2.3)	0.9	2 (2.9)	0 (0.0)	0.9
Hematogenous osteoarticular infection	1 (2.5)	1 (2.3)	0.9	1 (1.4)	1 (7.7)	0.5
Pulmonary emboli	2 (4.7)	0 (0.0)	0.3	2 (2.9)	0 (0.0)	0.6
All-cause deaths at 30 days	6 (15.0)	4 (9.3)	0.5	9 (12.9)	1 (7.7)	0.5
MSSA CRBSI–attributable deaths at 30 days	1 (2.5)	1 (2.3)	0.4	2 (2.9)	0 (0.0)	0.4

*Values are geometric mean ± SD or no. (%). MSSA CRBSI: methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection.
†Some patients could be included in >1 category.
‡Parenteral cloxacillin, cefazolin, amoxicillin/clavulanate, piperacillin/tazobactam, imipenem, or meropenem.

with high MICs for vancomycin. This combined evidence suggests that the theoretically increased pathogenicity of these strains might exert a local vascular effect but does necessarily imply a higher risk for severe sepsis or death.

The role of MICs for daptomycin as a prognostic marker in episodes of MSSA bacteremia has not been extensively investigated. In a study by Cervera et al., which included only patients with endocarditis caused by MSSA, MICs for daptomycin were not related to increased mortality rates (*8*). We have established a cutoff value for the MIC for daptomycin (>0.5 μg/mL) that also identified patients with an increased risk for development of complicated MSSA CRBSI, particularly septic thrombophlebitis. In accordance with lack of a correlation for the MIC for vancomycin, mortality rates did not differ according to MICs for daptomycin. Only 11 isolates (13.2% of the cohort) had

Table 4. Comparative analysis of patients with methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection with or without complicated bacteremia*

Variable	Complicated bacteremia		p value
	No, n = 5	Yes, n = 26	
Age, y	61.4 ± 16.0	59.3 ± 21.0	0.1
Male sex	32 (56.1)	17 (65.4)	0.5
Recruiting center			
1	15 (26.3)	17 (65.4)	0.01
2	14 (24.6)	5 (19.2)	0.8
3	2 (3.5)	0 (0.0)	1.0
4	11 (19.3)	1 (3.8)	0.2
5	15 (26.3)	3 (11.5)	0.2
Prognosis of underlying disease			
Not fatal	24 (42.1)	7 (26.9)	0.2
Fatal	29 (50.9)	15 (57.7)	0.6
Rapidly fatal	4 (7.0)	4 (15.4)	0.3
Charlson comorbidity index	3.6 ± 2.4	1.9 ± 1.9	0.3
Previous conditions			
Diabetes	20 (35.1)	12 (46.2)	0.3
Malignancy	29 (50.9)	16 (61.5)	0.6
Valvular prosthesis	1 (1.8)	0 (0.0)	1.0
Ostheoarticular prosthesis	1 (1.8)	2 (7.7)	0.2
Renal failure requiring hemodialysis	7 (12.3)	3 (11.5)	1.0
Type of intravascular catheter			
Peripheral venous	23 (40.4)	9 (34.6)	0.8
Nontunneled (temporary) central venous	17 (29.8)	8 (30.8)	1.0
Peripherally inserted central	7 (12.3)	3 (11.5)	1.0
Permanent central venous	10 (17.5)	6 (23.1)	0.6
Pitt score at bacteremia onset	1.1 ± 1.4	1.7 ± 1.9	0.1
Severe sepsis or septic shock	11 (19.3)	6 (23.1)	0.3
Empiric treatment including†			
Glycopeptides	24 (42)	8 (30.7)	0.4
Antistaphylococcal β-lactams‡	31 (54.3)	12 (46.1)	0.6
Other antistaphylococcal antimicrobial drugs	1 (1.7)	3 (11.5)	0.2
Daptomycin	8 (14)	9 (34.6)	0.06
Daptomycin monotherapy	5 (8.8)	8 (30.8)	0.02
None or noneffective antimicrobial drugs	10 (17.5)	2 (7.7)	0.4
Antimicrobial regimen			
Glycopeptides followed by antistaphylococcal β-lactam	19 (33.3)	6 (23.1)	0.4
Only antistaphylococcal β-lactams	23 (40.4)	7 (26.9)	0.3
Daptomycin followed by antistaphylococcal β-lactam	4 (7.0)	8 (30.8)	0.01
Only daptomycin	1 (1.8)	0 (0.0)	1.0
Glycopeptides followed by daptomycin plus antistaphylococcal β-lactam	6 (10.5)	1 (3.8)	0.4
Daptomycin plus antistaphylococcal β-lactam	3 (5.3)	4 (15.4)	0.2
Other	1 (1.8)	0 (0.0)	1.0
Timing of catheter removal			
Same day or before of sampling first blood cultures	36 (63.2)	11 (42.3)	0.1
1 day after sampling	6 (10.5)	6 (23.1)	0.1
2 days after sampling	10 (17.5)	7 (26.9)	0.4
3 days after sampling	5 (8.8)	2 (7.7)	1.0
Venous Doppler ultrasound examination	21 (36.8)	20 (76.9)	0.001
Echocardiogram	36 (63.2)	25 (96.2)	0.01
All-cause deaths at 30 days	7 (12.3)	3 (11.5)	1.0
MSSA CRBSI–attributable deaths at 30 days	0 (0.0)	2 (7.7)	0.09

*Values are geometric mean ± SD or no. (%). MSSA CRBSI: methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection.
†Some patients could be included in >1 category.
‡Parenteral cloxacillin, cefazolin, amoxicillin/clavulanate, piperacillin/tazobactam, imipenem, or meropenem.

simultaneously high MICs for vancomycin and daptomycin, and multivariate analysis demonstrated that both variables had an independent effect on the risk for development of complicated MSSA CRBSI.

It has been reported that MRSA isolates, mostly hVISA isolates, with higher MICs for vancomycin also have increased MICs for daptomycin (*21–24*). However, other studies have failed to confirm such an association (*25–27*). Pillai et al. found that some MSSA strains obtained from a patient with persistent bacteremia treated with vancomycin showed increasing MICs for daptomycin (*28*). In vitro studies suggested that patients infected with MSSA strains that have high MICs for vancomycin could have poor responses not only to vancomycin (*29*) but also to other antistaphylococcal agents, such as cloxacillin or daptomycin (*30*). In a recent in vitro study, daptomycin

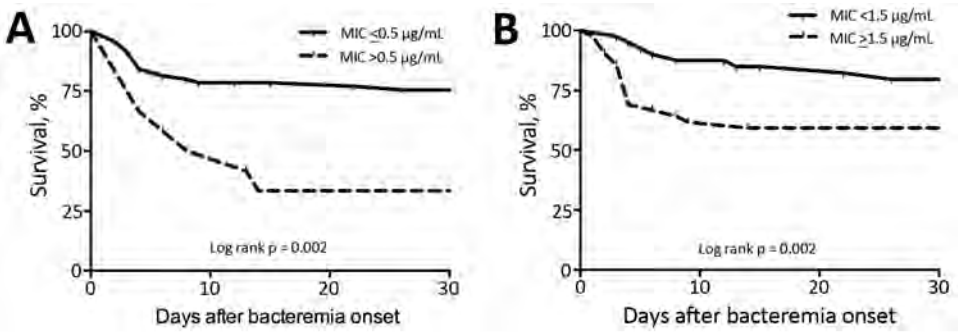

Figure. Event-free Kaplan-Meier survival curves for complicated bacteremia according to E-test MICs for daptomycin (A) and vancomycin (B) for complicated catheter-related bloodstream infections with a methicillin-sensitive *Staphylococcus aureus* isolate.

resistance was associated with increased thickness of bacterial cell wall peptidoglycan and a slightly delayed transition to the postexponential growth phase because of alterations in bacterial metabolism (*31*). These findings suggest a deleterious fitness cost in terms of invasiveness (*31*) but also a blockage in the adhesive phase of *S. aureus* that might be linked to an increased trend in local complications. High MICs for vancomycin in isolates with the hVISA phenotype have been shown to be associated with increased persistence, rather than increased mortality rates and sepsis in patients (*32*). Although we did not detect any hVISA isolates in our study, we postulate that a high MIC for vancomycin could be an essential step in acquisition of this phenotype, and that this step might be the common underlying pathogenic mechanism.

Apart from the limited number of events (which prevented including all explanatory variables into a single multivariate model), some limitations of the study also deserve specific consideration. Despite its multicenter nature, 1 specific participating center provided most of the results because this center recruited most of the patients and most patients who had complicated bacteremia. However, all multivariate models were adjusted by the recruiting center to minimize such potential bias. Antimicrobial drug susceptibility testing was not performed in parallel with the reference broth microdilution method, which could have enabled us to confirm the poor correlation previously reported between this technique and the E-test (*11*).

Conversely, interobserver reproducibility of the E-test for assessing vancomycin MICs has been recently questioned (*33*). In our study, MIC testing was performed over a continuous period, and E-test results were read by 2 independent observers who were blinded to patient outcome, an approach that theoretically would reduce intraassay variability and facilitate interpretation of results. Finally, real-life applicability of our findings and potential prognostic value of performing E-tests for daptomycin for all MSSA isolates from patients with CRBSI remain to be established with support of cost-effectiveness analysis. Although we did not specifically address some variables that had been postulated to be related with higher MICs for vancomycin or daptomycin, such as inoculum size (*34*) or use of suboptimal dosing of daptomycin for cases of persistent bacteremia (*35*), we set a priori inclusion criteria to homogenize the clinical management of patients analyzed (including uniform dosing of daptomycin and vancomycin).

In summary, our data suggest that patients with MSSA CRBSI caused by strains with MICs ≥1.5 μg/mL for vancomycin or >0.5 μg/mL for daptomycin determined by E-test are more prone to show development of complications, particularly septic thrombophlebitis. However, these patients do not appear to have an increased risk for severe sepsis or death. On the basis of these preliminary findings, patients with CRBSI cause by MSSA isolates with high MICs for vancomycin or daptomycin should be carefully evaluated to exclude local complications and eventually individualize duration of therapy. These prospects would merit confirmation in future intervention studies.

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Table 5. Univariate and multivariate analyses of risk factors for development of complicated methicillin-sensitive *Staphylococcus aureus* catheter-related bloodstream infection*

Variable	Univariate, HR (95% CI)	Multivariate, HR (95% CI)†
Recruiting center 1	3.6 (1.6–8.1)	–
Any daptomycin-containing empiric therapy	2.5 (1.1–5.7)	–
Daptomycin monotherapy as empiric therapy	3.1 (1.3–7.1)	–
Vancomycin MIC ≥1.5 μg/mL	2.6 (1.1–5.9)	2.4 (1.2–5.5)
Daptomycin MIC >0.5 μg/mL	3.4 (1.6–8.2)	2.4 (1.1–5.9)

*HR, hazard ratio; –, corresponding variables were not retained in the final model.
†Various models, including a maximum of 3 variables, were explored that combined all statistically significant variables identified at the univariate level. Only variables that were constantly retained in these models are shown with minimum HR values obtained.

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Population-Level Effect of Cholera Vaccine on Displaced Populations, South Sudan, 2014

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Following mass population displacements in South Sudan, preventive cholera vaccination campaigns were conducted in displaced persons camps before a 2014 cholera outbreak. We compare cholera transmission in vaccinated and unvaccinated areas and show vaccination likely halted transmission within vaccinated areas, illustrating the potential for oral cholera vaccine to stop cholera transmission in vulnerable populations.

In December 2013, violence erupted in Juba, South Sudan, and quickly spread throughout the country. By the end of 2014, one in five persons within the country had been displaced, and many sought refuge in protection of civilians (PoC) sites inside United Nations (UN) Mission bases and in spontaneous internally displaced persons (IDP) settlements. Within 6 weeks of the start of the violence, South Sudan Ministry of Health requested vaccine from the global oral cholera vaccine stockpile to target 163,000 IDPs in 6 camps throughout the country, but not persons in the broader host communities (*1*).

In April 2014, two months after vaccine deployment, South Sudan confirmed the first case of cholera in the country since 2009; ≈4 weeks later, officials declared a cholera outbreak. Over 5 months, 6,269 suspected cholera cases were reported, including 156 deaths. Most cases occurred outside vaccinated camps, often in communities or camps surrounding vaccinated populations.

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Several studies have demonstrated the individual-level (direct) effects of oral cholera vaccination (*2*–*4*), but few have estimated the overall population-level effect (a combination of direct and indirect effects), which is critical to determining costs and benefits. To estimate the overall effect, the observed epidemic in vaccinated areas must be compared with a counterfactual epidemic that is modeled or based on an observed suitable control population.

We used detailed epidemiologic data from the 2014 vaccination campaigns and the subsequent cholera outbreak in South Sudan to determine how vaccine use may have altered the epidemic course in vaccinated areas. We compared epidemics in 2 areas that included vaccinated and unvaccinated populations: 1) PoC sites (vaccinated) and the community (unvaccinated) in Juba; and 2) Malakal PoC (vaccinated) and Wau Shilluk IDP (unvaccinated), 2 similar camps separated by a river.

The Study

The South Sudan Ministry of Health and World Health Organization implemented a clinic-based cholera surveillance system that captured basic patient data, laboratory results (if available), and outcomes. A suspected cholera case-patient was defined as anyone with acute watery diarrhea (diagnosed by a clinician); suspected cases were considered confirmed if the patient had a culture-positive fecal sample. Our analyses include all suspected cases.

We considered 5 populations in our comparisons, 3 in Juba County and 2 in Malakal County. In Juba, displaced persons were largely confined to 2 camps: 1) Tongping PoC camp (population 14,015) near the center of Juba; and 2) the UN House PoC camp (population 17,627) on the outskirts the city. We assumed all camp occupants were at risk for cholera and that, in the Juba community, only those residents without access to improved sanitation were at risk (*5*,*6*) (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-1592-Techapp1.pdf).

Two-dose vaccine coverage among those eligible for vaccination (based on age and pregnancy status) was 93% in Tongping PoC and 95% at UN House; the remaining Juba population was not vaccinated (*1*). In Malakal, we compared an informal unvaccinated IDP settlement, Wau Shilluk (population 39,000; online Technical Appendix), with an official PoC site, Malakal PoC camp (population 17,000; online Technical Appendix). Two-dose vaccine coverage in Malakal was 92.2% based on a coverage survey using systematic random sampling (*1*).

Table. Effect of oral cholera vaccine by location, South Sudan, 2014*

Variable	Location				
	Juba†	Tongping	UN House	Wau Shilluk†	Malakal
Setting type	Community	PoC Camp	PoC Camp	IDP camp	PoC camp
Population vaccinated	No	Yes	Yes	No	Yes
Population at risk	387,512	14,015	17,627	39,000	17,000
No. cases/10,000 persons	53.4	51.3	48.8	236.4	38.8
No. cases/10,000 children <5 y of age	56.0	186.5	146.5	–	–
Risk ratio, children <5 y compared with those ≥5 y of age	1.0	3.6	3.0	–	–
No. days with R_t >1	16‡	2‡	2‡	14‡	2‡
Maximum R_t	2.4	1.5	1.5	2.2	1.9

*IDP, internally displaced person; PoC, protection of civilian; R_t, reproductive number; UN, United Nations; –, no age-specific population data available.
†Reference population.
‡Significant difference ($p<0.0001$) in number of days with R_t >1, compared with reference population.

We estimated the time-varying reproductive number of cholera within each location (online Technical Appendix) (*7*). We assumed that the median generation time for cholera followed a gamma distribution with a median of 5 days and that all infectious cases were clinically apparent. We calculated 95% CIs by using a multiple imputation and bootstrapping routine, in which we first stochastically imputed missing or inconsistent symptom onset times and then resampled observations with replacement (online Technical Appendix).

The cholera attack rate in the Juba community was 53.4 cases/10,000 persons at risk (i.e., 2,229 cases/387,512 persons at risk), compared with 49.9 cases/10,000 persons at risk in the Juba camps (i.e., 158 cases/31,642 persons at risk). Although the overall attack rates were similar, the age distribution in camps differed markedly from those in the community. In the community, the risk for cholera among children <5 and those ≥5 years of age was nearly identical (risk ratio [RR] 1.0), but in the camps, the risk was substantially higher among children <5 years of age (Table; Figure 1). These age-specific differences in attack rates between camps and the community did not appear to be explained by population structure, age-specific vaccination coverage, or circulation of another diarrheal pathogen in the camps (online Technical Appendix); the differences point toward possible lower vaccine effectiveness among young children.

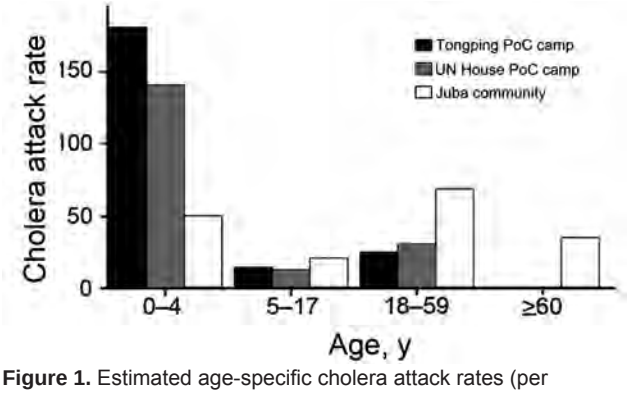

Figure 1. Estimated age-specific cholera attack rates (per 100,000 population) at different locations in Juba, South Sudan, 2014. PoC, protection of civilians; UN, United Nations.

The response mounted to oral vaccines is weaker in children than adults (*8*), although considerable uncertainty remains regarding the response to the oral cholera vaccine.

The difference in the estimated cumulative cholera attack rates between the unvaccinated Wau Shilluk IDP camp (236.4 cases/10,000 persons at risk) and the vaccinated Malakal PoC camp (38.8 cases/10,000 persons at risk) was even more striking (incidence rate ratio 6.1) (Table). Age-specific population figures were unavailable for Wau Shilluk.

Although differences in attack rates suggest a likely reduction in cholera risk in vaccinated areas and the possibility of age-dependent vaccine protection, these estimates are uncertain and should be cautiously interpreted. An alternative approach to understanding the effect of vaccination is to compare observed cholera transmission dynamics within vaccinated and unvaccinated populations.

The epidemic curves within vaccinated camps in Juba had no distinct peak and suggest a series of cholera introductions with little to no onward transmission (Figure 2). We estimated that the daily reproductive number (R_t; i.e., average number of secondary cases from a case becoming symptomatic on day *t*; online Technical Appendix) in vaccinated camps was ≤1 for most of the epidemic. Each vaccinated camp had only 2 days on which the 95% CI of R_t was above unity. This finding contrasts with our estimates in unvaccinated areas, where despite conditions that may have been less suitable for transmission, R_t remained >1 for a sufficient and significantly longer time for an epidemic to progress ($p<0.0001$; Table; online Technical Appendix).

Conclusions

We show that cholera vaccination campaigns likely played a key role in curtailing cholera transmission in vaccinated areas within South Sudan. The age-specific transmission patterns within the vaccinated camps in Juba suggest that vaccinated young children were less protected in the camps, although further investigation is needed to explore this and other possible explanations, including age-specific differences in care-seeking behavior between populations.

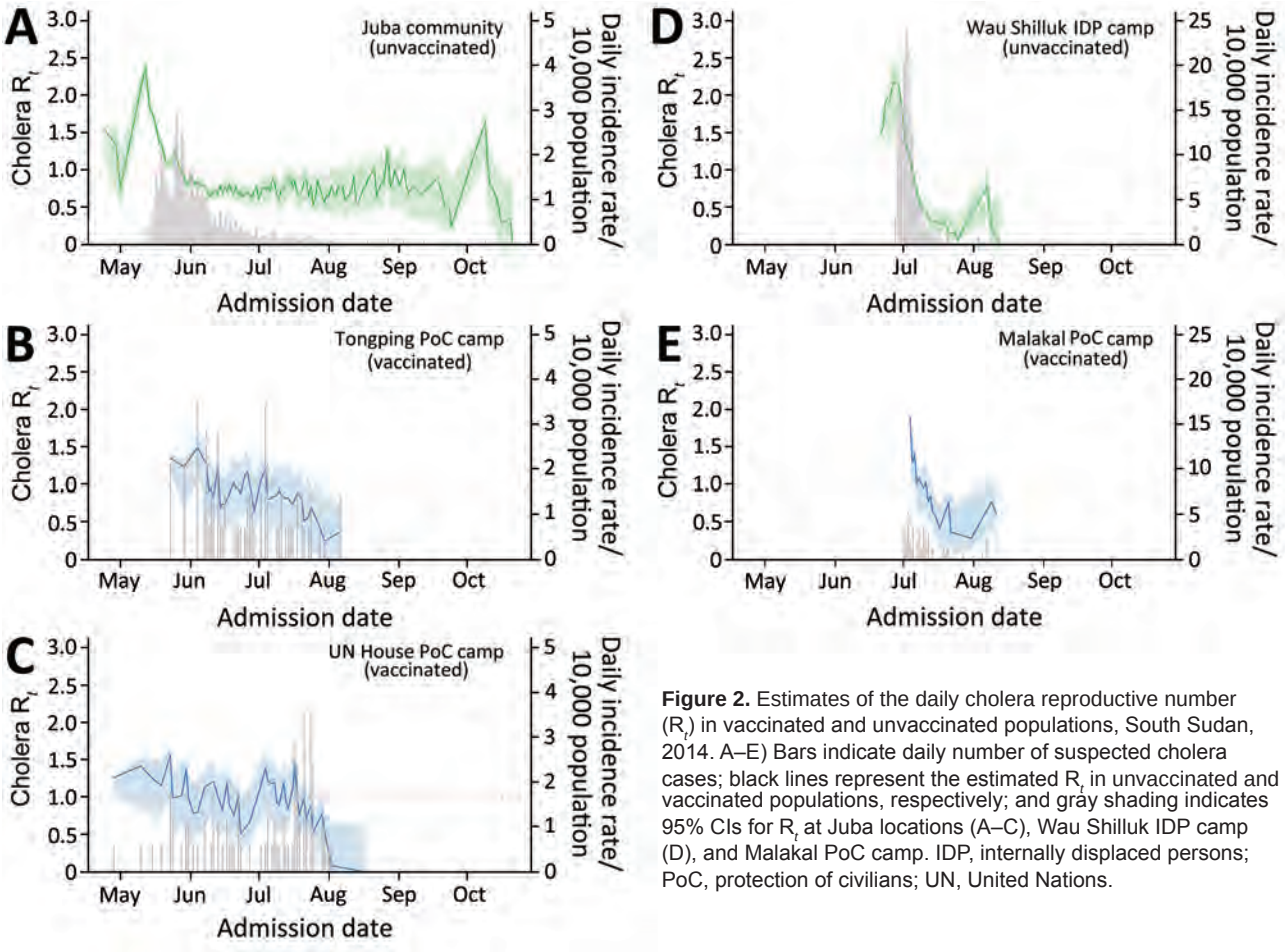

Figure 2. Estimates of the daily cholera reproductive number (R_t) in vaccinated and unvaccinated populations, South Sudan, 2014. A–E) Bars indicate daily number of suspected cholera cases; black lines represent the estimated R_t in unvaccinated and vaccinated populations, respectively; and gray shading indicates 95% CIs for R_t at Juba locations (A–C), Wau Shilluk IDP camp (D), and Malakal PoC camp. IDP, internally displaced persons; PoC, protection of civilians; UN, United Nations.

Our study had several limitations. Analyses were based on suspected cases, which were defined by using a sensitive, but less specific, case definition; thus, many included cases were likely to be false positives. Our estimates of cholera attack rates depended on estimates of the population at risk in each area. We used the most reliable and up-to-date sources from agencies with an operational presence on the ground; however, the sizes of the dynamic community and camp populations used in the analyses were uncertain, and this uncertainty was not accounted for in the models. Last, we estimated the time-varying reproductive number of cholera by assuming a fixed generation time throughout the epidemic, which may not reflect reality due to the possibility of differences in care-seeking behavior and differential contraction of generation intervals between populations with an increasing prevalence of cholera (*9*).

Our findings provide evidence of the population-level effects of oral cholera vaccine. More work is needed to quantify this effect across multiple settings in reactive and preemptive deployments of the vaccine. High-quality surveillance and capacity to confirm suspect cases can greatly improve the possibility of making future estimates.

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Experimental Infection and Response to Rechallenge of Alpacas with Middle East Respiratory Syndrome Coronavirus

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We conducted a challenge/rechallenge trial in which 3 alpacas were infected with Middle East respiratory syndrome coronavirus. The alpacas shed virus at challenge but were refractory to further shedding at rechallenge on day 21. The trial indicates that alpacas may be suitable models for infection and shedding dynamics of this virus.

Middle East respiratory syndrome coronavirus (MERS-CoV) was first reported in September 2012 (*1*); since then, >1,600 confirmed cases have been reported to the World Health Organization (http://www.who.int/csr/don/29-february-2016-mers-saudi-arabia/en). The role of domestic animals as an intermediate host for humans was initially suggested by case histories of infected patients who had visited farms or tended sick animals shortly before onset of infection (*2*). This suggestion was given credence by a study of camel serum samples that showed high levels of neutralizing antibodies in disparate camel populations (*3*); the findings were subsequently confirmed by virus detection and sequencing (*4*).

Infection trials in camels have been limited (*5*,*6*), mainly because of difficulties in housing and handling the animals in a high-containment facility, which is necessary because the virus has a Biosafety Level 3 classification (*7*). However, the alpaca, a close relative within the *Camelidae* family, may provide a temperamentally suitable and valuable animal model for MERS-CoV infection, particularly for developing and testing vaccine candidates for camels. We sought to assess whether alpacas could be infected by means of a natural (oronasal) route, to determine whether viral shedding occurred after reinfection, and to evaluate the development of serologic markers of protection.

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The Study

We obtained 3 adult female alpacas (*Vicugna pacos*) from a commercial supplier in Victoria, Australia, and housed them in the Biosafety Level 3 containment facility at the CSIRO Australian Animal Health Laboratory. Before experiments, the alpacas were allowed to acclimatize for 6 days; during this time, intrauterine temperature data loggers were implanted according to a previously published procedure (*8*). We found no previous MERS-CoV challenge trial reported in alpacas, so we chose a preliminary dose and rechallenge time on the basis of our experience with other virus infection trials for other emerging infectious diseases (*8*).

We used a camel isolate of MERS-CoV (Dromedary_MERS-CoV_Al-Hasa_KFU-HKU13/2013; GenBank accession nos. KJ650295–KJ650297) for infection; the isolate was prepared in Vero cells as described (*9*). The 3 alpacas were exposed oronasally to a 10^6 50% tissue culture infective dose of MERS-CoV in 5 mL of phosphate-buffered saline. The animals were monitored for 21 days, reexposed to a replicate challenge of MERS-CoV, and observed for 14 more days. Clinical samples of blood (in EDTA for obtaining serum) and swabs (deep and superficial nasal, oral, rectal, and urogenital) were collected immediately before inoculation and thereafter on days 3, 5, 7, 10, 12, 14, 21, 26, 28, 31, 33, and 35. Alpacas were electively euthanized, 1 on day 33 and the others on day 35.

The animals remained clinically healthy except for a reduced condition score that occurred by day 18 in 1 animal (alpaca 2); no signs of upper or lower respiratory tract disease appeared in any animal. Increased temperature was noted in alpaca 2 during days 17–20, but fever (rectal temperature >39°C) was not recorded. Gross abnormalities at postmortem examination were found only in alpaca 2 and comprised extensive adhesions of the caudal sac of compartment 1 of the stomach to the umbilicus; clinical findings in this animal were attributed to this lesion.

RNA extraction and real-time PCR were performed by following specimen-handling procedures established for Hendra virus (*8*) and were used to identify shedding patterns after each challenge. After initial challenge,

viral RNA was detected in each animal from oral and deep and superficial nasal swab samples taken on days 3–12 (Table 1).

Virus isolation was undertaken with Vero cells by using published protocols (*9*) and was successful for all 3 animals from all types of samples. Virus recovery was successful from oral and superficial nasal swab samples through day 12; deep nasal swab samples were positive only through day 10. All urogenital and rectal swab samples were negative by both real-time PCR and virus isolation. After rechallenge, viral RNA was not detected with confidence from any sample (Figure).

Serum samples were assessed for immunologic responses by using a virus neutralization test (VNT) and a Luminex bead assay to the nucleocapsid protein. We used in-house assays modeled after those previously developed to assess the serologic status of feral camels in central Australia (*10*). All animals were seronegative by both Luminex and VNT before challenge. Antibody was first detected by Luminex on day 10 or day 12 in each animal (Table 2); neutralizing antibody titers were 1:20 to 1:40 in alpaca 2 from day 10. Neutralizing antibody titers of 1:10 to 1:20 were detected in alpaca 1 from day 21 on but not in alpaca 3 at any time during the study. For controls, we used MERS-CoV positive and negative serum samples from Egypt and Australia (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/6/16-0007-Techapp1.pdf).

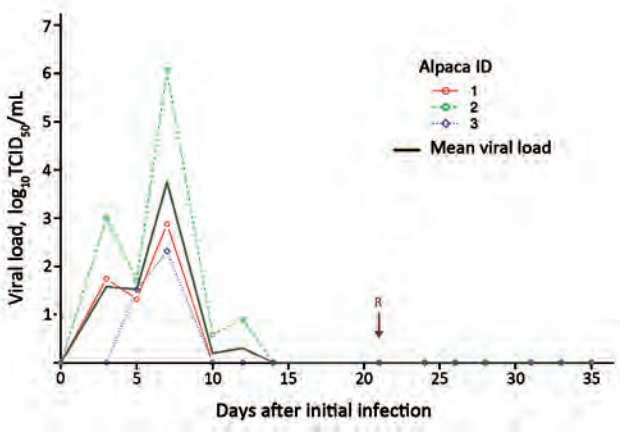

Figure. Virus shedding of MERS-CoV from 3 infected alpacas as detected from the deep nasal swab samples by day after initial infection and reinfection. Viral load was estimated from real-time cycle threshold values and a calibration experiment. Arrow indicates day 21, when the animals were reinfected with MERS-CoV. MERS-COV, Middle East respiratory syndrome coronavirus; TCID, tissue culture infective dose.

Conclusions

Our study confirms that alpacas are susceptible to MERS-CoV infection; this finding is consistent with a previous report showing that alpaca kidney cell lines possessing the dipeptidyl peptidase-4 receptor could be infected in vitro (*11*). Our challenge/rechallenge trial was planned as a first stage in the assessment of the alpaca as a potential surrogate for camels for MERS-CoV vaccine testing. Consequently, the trial was not designed for direct comparison with 2 previous MERS-CoV challenge trials reported in camels (*5*,*6*). Our trial used a lower challenge dose and a different timeframe for observation; nevertheless, some preliminary comparative observations may be useful. In the previous studies, as in ours, the animals were inoculated

Table 1. Virus shedding in 3 alpacas infected with MERS-Cov, as measured by virus isolation and real-time PCR for each sample day*

	Cycle threshold value (virus isolation result)†‡									No. positive/no. tested	
	Deep nasal swab sample			Oral swab sample			Superficial nasal swab sample				
Dpi	Alpaca 1	Alpaca 2	Alpaca 3	Alpaca 1	Alpaca 2	Alpaca 3	Alpaca 1	Alpaca 2	Alpaca 3	Real-time PCR	Virus isolation
0	U (–)	U (–)	U (–)	U (–)	40.8 (–)	U (–)	U (–)	U (–)	U (–)	0/3	0/3
3	**33.4 (+)**	29.0 (–)	U (–)	**34.2** (–)	**31.7** (–)	42.3 (–)	**35.4** (–)	40.7 (–)	U (–)	2/3	1/3
5	34.9 (–)	**33.5** (–)	**34.2** (–)	**32.0** (–)	**35.4** (–)	**32.0 (+)**	**35.0** (–)	**33.0** (–)	**32.5** (–)	3/3	1/3
7	**29.4 (+)**	**18.2** (–)	**31.4 (+)**	**32.7** (–)	**30.1 (+)**	**28.3 (+)**	**31.9** (–)	**28.5 (+)**	**38.6 (+)**	3/3	3/3
10	41.0 (–)	**37.5 (+)**	U (–)	41.3 (–)	**38.0** (–)	**30.5 (+)**	**39.9** (–)	36.0 **(+)**	U (–)	3/3	2/3
12	42.0 (–)	**36.4** (–)	U (–)	U (–)	U (–)	**37.3 (+)**	42.0 (–)	**39.5 (+)**	U (–)	2/3	2/3
14	U (–)	42.2 (–)	U (–)	43.0 (–)	44.0 (–)	43.0 (–)	U (–)	U (–)	U (–)	0/3	0/3
21	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	0/3	0/3
24	U (–)	U (–)	U (–)	40.8 (–)	U (–)	U (–)	U (–)	43.2 (–)	U (–)	0/3	0/3
26	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	0/3	0/3
28	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	0/3	0/3
31	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	0/3	0/3
33	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	U (–)	43.1 (–)	U (–)	0/3	0/3
35§	U (–)	NA	U (–)	U (–)	NA	U (–)	U (–)	NA	U (–)	0/2	0/2

*Bold indicates positive results (cycle threshold ≤40). Gray shading indicates that ≥1 animal was positive for the sample collected on that day. Dpi, day postinfection for challenge (initial infection); MERS-CoV, Middle East respiratory syndrome coronavirus; NA, not available; U, undetermined; –, negative; +, positive.
†Real-time PCR cycle threshold values are an average of the duplicates, except when 1 result was undetermined; then, only the single numeric value is shown.
‡The starting dilution was 1:10; the threshold for a positive result was a dilution of ≥1:20.
§Alpaca 2 was euthanized at day 33, leaving only 2 animals in the study at day 35.

Table 2. Serologic responses in 3 infected alpacas, as measured by virus neutralization tests and Luminex bead assays for selected sample days*

	Serologic test results							
	VNT titer†			Luminex assay MFI‡			No. positive/no. tested)§	
Dpi	Alpaca 1	Alpaca 2	Alpaca 3	Alpaca 1	Alpaca 2	Alpaca 3	VNT	Luminex
0	Negative	Negative	Negative	787	889	167	0/3	0/3
3	Negative	Negative	Negative	373	814	152	0/3	0/3
5	Negative	Negative	Negative	418	945	223	0/3	0/3
7	Negative	Negative	Negative	272	932	249	0/3	0/3
10	Negative	**1:40**	Negative	478	**2,869**	58	1/3	1/3
12	Negative	**1:40**	Negative	928	**10,274**	331	1/3	1/3
14	Negative	**1:40**	Negative	1,041	**7,658**	899	1/3	1/3
21	1:10	**1:40**	Negative	877	**6,893**	629	1/3	1/3
24	**1:20**	**1:20**	Negative	1,506	**3,324**	678	2/3	1/3
26	**1:20**	**1:20**	Negative	853	**4,161**	667	2/3	1/3
28	**1:20**	**1:20**	Negative	773	**4,682**	724	2/3	1/3
31	**1:20**	**1:40**	Negative	548	**11,259**	649	2/3	1/3
33	1:10	**1:20**	Negative	688	**6,090**	455	1/3	1/3
35§	1:10	NA	Negative	510	NA	586	0/2	0/2

*Bold indicates positive results. Gray shading indicates that ≥1 animal was positive for the sample collected on that day. Dpi, day postinfection for challenge (initial infection); MFI, median fluorescent intensity; VNT, virus neutralization test.
†The starting dilution was 1:10, and the threshold for a positive result was a dilution of ≥1:20.
‡The MFI threshold for a positive result for the Luminex assay was 2,500.
§Alpaca 2 was euthanized at day 33, leaving only 2 animals in the study at day 35.

by the oronasal route, and live virus was detected through day 7 postinfection. Similarly, neutralizing antibodies were detected beginning 7–8 days postinfection. However, findings in the trials with camels differed considerably from findings in our trial. The trials with camels detected live virus from nasal washes at days 1–3, a nasal discharge, and transient temperature rises; viral RNA was detected by real-time PCR for an extended period. Furthermore, the VNT titers for camels were much higher than those for the alpacas in our study. These differences possibly represent underlying dissimilarities in immune responses to MERS-CoV for the 2 species but may also result from the higher infecting dose (10^7 50% tissue culture infective dose) used in the camel studies.

Our study showed that alpacas secreted live virus after oronasal infection and that the immune response to the initial infection prevented further excretion following reinfection. An underlying assumption in our trial is that the initial infection equates to natural vaccination and that the lack of viral excretion thus follows an induced immune memory response. However, our results indicate that this immunologic response is complex; although a strong serologic response developed in only 1 alpaca, all 3 alpacas were refractory to reinfection.

This study has several limitations. First, it was a preliminary study with only 3 animals and functioned more as proof of concept than a definitive study of the use of alpacas as a model for studying infection dynamics of MERS-CoV in camelids. Second, our observation period of 21 days before rechallenge is informative but does not provide complete information on duration of protective immunity. Future studies should have a larger sample and a longer period of study postinoculation. Third, our study did not seek to understand the pathogenesis of infection; we did not conduct histopathology or immunohistochemistry to understand the site of initial viral replication and the role of mucosal immunity in mounting an effective immune response upon infection.

Notwithstanding these limitations, we believe that the alpaca might be a useful model that could greatly facilitate the development and testing of vaccine candidates. We recommend further research and trials to substantiate this potential.

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Mr. Crameri is a virologist with the CSIRO Australian Animal Health Laboratory. He has pioneered work in the high biocontainment facility at CSIRO and worked on Hendra, Nipah, SARS, MERS, Ebola, and many other viruses with high impact to human and animal health.

Note Added in Proof: Adney et al. also report infection, replication, and transmission of Middle East respiratory syndrome coronavirus in alpacas in this issue of Emerging Infectious Diseases (*12*).

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Scarlet Fever Upsurge in England and Molecular-Genetic Analysis in North-West London, 2014

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Scarlet fever notifications surged across the United Kingdom in spring 2014. Molecular epidemiologic investigation of *Streptococcus pyogenes* infections in North-West London highlighted increased *emm*4 and *emm*3 infections coincident with the upsurge. Unlike outbreaks in other countries, antimicrobial resistance was uncommon, highlighting an urgent need to better understand the drivers of scarlet fever activity.

An unprecedented rise in scarlet fever occurred in England in spring 2014, with >13,000 notifications, for an overall population rate of 24.5/100,000 persons (*1*,*2*). We analyzed clinical notification data for North-West London (population ≈1,900,400) during 2009–2014 and determined *emm* genotypes of *Streptococcus pyogenes* causing upper respiratory tract (URT) infections during 2009–2014. We focused on peak periods of scarlet fever notification.

The Study

During weeks 10–20 (March–May) 2014, scarlet fever notifications in North-West London increased 3–8-fold compared with the same period in previous years (Figure, panel A). Although Health Protection regulations in England require clinicians to report suspected cases of scarlet fever, molecular surveillance of noninvasive *S. pyogenes* is not feasible because testing for *S. pyogenes* is not routinely advised for patients with a sore throat in the United Kingdom (*3*). Nonetheless, a limited number of URT swab specimens are submitted by clinicians for culture. Since 2009, we have stored all *S. pyogenes* URT isolates identified in our West London diagnostic laboratory, which serves a population of ≈2 million, overlapping with the North-West London region.

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Molecular testing, with standard DNA extraction, *emm* typing, and superantigen typing methods (*4*), was performed on all 404 viable *S. pyogenes* URT isolates identified. Of all isolates obtained from March through May 2009–2013 (n = 72), predominant *emm* genotypes were *emm*12 (26%), *emm*89 (13%), and *emm*1 (14%) (Figure, panel B). These proportions were almost identical to proportions for all 308 isolates obtained throughout 2009–2013 (Figure, panel B). In contrast, during March–May 2014 (n = 96), dominating *emm* types changed, with a borderline significant increase in *emm*3 (from 10% in March–May 2009–2013 to 20% in 2014; $\chi^2_{(1df)} = 3.766$, p = 0.0523), and a significant >5-fold increase in *emm*4 (from 3% to 17%; $\chi^2_{(1df)} = 9.478$, p = 0.0021). Among 96 URT samples submitted in March–May 2014, a total of 42 were from children ages ≤5 years. *Emm*4 was significantly associated with age ≤5 years ($\chi^2_{(1df)} = 6.046$, p = 0.0139), and the rise was largely attributable to disease in this age group (12/17 *emm*4 isolates).

Isolates from March–May 2014 were categorized by at least one of the following clinical features (provided by submitting physician): 1) tonsillitis, pharyngitis, or sore throat and no mention of scarlet fever (n = 44); 2) any mention of scarlet fever, regardless of other information (n = 16); 3) any other illness (n = 6); and 4) no details provided (n = 30). The 16 scarlet fever–associated isolates were limited to patients ages 1.25–11 years, a significant proportion of whom were ≤5 years (12/16; $\chi^2_{(1df)} = 7.619$, p = 0.0058); 7/16 were *emm*3 and 3/16 were *emm*4. The remainder were *emm*12 (3), *emm*28 (2), and *emm*87 (1). On the basis of these limited data, *emm*3 was significantly associated with scarlet fever in 2014 ($\chi^2_{(1df)} = 5.964$, p = 0.0146).

Clinical data were not collected in earlier years routinely, although in 2009 a total of 3/3 isolates from scarlet fever case-patients were *emm*3. Scarlet fever–associated *emm*4 strains from 2014 (n = 3) carried superantigens *speC*, *ssa*, and *smeZ*; the same superantigen profile was found in *emm*4 strains from patients for whom scarlet fever was not mentioned (n = 14). All 7 scarlet fever–associated *emm*3 strains carried *speA*, *ssa*, *speG*, and a known mutation in *smeZ*.

Antimicrobial drug resistance was identified in 10/96 URT isolates from 2014; however, none of these isolates were associated with scarlet fever and none were *emm*4.

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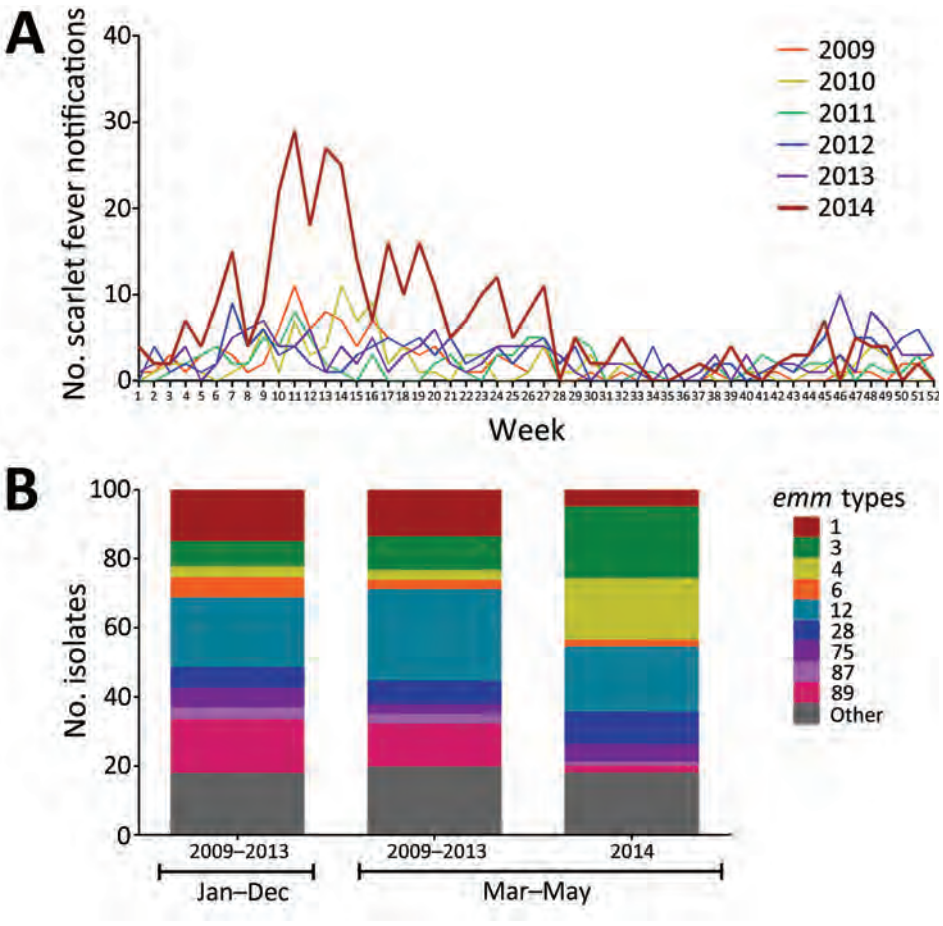

Figure. Increase in North-West London scarlet fever notifications and association with *emm*4 and *emm*3, 2014. A) Weekly scarlet fever notifications in North-West London during 2009–2014. During weeks 10–20 (March–May) 2014, the number of notifications substantially increased. B) *emm* genotyping of 404 upper respiratory tract *Streptococcus pyogenes* isolates. Isolates were available from March 2009 through May 2014, inclusive. A total of 308 isolates were from 2009–2013; however, of these, 134 were from 2009, and 174 were from 2010–2013, reflecting a fall in submission rates and affecting the availability of strains for study. Thus, isolates from 2009–2013 were considered a single group (January–December) and the following *emm*-types were identified: *emm*12 20% (62/308), *emm*89 16% (48/308), *emm*1 15% (47/308), *emm*3 7% (22/308), *emm*28 6% (19/308), *emm*4 3% (10/308). A similar pattern was observed during March–May in 2009–2013 (n = 72). A total of 96 isolates were available from 2014, all of which were from March–May, submitted following alerts to clinicians regarding scarlet fever activity. During March–May 2014, isolates typed as *emm*4 increased significantly, from 3% (2/72) to 17% (17/96), ($\chi^2_{(1df)}$ = 9.478. p = 0.0021). A borderline significant increase occurred in *emm*3, from 10% (7/72) in 2009–2013 to 20% (20/96) in 2014 ($\chi^2_{(1df)}$ = 3.766, p = 0.0523). This constituted a 3-fold increase in *emm*3 and *emm*4 combined, from 13% in 2009–2013 to 37% in 2014 (March–May).

Erythromycin resistance was found in 2/20 non–scarlet fever *emm*3 isolates, in combination with clindamycin resistance in 1 isolate.

Conclusions

An increase in *emm*3 and *emm*4 *S. pyogenes* URT isolates was detected in North-West London, during the period in 2014 when scarlet fever notifications peaked. The increase in *emm*4 infections was also found predominantly in 4- to 5-year-old children, the group we and others found to be most at risk for scarlet fever (*1*). The percentage of children 4 years old in North-West London (an urban population) is similar to the national average of 1.3%; therefore, our findings are probably relevant to the rest of the United Kingdom.

*Emm*4 isolates accounted for only 3/16 cases in which scarlet fever was mentioned, although, because of the study's retrospective nature and paucity of clinical data supplied, we cannot dismiss the possibility that other *emm*4 isolates were also associated with scarlet fever. On the basis of the limited analysis of isolates from infections in which scarlet fever was mentioned, we found an association between scarlet fever and *S. pyogenes emm*3 strains.

The results of our historical comparison must be interpreted with caution; obtaining swab samples from patients with URT infections in England is not routine. Thus, the 2009–2013 samples may reflect persistent infections, in contrast to 2014 samples, when clinicians were encouraged to submit swab specimens for scarlet fever case-patients. Furthermore, the number of strains available for *emm* typing was limited. Nonetheless, this was the only collection of strains available to us that permitted historical comparison.

Both *emm*3 and *emm*4 *S. pyogenes* strains have been associated with scarlet fever (*5*). In the Far East, *emm*1 and *emm*4 isolates were the leading causes of scarlet fever in the late 1990s (*6*), although more recently, antimicrobial

drug–resistant *emm*12 *S. pyogenes* has dominated in this region (*7*–*9*). We found that the proportion of *emm*12 isolates fell during the scarlet fever surge and found no antimicrobial drug resistance among *emm*3 or *emm*4 isolates associated with scarlet fever.

*Emm*4 isolates are associated with pharyngitis in children (*10*,*11*); these isolates are entirely acapsular, a phenotype linked to enhanced adhesion to surfaces (*12*). Whether this characteristic can increase persistence and transmission is unknown. Surges in scarlet fever are believed to require a population susceptible to pharyngeal infection with specific strain types and specific superantigens. Both *emm*3 and *emm*4 strains in our study possessed 2 prophage-associated superantigens, either SPEA and SSA, or SPEC and SSA. Although these toxin genes were found in *emm*3 and *emm*4 strains not associated with scarlet fever, the probability of triggering scarlet fever may be enhanced through production of 2 such superantigens. An association between these superantigens and scarlet fever has been reported (*13*).

Periodic increases in scarlet fever are well recognized, although the magnitude of the upsurge in the United Kingdom was unexpected. Consultation rates for sore throat diminished in the 1990s (*14*), and the 2008 UK national guidelines advise against diagnostic testing and recommend a policy of nonprescribing or delayed prescribing for sore throat when the Centor score is <3 (*3*). These recommendations contrast with those of North America and of some European countries (*15*). Whether exceeding a threshold level of community *S. pyogenes* transmission is required for such a marked upsurge is unclear; increased scarlet fever activity was not reported elsewhere in Europe, to our knowledge. Apart from natural fluctuations in population immunity, emergence of hypertransmissible lineages, acquisition of novel phage-encoded toxins, or antimicrobial drug resistance may contribute to scarlet fever surges (*6*,*7*). Notably, isolates we found associated with scarlet fever were not resistant to common antimicrobial agents.

As part of the national response, clinicians were advised to treat scarlet fever to minimize complications and reduce transmission. Whether use of more refined molecular diagnostics could assist future community prevention and management of *S. pyogenes* infection will require careful evaluation. Increased scarlet fever activity has continued in England in 2015 and 2016, underscoring the need for ongoing surveillance and further investigation.

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Possible Case of Novel Spotted Fever Group Rickettsiosis in Traveler Returning to Japan from India

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A 60-year-old woman experienced fever, headache, rash, and altered vision after returning to Japan from India. Testing detected elevated antibody titers to spotted fever group rickettsia; PCR on blood yielded positive results for the rickettsial outer membrane protein A gene. We isolated a unique rickettsial agent and performed a full-genome analysis.

Various types of spotted fever group (SFG) rickettsioses have been reported worldwide (*1*). Common symptoms include fever, headache, intense myalgia, and skin rash (*2*). Even though cases of spotted fever have occurred in Japan, physicians often face difficulty diagnosing the disease in febrile patients because of the unusual or unfamiliar symptoms associated with SFG rickettsia.

In January 2011, a 60-year-old woman from Japan who was undergoing treatment for diabetes mellitus stayed in a suburban area of Bangalore in South India for 1 week. She went camping and did other outdoor activities. She recalled that there were many opportunities to be bitten by insects; however, she could not specify the types of insects to which she may have been exposed. On her first day back in Japan (day 1), she experienced general malaise and loss of appetite. A fever (38°C) and skin rash appeared on day 7. She consulted a local clinic and underwent evaluation. Blood test results indicated thrombocytopenia (91×10^9 thrombocytes/L), liver dysfunction (elevated aspartate aminotransferase [92 U/L] and alanine transaminase [97 U/L]), and elevated C-reactive protein levels (206 mg/L). Splenomegaly was evident on abdominal sonography. On day 12, the woman was admitted to Miyazaki University Hospital (Miyazaki, Japan) with a high fever (39.4°C), headache, altered vision with eye floaters, and rash (Figure 1, panel A). Proteinuria, glycosuria, thrombocytopenia (92×10^9 thrombocytes/L), liver dysfunction (elevated aspartate aminotransferase [95 U/L] and alanine aminotransferase [93 U/L]), and hyponatremia (sodium 127 mmol/L) were observed, and levels of serum procalcitonin (1.9 ng/mL) and C-reactive protein (198 mg/L) were elevated. Her diabetes was poorly controlled (glucose 40.5 mmol/L).

A skin biopsy of the rash was performed on day 13; infiltration of inflammatory cells (mainly lymphocytes) around the capillaries, associated with hemorrhagic changes, was observed (Figure 1, panel B). No eschars were detected. Given the patient's travel history to India and her signs and symptoms, we considered acute infectious diseases such as dengue fever, chikungunya fever, and typhoid fever. Ceftriaxone (2 g/d) and levofloxacin (500 mg/d) were administered. Subsequently, the fever subsided, and platelet count, liver function, and C-reactive protein level returned to normal. However, laboratory findings ruled out the principal tropical infectious diseases. Blood smear specimens tested negative for malaria parasites. Blood culture tested negative for bacteria, thus ruling out typhoid fever. Dengue and chikungunya virus infections were ruled out on the basis of serologic tests and antigen detection. Although the rash, bilateral lower limb edema, and visual alterations with eye floaters persisted, the patient was discharged on day 27.

A serum sample revealed apparent positivity to *Rickettsia conorii* Malish 7 and *R. japonica* YH on day 19 (Table). Therefore, SFG rickettsia was strongly suspected. By day 40, the patient's rash and edema persisted, and her visual alterations had become more severe. Because persistent SFG rickettsiosis was suspected, additional oral treatment with minocycline (100 mg/d) was administered for 14 days. By day 48, the remaining symptoms resolved.

PCR test results for the acute-phase whole blood sample collected on day 13 were positive for the outer membrane protein A (*ompA*) gene for SFG rickettsia (*3*,*4*). Direct nucleotide sequencing of PCR products (GenBank accession no. LC089865) yielded a profile different from any known *Rickettsia* spp. but identical to that of *Rickettsia* sp. CMCMICRO 1–4 (GenBank accession nos. HM587248.1–HM587251.1) (*5*). We successfully isolated an SFG rickettsial agent, designated as strain Tenjiku01,

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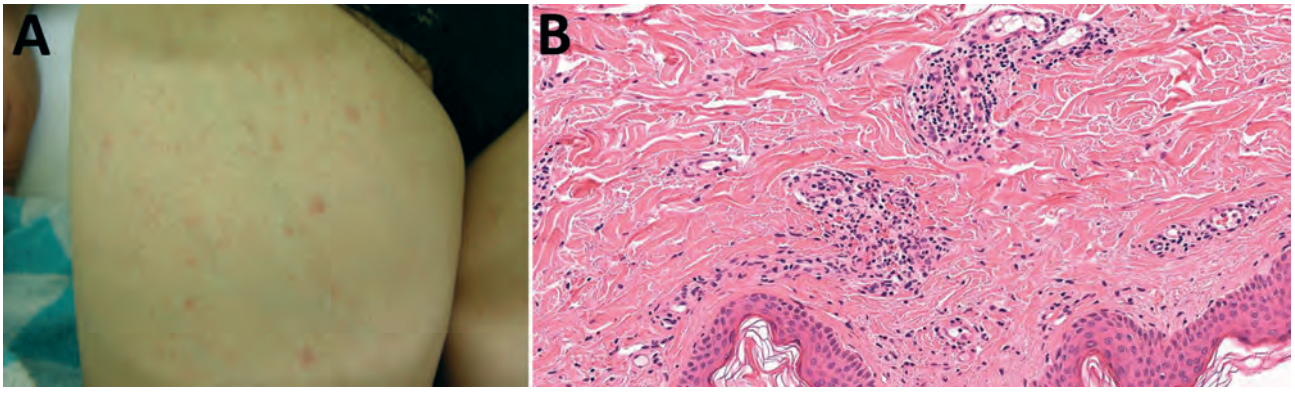

Figure 1. Rash and skin biopsy of a 60-year-old female traveler who had returned to Japan from India, January 2011. A) Rash on the inner side of the right thigh. B) Skin biopsy showing infiltration of inflammatory cells (mainly lymphocytes) around the capillaries, associated with hemorrhagic changes (hematoxylin-eosin staining, original magnification ×400).

from acute-phase whole blood (collected on day 13) using the shell vial method with L929 cells. The *ompA* sequence of the isolate was identical to that of the clinical sample (online Technical Appendix 1 Figure, http://wwwnc.cdc.gov/EID/article/20/6/15-1985-Techapp1.pdf). In addition to partial sequencing of PCR products (GenBank accession nos.: *16S rRNA,* LC089861; *17K-Da*, LC089862; *geneD*, LC089863; *gltA*, LC089864), we performed whole-genome analysis. The partial sequence of *ompA* from our PCR products was 100% similar to the CMCMICRO *ompA* sequence (*5*) and 98.4% similar to the *Candidatus* R. kellyi *ompA* sequence (*6*). Other genes with high sequence homology were as follows: *gltA* with uncultured *Rickettsia* sp. LIC4275 (99.7% homology, accession no. KT153042); *rrs* with *R. slovaca* 13-B (99.7%, NR_074462); *sca4* (geneD) with *R. slovaca* 13-B (98.4%, CP002428); and 17K-Da gene with *R. honei* RB (99.5%, AF060704). The draft genome sequence (≈1.3 Mb, 32 contigs) was obtained with next-generation sequencing (accession nos. BCMR01000001–BCMR01000032). The outer membrane protein B sequence, which was extracted from the contig by next-generation sequencing, was 96.98% similar to that of *R. slovaca* D-CWPP (accession no. CP003375). Results of pan-genome analysis suggested that 586 core genes were shared among 34 *Rickettsia* spp. genomes, and the gene components of Tenjiku01 were highly similar to those of the SFG group (data not shown). A maximum-likelihood phylogenetic tree of concatenated amino acid sequence alignments of the core genes, constructed by using RAxML software version 8.2.0 (http://sco.h-its.org/exelixis/web/software/raxml), indicated that Tenjiku01 belongs to the SFG group and is closely related to *R. honei* RB (Figure 2). Moreover, blastp matrix analysis (http://blast.ncbi.nlm.nih.gov) of 73 *Rickettsia* spp. indicated that 315 core genes of Tenjiku01 showed 98.21%–98.95% homology to those of *R. japonica*, *R. argasii*, *R. heilongjiangensis*, *R. honei*, *R. parkeri*, *R. sibirica*, *R. africae*, and *R. conorii* (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/22/6/15-1985-Techapp2.xlsx).

At first, this patient's infection partially responded to levofloxacin and ceftriaxone therapy. The efficacy of levofloxacin in treating *Rickettsia* spp. has been described previously (*7*). Therefore, levofloxacin, not ceftriaxone, probably was effective in this patient. After detection of SFG rickettsia antibodies, we performed PCR analysis, which resulted in the final diagnosis; the patient's remaining symptoms were then successfully treated with minocycline.

Recently, many types of SFG rickettsia, except *R. japonica*, have occurred in Japan (*4*,*8*). An even greater variety of rickettsioses has been reported worldwide (*1*,*9*,*10*), and the incidence of imported rickettsioses has increased in Japan (*11*–*13*).

In our patient, antibody titers to *R. conorii* and *R. japonica* were elevated on day 19, and the titers to *R. conorii* were higher than those against *R. japonica*. Therefore, we

Table. IgG titers to rickettsiae detected by indirect immunofluorescence assay conducted on a serum sample from a 60-year-old female traveler who had returned to Japan from India, January 2011*

Species	Day 13	Day 19	Day 40
Rickettsia japonica (YH)	–	1:640	1:640
R. conorii (Malish7)	–	1:2,560	1:5,120
R. typhi (Wilmington)	–	1:40	1:160
R. prowazekii (Brainl)	–	1:40	1:80
Orientia tsutsugamushi (Karp)	–	–	–
O. tsutsugamushi (Gilliam)	–	–	–

*–, negative.

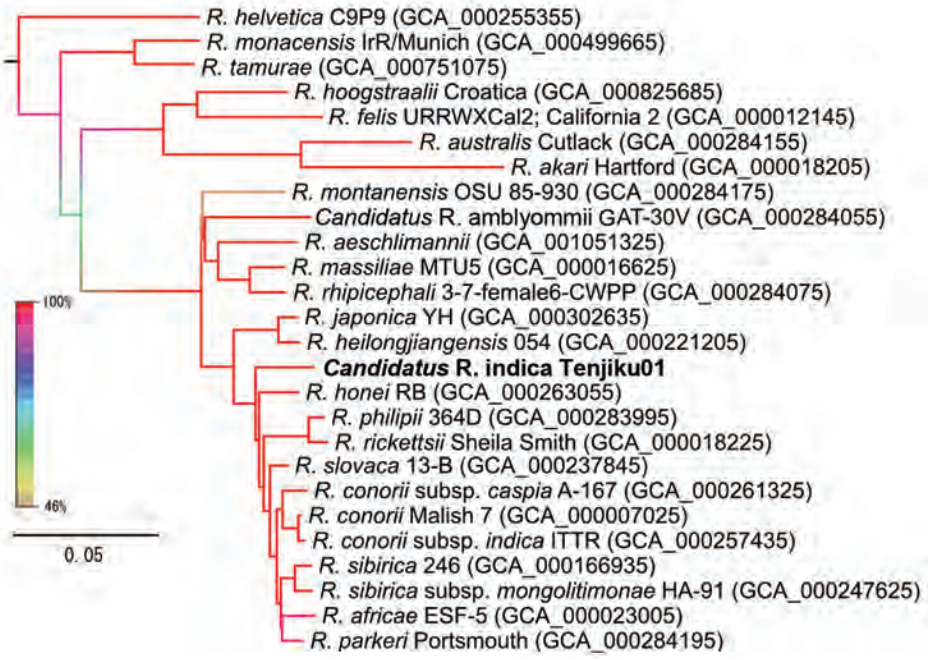

Figure 2. Maximum-likelihood phylogenetic tree of concatenated core genes in 26 *Rickettsia* spp. strains constructed by using RAxML software version 8.2.0 (http://sco.h-its.org/exelixis/web/software/raxml) with 1,000-fold bootstrapping. Boldface indicates isolate from this study. The color of each branch represents the bootstrapping value. GenBank assembly accession numbers are given in parentheses. Scale bar indicates amino acid changes per position.

suspected that the causative pathogen was closely related to *R. conorii*.

The *ompA* PCR products amplified from clinical samples were identical to the sequences of *Rickettsia* sp. CMCMICRO registered in India. Prakash et al. tested skin biopsy samples for SFG rickettsial genes and concluded that novel species of SFG rickettsia (CMCMICRO1–8) were in their area (*5*). However, isolation of SFG rickettsia has not been reported thus far. Moreover, data on other genes, such as *17KDa*, *gltA*, and *gene D*, are lacking (*5*). Little sequence homology was observed between the *ompA* sequence in our case and that of *Candidatus* R. kellyi (*6*), which is considered to be the most closely related to *Rickettsia* sp. CMCMICRO, according to Fournier's criteria (*14*). In our case, we successfully isolated SFG rickettsia, *Rickettsia* sp. strain Tenjiku01, from the clinical sample. Comparative genomics suggested that Tenjiku01 could be a novel species because the phylogenetic distance between Tenjiku01 and *R. honei* RB was longer than that between Tenjiku01 and other similar species.

Conclusions

We successfully diagnosed imported SFG rickettsiosis in a traveler returning to Japan from India on the basis of serology and molecular laboratory techniques. If a patient reports a recent history of travel abroad, physicians should consider SFG rickettsia in the differential diagnosis. Our analysis will help elucidate a variety of rickettsial pathogenicities and biologic characteristics reported worldwide. On the basis of our findings, we propose this isolate as a novel species, *Candidatus* Rickettsia indica.

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Shigella Antimicrobial Drug Resistance Mechanisms, 2004–2014

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To determine antimicrobial drug resistance mechanisms of *Shigella* spp., we analyzed 344 isolates collected in Switzerland during 2004–2014. Overall, 78.5% of isolates were multidrug resistant; 10.5% were ciprofloxacin resistant; and 2% harbored *mph(A)*, a plasmid-mediated gene that confers reduced susceptibility to azithromycin, a last-resort antimicrobial agent for shigellosis.

Shigella spp. are the etiologic agents of acute invasive intestinal infections clinically manifested by watery or bloody diarrhea. Shigellosis represents a major burden of disease, especially in developing countries, and is estimated to affect at least 80 million persons, predominantly children, each year (*1*). Disease may be caused by any of the 4 *Shigella* species: *S. dysenteriae, S. flexneri*, *S. boydii*, *and S. sonnei*. In industrialized countries, the most common species is *S. sonnei*, but this species is spreading intercontinentally to developing countries as a single, rapidly evolving lineage (*2*). By contrast, in developing countries, the predominant species is *S. flexneri*, which is characterized by long-term persistence of sublineages in shigellosis-endemic regions with inadequate hygienic conditions and unsafe water supplies (*3*). More rarely isolated are *S. dysenteriae*, responsible for large epidemics in the past, and *S. boydii* (*4*). Although shigellosis is principally a self-limiting disease, the World Health Organization guidelines recommend antimicrobial drug treatment as a means of reducing deaths, disease symptoms, and organism-excretion time; the current drug of choice is ciprofloxacin (*1*). Of growing concern is multidrug resistance, and in particular the increasing rate of resistance to ciprofloxacin reported for *Shigella* isolates from Asian and African regions (*5*). Furthermore, resistance to recommended second-line antimicrobial drugs, such as the third-generation cephalosporin ceftriaxone and the macrolide azithromycin, is emerging (*1*).

The Study

To determine antimicrobial drug resistance profiles, we analyzed clinical isolates representing 344 *Shigella* spp. collected during 2004–2014. We focused on molecular resistance mechanisms that promote resistance to currently recommended antimicrobial drugs.

We performed susceptibility testing by using the Kirby–Bauer disk-diffusion method. Results were interpreted according to Clinical and Laboratory Standards Institute performance standards (*6*). All 344 isolates were screened for plasmid-mediated quinolone resistance (PMQR) genes (*7*). A subset of 34 isolates eliciting reduced susceptibility to nalidixic acid, ciprofloxacin, or both, and representing different years of isolation was subjected to PCR-based detection of mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes (*7*). Isolates showing an extended-spectrum β-lactamase (ESBL) phenotype were screened by PCR for the presence of genes belonging to the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ families, by using primers described previously (*8*). All 344 isolates were analyzed for *mph(A)* by PCR by using previously published primers (*9*). Resulting amplicons were purified and sequenced. For database searches, we used blastn (http://www.ncbi.nlm.nih.gov/blast/).

Multidrug resistance was defined as resistance to ≥3 classes of antimicrobial agents. Multidrug resistance was detected in 150 (83.8%) of the *S. sonnei*, 84 (78.5%) of the *S. flexneri*, 20 (60.6%) of the *S. dysenteriae*, and 16 (64%) of the *S. boydii* isolates (Table 1).

Resistance to nalidixic acid was detected in all species, but none of the *S. dysenteriae* and *S. boydii* isolates were resistant to ciprofloxacin (Table 1). The time distribution and the frequency of ciprofloxacin-resistant *S. sonnei* isolates showed a rising tendency (Figure). A similar tendency was noted for ciprofloxacin-resistant *S. flexneri* isolates, which, however, revealed higher variability throughout the study period (Figure). No ciprofloxacin-resistant isolates were found before 2008. In total, 27 (15%) *S. sonnei* and 9 (8.4%) *S. flexneri* isolates were resistant to ciprofloxacin.

The *qnrS1* gene was found in 13 (3.8%) of the strains: 4 *S. dysenteriae*, 4 *S. flexneri*, 4 *S. boydii*, and 1 *S. sonnei*. Other PMQR genes included *qnrB19*, detected in *S. sonnei* (n = 1), and *qnrB4*, detected in combination with *qepA* in *S. sonnei* (n = 1). Of the 15 PMQR-positive isolates, only 2 were resistant to nalidixic acid and ciprofloxacin, illustrating the potential for development of resistance in susceptible strains (Table 2, http://wwwnc.cdc.gov/EID/article/22/6/15-2088-T2.htm).

Most of the 34 isolates analyzed for mutations in their QRDR carried mutations in the *gyrA* and *parC* genes (Table 2). Most frequently observed was the first-step amino acid substitution within GyrA at Ser83Leu (n = 14), which

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Table 1. Antimicrobial drug resistance of 344 *Shigella* spp. isolates, Switzerland, 2004–2014

Agent	No. (%) isolates			
	S. sonnei, n = 179	*S. flexneri*, n = 107	*S. dysenteriae*, n = 33	*S. boydii*, n = 25
Ampicllin	31 17.3)	73 (68.2)	19 (57.6)	12 (48)
Amoxicillin/clavulanic acid	2 (1.1)	1 (0.9)	0	0 (0)
Cephalothin	12 (6.7)	0	0	0
Cefotaxime	8 (4.5)	0	0	0
Nalidixic acid	49 (27.4)	15 (14)	2 (6)	2 (8)
Ciprofloxacin	27 (15)	9 (8.4)	0	0
Azithromycin*	2 (1.1)	5 (4.7)	0	0
Trimethoprim	172 (96)	70 (65.4)	20 (60.6)	15 (60)
Sulfamethoxazole	151 (84.4)	71 (66.4)	19 (57.6)	16 (64)
Kanamycin	1 (0.5)	1 (0.9)	0	0
Gentamicin	4 (2.2)	0	0	0
Streptomycin	163 (91)	81 (75.7)	24 (72.7)	18 (72)
Tetracycline	145 (81)	83 (77.6)	22 (66.6)	13 (52)
Chloramphenicol	6 (3.4)	56 (52.3)	9 (27.3)	2 (8)

*For azithromycin, no Clinical and Laboratory Standards Institute breakpoints for *Enterobacteriaceae* exist. Isolates harboring *mph(A)* were regarded as resistant.

was associated with resistance to nalidixic acid. The double substitutions within GyrA at Ser83Leu/Asp87Gly (n = 11) and Ser83Leu/Asp87Asn (n = 2) occurred invariably in combination with the substitution in ParC (Ser80Ille) and occurred in ciprofloxacin-resistant isolates. In addition, some unusual genotypes were detected; strains containing only second-step mutations within GyrA were observed for Asp87Tyr (n = 4) and Asp87Asn (n = 1) and were associated with resistance to nalidixic acid. The substitution ParC(Ala85Ser) was observed in nalidixic acid–resistant *S. boydii* isolates with Gly(Ser80Leu) (n = 2) (Table 2).

Our data document an ongoing trend toward dominance of *S. sonnei*, which is reflective of a current global shift in the epidemiologic distribution of this species (*10*). Of the 18 patients for whom travel to India was documented, isolates from 55.6% were resistant to ciprofloxacin, a finding that supports previous reports of importation of ciprofloxacin-resistant *Shigella* from India to Europe and the United States (*11*,*12*) and emphasizes the need to obtain travel information from patients receiving treatment for shigellosis. Furthermore, therapeutic efficiency of fluoroquinolones may be decreased because of the presence of PMQR determinants in phenotypically susceptible strains. PMQR genes are of concern because they not only promote mutations within the QRDR, resulting in resistance to fluoroquinolones, but they may disseminate among other species of *Enterobacteriaceae*.

Besides ciprofloxacin, the third-generation cephalosporin ceftriaxone is recommended as an alternative for the treatment of shigellosis (*1*). Resistance to the broad-spectrum β-lactam ampicillin was observed in all *Shigella* species (Table 1); however, the ESBL phenotype (resistance to cefotaxime; Table 1) was restricted to *S. sonnei* and was found in 8 strains (4.5% of *S. sonnei* isolates). PCR

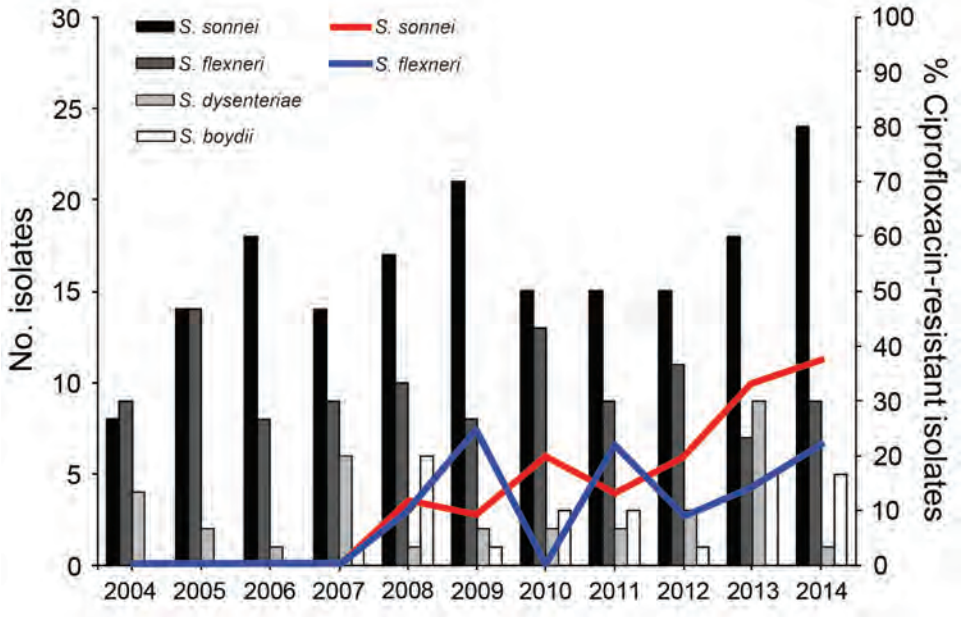

Figure. *Shigella* spp. isolated in Switzerland, 2004–2014, and percentages of ciprofloxacin-resistant *S. sonnei* and *S. flexneri*.

analysis confirmed the presence of $bla_{CTX\text{-}M}$ genes in all 8 isolates: $bla_{CTX\text{-}M\text{-}3}$ (n = 1), $bla_{CTX\text{-}M\text{-}14}$ (n = 2), and $bla_{CTX\text{-}M\text{-}15}$ (n = 5) (Table 2). The establishment of $bla_{CTX\text{-}M}$–harboring *Shigella* as an additional reservoir of these widely disseminated resistance determinants poses a threat to the treatment of shigellosis, especially because all ESBLs detected in this study were CTX-M enzymes, which are also potent ceftriaxone hydrolyzers (*13*).

Screening of the 344 *Shigella* isolates for the presence of *mph(A)* revealed 7 (2%) positive strains: 2 *S. sonnei* and 5 *S. flexneri* (Table 2). *Shigella* species exhibiting reduced susceptibility to azithromycin are of great concern because azithromycin, in combination with colistin, has recently been found to represent a potentially invaluable option for the treatment of gram-negative rods expressing MDR, including carbapenem-resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* (*14*). Hence, judicious use of this particular drug and susceptibility monitoring are warranted. Furthermore, our data show that *mph(A)* may be present in isolates displaying MICs as low as 12 μg/mL, highlighting the urgency with which azithromycin susceptibility breakpoints and interpretive criteria for *Enterobacteriaceae* are needed.

Conclusions

Treatment of shigellosis with currently recommended antimicrobial drugs is increasingly threatened by the emergence of ciprofloxacin resistance, ESBLs, or plasmid-mediated azithromycin resistance in multidrug-resistant *Shigella* isolates. Because azithromycin is a last-resort antimicrobial agent used to treat shigellosis, the emergence of *mph(A)* among *Shigella* spp. is cause for concern.

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MERS-CoV Antibodies in Humans, Africa, 2013–2014

Anne Liljander,[1] Benjamin Meyer,[1] Joerg Jores, Marcel A. Müller, Erik Lattwein, Ian Njeru, Bernard Bett, Christian Drosten, Victor Max Corman

Dromedaries in Africa and elsewhere carry the Middle East respiratory syndrome coronavirus (MERS-CoV). To search for evidence of autochthonous MERS-CoV infection in humans, we tested archived serum from livestock handlers in Kenya for MERS-CoV antibodies. Serologic evidence of infection was confirmed for 2 persons sampled in 2013 and 2014.

Middle East respiratory syndrome coronavirus (MERS-CoV) infection causes severe respiratory illness in humans. Some cases have been sporadic, but others have been part of nosocomial outbreaks mainly on the Arabian Peninsula (*1*), where dromedary camels widely carry the virus and human infections have been directly linked to contact with camels (*2*–*4*). As of January 2016, at least 1,625 acute cases in humans and 586 deaths from MERS-CoV infection have been documented (*5*). In a geographically comprehensive, age-stratified sample representing the population of Saudi Arabia, antibodies against MERS-CoV were detected in ≈0.15%, indicating sporadic infections without severe disease (*6*).

Antibodies against MERS-CoV have also been detected in dromedaries in several countries in Africa (e.g., Nigeria, Egypt, Kenya) in samples collected as long as 30 years ago (*7*–*10*). East Africa harbors >70% of the world's dromedary population; predominantly unilateral trade is conducted from Africa to the Arabian Peninsula (*11*). The basal phylogenetic clustering of viral sequences from camels in Africa suggests an African origin of MERS-CoV (*9*,*10*).

To our knowledge, evidence for autochthonous human infections in Africa has not been reported. To search for evidence of previous MERS-CoV infection, we tested archived human serum samples collected from 1,122 livestock handlers in Kenya during 2013 and 2014. This work was done in compliance with national regulations and was approved by the ethical committee of African Medical Research and Foundation, Kenya (AMREF-ESRC P65/2013).

The Study

The serum samples were collected as part of a household survey conducted during 2013–2014 in 2 eastern counties of Kenya, Garissa and Tana River (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/16-0064-Techapp1.pdf). Of those for whom information about sex and age was known, 603 were female and 407 were male, and median age was 27 (range 5–90) years. Occupational data were available for 650 (57.9%) participants; the 3 largest occupational groups represented were pastoralist (20.6%), farmer (17.0%), and student (11.4%). The households of nearly all participants kept or owned livestock, mainly goats, sheep, cattle, and donkeys. Although camel husbandry was not common among participants, camels are widespread in this region. The average camel density (calculated on the basis of census data from 2000–2013) was 1.68 and 1.98 camels/km^2 in Garissa and Tana River County, respectively (*7*).

We analyzed serum samples for antibodies against MERS-CoV by using a commercial anti–MERS-CoV recombinant ELISA (rELISA; EUROIMMUN AG, Lübeck, Germany), which is based on the recombinant MERS-CoV spike protein subunit 1 and specifically detects IgG. Samples were tested at a dilution of 1:100; an optical density ratio of 0.3 was set as a cutoff (*6*,*12*). The assay conditions used were the same as those used during a nationwide serologic study in Saudi Arabia (*6*). A total of 16 (1.40%) samples had positive results by rELISA (Table, Figure 1). The proportion of seropositive specimens in both counties in Kenya did not differ significantly (Fisher exact test, $p = 0.07$).

We subsequently tested all samples positive by rELISA by using a highly specific MERS-CoV plaque-reduction neutralization test (PRNT) as recommend by the World Health Organization (*6*,*13*). Of note, the MERS-CoV strain EMC/2012 used for PRNT may genotypically differ from putatively circulating MERS-CoV strains from Africa. However, there is no serotypic discrimination between strains because the ability of human serum to neutralize

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[1]These authors contributed equally to this article.

Table. Seropositivity for Middle East respiratory syndrome coronavirus in samples from humans in Kenya, 2013–2014*

County	No. samples tested	No. (%) positive by rELISA	No. (%) positive by rELISA and with ≥50% plaque reduction at 1:20 dilution
Garissa	559	4 (0.72)	0
Tana River	563	12 (2.13)	2 (0.36)
Total	1,122	16 (1.43)	2 (0.18)

*rELISA, recombinant ELISA.

diverse MERS-CoV strains, including the EMC/2012 strain, does not differ (*14*).

For the PRNT, dilutions starting at 1:10 were used and titers resulting in 50% ($PRNT_{50}$) and 90% ($PRNT_{90}$) plaque reduction were recorded. The 1:20 dilution was the lowest possible diagnostically significant titer (*6*). The $PRNT_{50}$ end point was considered confirmation of positivity by rELISA because this end point was found to be most sensitive and still specific during investigations of antibody responses in reverse transcription PCR–confirmed MERS-CoV–positive samples from patients in South Korea (*14*). Of the 16 samples positive by rELISA, 2 (0.18%) had reproducible MERS-CoV $PRNT_{50}$ titers of 1:20 and 1:40 (Table; Figure 2); 1 of these samples also had a titer of 1:40 when the more stringent $PRNT_{90}$ end-point criterion was used (Figure 2). For controls, we conducted PRNT testing of 22 samples negative by rELISA from persons originating from the same region as the 2 samples positive by PRNT. None of these 22 samples showed neutralizing activity at a 1:20 dilution (Figure 1).

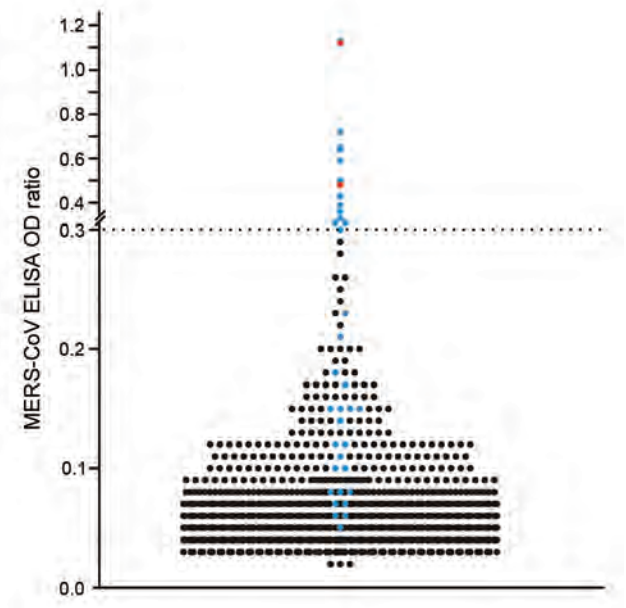

Figure 1. Plot of all individual optical density (OD) ratios obtained from recombinant ELISA testing of human serum samples for Middle East respiratory syndrome coronavirus (MERS-CoV) antibodies, Africa, 2013–2014. All 16 samples exceeding the cutoff of 0.3 and 22 other samples showing an OD ratio below the cutoff were subsequently tested in a plaque-reduction virus neutralization (PRNT) test; these samples are shown in blue, and the 2 samples positive by PRNT are shown in red. The horizontal dashed line represents the cutoff value as determined in a nationwide, cross-sectional serologic study in Saudi Arabia (*6*).

The 2 samples positive by PRNT were from a woman (26 years of age) and a man (58 years of age) from Tana River County. The woman kept goats, sheep, cattle, and donkeys; the man kept goats and donkeys. Both persons had low antibody titers, and neither reported any recent clinical symptoms, indicating that their MERS-CoV infections probably occurred well before the time of sampling and that the infections may have been mild or subclinical. Because data about persistence of MERS-CoV antibodies after asymptomatic infection are not available, it can only be speculated when and where these infections were acquired. Neither the 2 MERS-CoV antibody–positive persons nor most of the other tested persons owned dromedaries. Nevertheless, camels roam in both counties (*7*), and humans have regular contact with camels and are likely to consume camel products.

Our study has several limitations. First, we were not able to test samples from persons who had close contact with camels, such as camel pastoralists. Second, although we used well-validated methods and a 2-step approach recommended by the World Health Organization for MERS-CoV diagnostics (*13*), our results should be confirmed by larger studies that may enable direct virus detection.

Conclusions

The absence of autochthonous human MERS-CoV infections in Africa has triggered hypotheses regarding differences in disease transmission between Africa and the Arabian Peninsula and has raised doubts regarding the role of camels as a source of infection. Our study provides evidence for unrecorded human MERS-CoV infections in Kenya. The proportion of seropositive specimens that we found is comparable to previously reported proportions of unrecorded infections in the general population in Saudia Arabia (1.52% vs. 1.43% positivity by rELISA and 0.15% vs. 0.18% positivity by PRNT for Kenya vs. Saudi Arabia, respectively) (*6*). Because of an apparently low infection rate and a bias toward reporting severe cases, the discovery of unreported MERS cases requires testing of large sample sizes with well-validated serologic methods (*6*). Although the number of samples we tested was approximately only one tenth of the number of samples tested during the Saudi Arabia study, the proportion of seropositive specimens may be similar in Kenya and Saudi

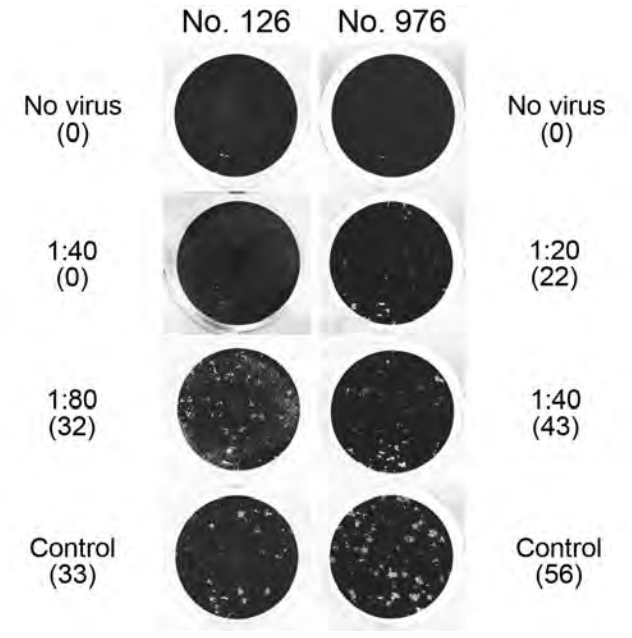

Figure 2. Middle East respiratory syndrome coronavirus (MERS-CoV) plaque-reduction neutralization test (PRNT) results for 2 serum samples positive by recombinant ELISA, showing virus neutralization activity against MERS-CoV strain EMC/2012 exceeding a titer of 1:10. Titers and number of plaques (in parenthesis) are shown next to the corresponding images. Sample no. 976 showed ≥50% plaque reduction up to a titer of 1:20, and sample no. 126 showed ≥90% plaque reduction up to a titer of 1:40. No serum was added to the control wells. Note that the image cannot represent the morphology and the contrast of plaques that was visible with direct inspection of cell culture plates with an appropriate light source, as was done for these experiments.

Arabia. The lack of a well-developed public health system in parts of Africa could lead to underdiagnosis of clinical cases and would therefore prevent case notification. Moreover, less accessible hospital care might preclude large nosocomial outbreaks as have been observed in countries on the Arabian Peninsula and in South Korea. Other possible explanations for the absence of confirmed and reported clinical cases of MERS-CoV infection in Africa include lesser virulence of strains from Africa and cultural differences that might cause persons of different age ranges to be exposed to the virus.

On the basis of the ability of MERS-CoV to infect a wide range of hosts in cell culture experiments (*15*), it remains to be excluded that other wild and livestock animals might act as additional sources of human MERS-CoV infection. It is paramount to characterize MERS-CoVs from humans, camels, or tentative other animal hosts in Africa. For increased understanding of any possible differences in pathogenicity and transmission potential, these MERS-CoV strains should be compared with isolates from the Middle East.

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Microcephaly in Infants, Pernambuco State, Brazil, 2015

Microcephaly Epidemic Research Group[1]

We studied the clinical characteristics for 104 infants born with microcephaly in the delivery hospitals of Pernambuco State, Brazil, during 2015. Testing is ongoing to exclude known infectious causes. However, microcephaly peaked in October and demonstrated central nervous system abnormalities with brain dysgenesis and intracranial calcifications consistent with an intrauterine infection.

In April 2015, Zika virus was identified in Brazil (*1*,*2*). In August, an increased incidence of microcephaly was detected in Pernambuco State. In November, the Brazilian Ministry of Health declared a relationship between the microcephaly epidemic and Zika virus infection during pregnancy (*3*) on the basis of accumulating evidence (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/16-0062-Techapp1.pdf). Since then, several reports of Zika virus–associated microcephaly have been published (*4*–*6*). As of December 12, 2015, a total of 2,401 suspected cases of microcephaly had been reported (including 29 stillbirths) in 549 municipalities in 20 states in Brazil; Pernambuco reported the most (874 cases) (*7*). In comparison, an annual mean of 156 microcephaly cases were reported in Brazil (through the routine birth notification system) during 2010–2014, including 9 in Pernambuco (*7*). We describe preliminary findings of 104 microcephaly cases in Pernambuco from the 2 hospitals to which infants with suspected cases were referred.

The Study

In August 2015, we began systematically collecting data prospectively and retrospectively (by reviewing hospital records dating to April 2015) on infants suspected to have microcephaly and referred to us. We defined microcephaly as head circumference below the third percentile for gestational age and sex using the Fenton growth chart (*8*). Our routine protocol for reviewing suspected microcephaly cases involved collecting data on maternal age and infant sex, gestational age, and birthweight. Laboratory testing was performed for 6 pathogens: serologic testing for dengue virus; and a nontreponemal test for toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus, and syphilis (collectively referred to in Brazil as ToRCHeS agents). Zika virus testing was not available at the time of the study. Brain imaging was performed when available by using computed tomography (CT) scans, magnetic resonance imaging, or transfontanellar ultrasonography. Hearing was assessed by using otoacoustic emission testing, and retinae were examined by using handheld fundoscopy. We asked mothers about rash during pregnancy, but no data were collected on the timing of the rash, other clinical symptoms, or environmental exposures. We excluded patients with suspected microcephaly who had a head circumference greater than the third percentile (40 infants), were missing chart data for head circumference (6 infants) or gestational age (4 infants), or tested positive for a congenital infection known to cause microcephaly (1 infant positive for cytomegalovirus identified by PCR). We plotted included cases by week of birth and compared variables of infants with microcephaly (head circumference 30–32 cm) and those with severe microcephaly (circumference <30 cm). We conducted this investigation as part of our routine clinical practice; it did not require human subject approvals.

Our final study comprised 104 infants with microcephaly. Cases increased from epidemiologic week 37 (mid-September) and peaked during weeks 40–43 (late October) (Figure 1). Seventy infants had severe microcephaly (Table; Figure 2, panel A) and a mean head circumference of 29 cm. Only 10% of case-infants were born prematurely, which was lower than the national estimates of the prevalence of premature birth (*9*). Mothers were a mean of 25 (range 15–43) years of age. Of the 100 mothers interviewed, 59 recalled having a rash during pregnancy. Testing for dengue and ToRCHeS was incomplete for more than half of the case-infants; the number tested for each of the 6 pathogens varied because of limited laboratory resources at the beginning of the epidemic (Table). Three infants tested positive for syphilis, 2 for dengue, and 1 for herpes simplex virus, but they also shared common features with the epidemic of congenital microcephaly (*10*).

As of January, 13, 2016, a total of 58 of the 104 infants had been investigated (54 by CT scan, 3 by magnetic resonance imaging, 1 by both). All 58 infants showed radiologic abnormalities, including calcifications (93%), mainly in the cortical/subcortical junction but also in the periventricular region, basal ganglia, thalamus, midbrain, and cerebellum. Sixty-nine percent showed evidence of malformations of cortical development, including

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[1]Members of the Microcephaly Epidemic Research Group who contributed data are listed at the end of this article.

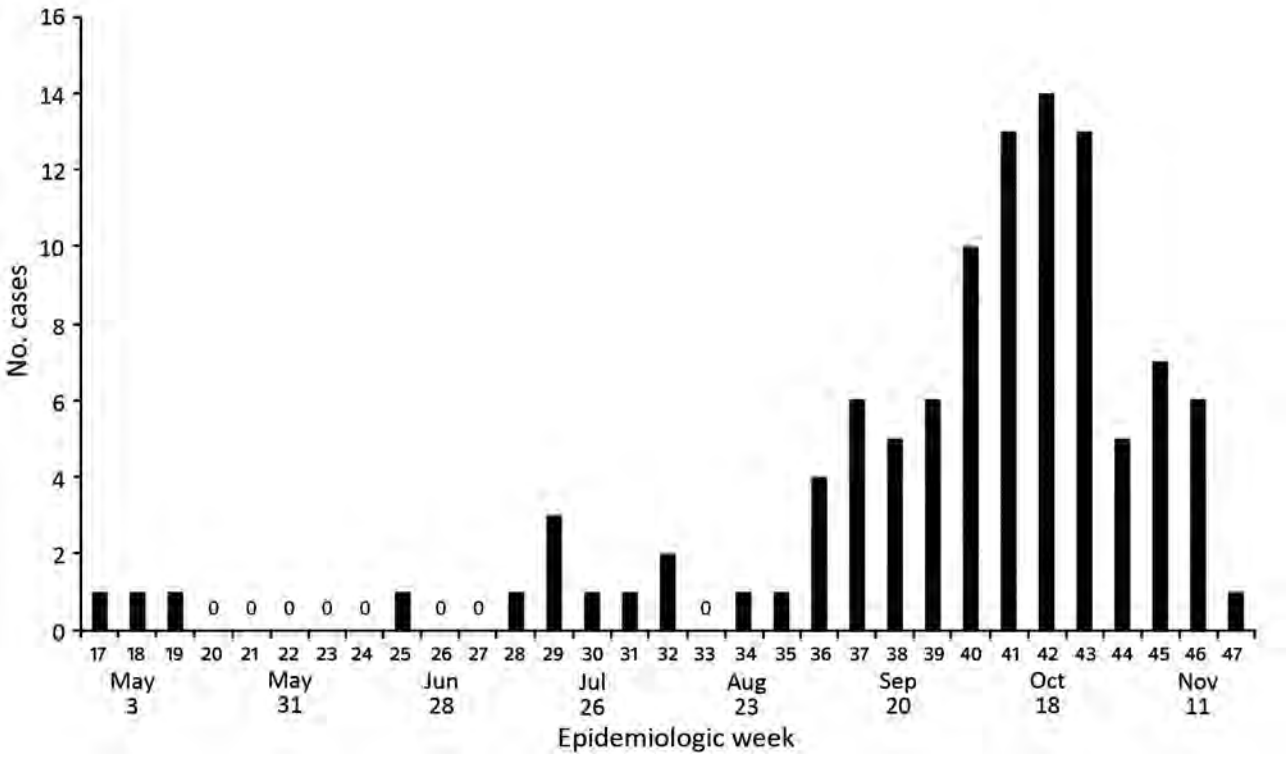

Figure 1. Microcephaly cases (based on Fenton growth chart criteria) at 2 referral hospitals, by week of birth, Pernambuco State, Brazil, 2015 (N = 104).

lissencephaly (relative smoothness of the brain surface) associated with pachygyria (poorly formed, broad cortical folds), agyria (no cortical folds), or both (Figure 2, panel B). Sixty-six percent had ventriculomegaly, an enlargement of the ventricles that can occur for several reasons, such as loss of brain volume or impaired outflow or absorption of cerebrospinal fluid from the ventricles. Of the 15 children with pending CT scans who had undergone ultrasonography, 14 had abnormalities, such as calcifications and brain atrophy. The child (at term) with a normal ultrasound had a head circumference of 30 cm, and the mother reported a rash during pregnancy.

Conclusions

Microcephaly among the 104 newborns peaked in October 2015 and demonstrated severe central nervous system abnormalities with brain dysgenesis and intracranial calcifications consistent with an intrauterine infection. Although we initially lacked Zika virus testing and completed only partial testing for ToRCHeS infections, the timing on the microcephaly and the history of rash in more than half of the pregnant mothers suggest an outbreak of congenital microcephaly caused by a congenital infection. Since our study was completed, less clinically detailed and more surveillance-oriented data have been published from the national reporting system (*11*). These data include the case-infants reported here and the reported occurrence of the 2015–2016 microcephaly cases, especially in Pernambuco. They highlight the temporal relationship between the preceding Zika virus transmission and the abrupt increase in prevalence of microcephaly at birth.

Our study focused on the clinical findings of 1 cohort, demonstrating a high proportion of calcifications and malformations of cortical development in infants with microcephaly and with severe microcephaly. Such malformations have commonly been observed in cases of intrauterine infections caused by cytomegalovirus and toxoplasmosis (*12*); more severe anomalies (e.g., lissencephaly) occur in infants whose mothers were infected before weeks 16–18 of pregnancy (*13*). The finding of intracranial calcifications predominantly in the cortical/subcortical parenchyma suggests a scan pattern distinct from that of other congenital infections, although further studies including comprehensive diagnostic testing for Zika virus and other known infectious causes of congenital central nervous system defects are needed to confirm this pattern.

Our study had several limitations. First, as previously noted, this study was preliminary, and Zika virus testing was not yet available. Second, personnel and laboratory resources were insufficient for ToRCHeS testing of more than half of the case-infants reported here; however, we collected blood and cerebrospinal fluid samples

Table. Characteristics of 104 newborns with microcephaly seen at 2 referral hospitals, Pernambuco State, Brazil, August–December 2015*

Finding	Severe microcephaly, no. positive/no. tested (%)	Microcephaly, no. positive/no. tested (%)	p value†
Mother			
Gestational age of newborn, N = 104			
At term, 37 wks to 41 wks 6 d	59/70 (84)	34/34 (100)	0.019
Preterm, <37 wks	10/70 (14)	0/34 (0)	
Postterm, ≥42 wks	1/70 (1)	0/34 (0)	
Self-reported rash during pregnancy, n = 100	41/68 (60)	18/32 (56)	0.702
Newborn‡			
Female sex, N = 104	38/70 (54)	16/34 (47)	0.489
Brain CT scan or MRI, n = 58			
Calcifications	43/45 (96)	11/13 (85)	0.208
Malformation of cortical development, including lissencephaly	32/45 (71)	8/13 (61)	0.517
Ventriculomegalia	32/45 (71)	6/13 (46)	0.102
Abnormal findings in transfontanellar ultrasound, n = 32	23/23 (100)	6/7 (86)	0.092
Abnormal findings in fundoscopy,§ n = 33	8/29 (28)	0/4 (0)	0.550
Abnormal findings in OAE,§ n = 23	2/16 (12)	0/7 (0)	1.000
Newborn serology			
Nontreponemal syphilis test, n = 62	2/42 (5)	1/20 (5)	1.000
Dengue virus IgM, n = 34	2/25 (8)	0/9 (0)	1.000
Cytomegalovirus IgM, n = 33	0/24 (0)	0/9 (0)	NA
Cytomegalovirus IgG, n = 34	19/23 (83)	8/11 (73)	0.245
Toxoplasmosis IgM, n = 44	0/37 (0)	0/7 (0)	NA
Toxoplasmosis IgG, n = 47	24/39 (61)	5/8 (62)	1.000
Rubella IgM, n = 54	0/42 (0)	0/12 (0)	NA
Rubella IgG, n = 88	55/56 (98)	28/32 (87)	0.396
Herpes IgM, n = 19	1/15 (7)	0/4 (0)	1.000
Herpes IgG, n = 18	13/14 (93)	4/4 (100)	1.000

*Severe microcephaly, head circumference <30 cm; microcephaly, head circumference 30–32 cm. CT, computed tomography; MRI, magnetic resonance imaging; NA, not applicable; OAE, otoacoustic emission test;.
†χ^2 comparing frequencies between the 2 groups.
‡Of the 98 infants with documented birthweight, average weight was 2,716 g (range 1,630–3,890 g) for term and postterm newborns and 1,918 g (range 1,135–2,580 g) for preterm.
§Of the 33 children examined by fundoscopy, 24% had abnormal findings, including rarefaction of the retinal pigment epithelium, atrophic macular lesions, macular scarring and macular reflection juxtafoveal changes. Of the 23 children tested with OAE, 9% had an abnormal result. The frequency of abnormal results in preterm vs. term and postterm newborns for OAE and fundoscopy was similar (<30%).

for future testing. Third, limited resources restricted the number of brain scans to slightly more than half of these infants. Fourth, the data on rash during pregnancy was collected postpartum and could be subject to recall bias. Nevertheless, a case–control study has been under way in Pernambuco since December 2015, supported by the Brazilian Ministry of Health and the Pan American Health Organization, to establish an association between microcephaly and Zika virus (primary hypothesis) and explore other infectious or noninfectious causes.

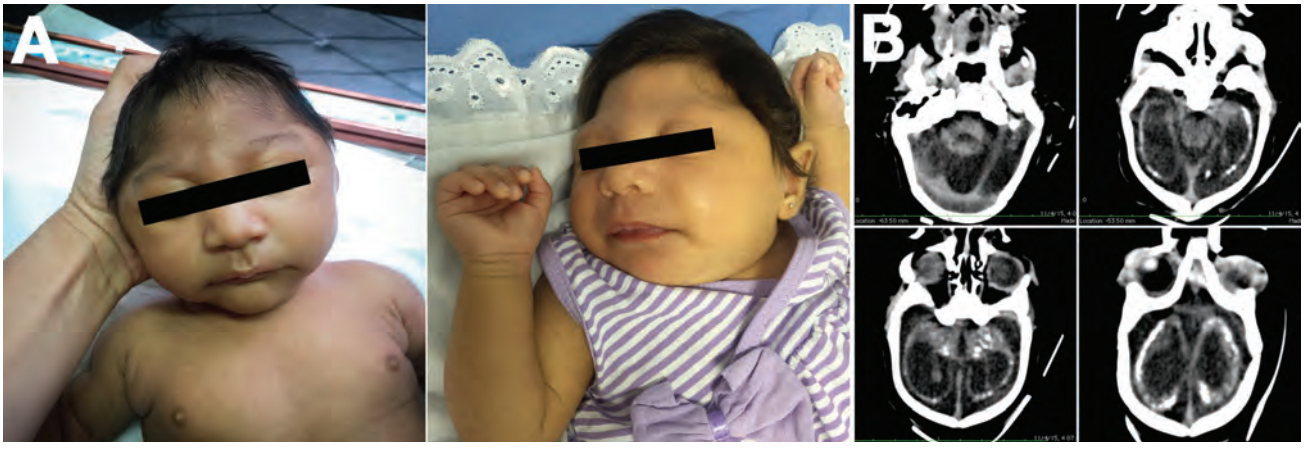

Figure 2. Microcephaly, Pernambuco State, Brazil, 2015. A) Two newborns in whom microcephaly was diagnosed during the epidemic. B) Brain computed tomography scan of a 43-day-old infant showing cerebellar hypoplasia, parenchymal calcifications, ventriculomegaly, and malformation of cortical development compatible with lissencephaly.

Although the ToRCHeS testing was incomplete, our findings suggest an outbreak of severe microcephaly in Pernambuco that peaked in October 2015. Our data, in conjunction with recent surveillance summaries from Brazil (*11*), are consistent with the timing of the Zika virus epidemic. Our findings illustrate the most severe end of the spectrum of defects affecting newborns. Other manifestations and complications in infants born to mothers infected with Zika virus during pregnancy will be described through close follow-up of these children. The government of Brazil plans to expand the support system for affected children. Further studies will define the proportion of congenital defects according to the gestational age of infection and describe the clinical outcomes.

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The Microcephaly Epidemic Research Group comprises experts from several institutions in response to the public health emergency. The main goals of the group are research development and epidemiologic data analysis.

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Prospective Validation of Cessation of Contact Precautions for Extended-Spectrum β-Lactamase–Producing *Escherichia coli*[1]

Sarah Tschudin-Sutter, Reno Frei, Friedbert Schwahn, Milanka Tomic, Martin Conzelmann, Anne Stranden, Andreas F. Widmer

After contact precautions were discontinued, we determined nosocomial transmission of extended-spectrum β-lactamase (ESBL)–producing *Escherichia coli* by screening hospital patients who shared rooms with ESBL-producing *E. coli*–infected or –colonized patients. Transmission rates were 2.6% and 8.8% at an acute-care and a geriatric/rehabilitation hospital, respectively. Prolonged contact was associated with increased transmission.

The rapid increase of extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* has challenged healthcare facilities worldwide regarding implementation of effective infection-control measures to limit further nosocomial spread (*1*). The benefits of routine enforcement of contact precautions must be balanced against additional costs, impediments to patient care, and exposure to ESBL-producing *E. coli* outside healthcare institutions.

At the University Hospital Basel (UHB), a university-affiliated tertiary care center in Basel, Switzerland, transmission rates of ESBL-producing *Escherichia coli* are low in contact patients exposed to patients colonized or infected with ESBL-producing *E. coli*. This low transmission rate challenges the routine use of contact precautions in nonepidemic settings (*2*). Based on our findings and recent data suggesting that ESBL-producing *E. coli* is predominantly acquired in the community (*3*), we abandoned contact precautions for patients infected or colonized with ESBL-producing *E. coli* at the UHB and an affiliated long-term care center, Felix Platter Hospital (FPH), in Basel. To validate this practice, we screened all patients who shared a hospital room with a patient with ESBL-producing *E. coli* to determine transmission rates.

The Study

UHB is an acute-care hospital with 735 beds, of which 8.7% are in rooms with 4 beds and the remaining are in rooms with 1–2 beds. FPH is a university-affiliated geriatric and rehabilitation center with 320 beds, of which 47.5% are in rooms with 4 beds and 52.5% are in rooms with 1–2 beds. In both facilities, the average distance between beds is 2 m. The 2 institutions share an infection-control team and microbiology laboratory. The study was approved by the local ethics committee as part of the quality assurance program; informed consent was waived.

FPH and UHB abandoned routine contact precautions for patients infected or colonized with ESBL-producing *E. coli* beginning January and June 2012, respectively; patients were included in this study through December 2013. We defined index patients as patients colonized or infected with an ESBL-producing *E. coli* in any specimen from any body site and contact patients as patients hospitalized for at least 24 hours in the same room as an index patient. Contact time was defined as the time index and contact patients shared a room before the contact patient was screened. Contact patients were prospectively screened once before discharge by swab sampling of the rectum and any open wounds or drainage sites; if Foley catheters were used, urine was also sampled and cultured. Transmission was considered to have occurred if ESBL screening results for the contact patient were positive and molecular typing by pulsed-field gel electrophoresis (PFGE) showed that the strain shared identity with the strain of the index patient.

We used standard culture methods with chromogenic medium (chromID ESBL; bioMérieux, Marcy l'Etoile, France) to detect ESBL-producing *E. coli*. We performed routine identification and susceptibility testing using the Vitek 2 System (bioMérieux, Durham, NC, USA) with cefpodoxime, ceftriaxone, and ceftazidime. We confirmed positive results by using Etest strips (bioMérieux, Marcy l'Etoile) containing cefotaxime or ceftazidime, with and without clavulanic acid. We used molecular typing by PFGE to determine the identity of strains.

We used the Fisher exact test and the Mann-Whitney U test for univariable comparisons. Logistic regression was performed to calculate odds ratios for transmission. Two-sided $p<0.05$ was considered significant.

During the study period, 231 contact patients (151 from UHB, and 80 from FPH) were exposed to 211 index

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[1]Preliminary results from this study were presented at the 25th European Congress of Clinical Microbiology and Infectious Diseases; April 25–28, 2015; Copenhagen, Denmark.

patients (178 from UHB, 33 from FPH). Contact patients were screened for ESBL-producing *E. coli* after a median contact time of 4 (interquartile range 3–6) days at UHB and 15 (interquartile range 9–23) days at FPH.

We recovered ESBL-producing *E. coli* from 24 contact patients (12 from each institution) and confirmed strain identity for 11, accounting for an overall transmission rate of 4.8% (11/231) (Figure 1). Transmission occurred in 2.6% (4/151) of contacts at UHB and 8.8% (7/80) at FPH (p = 0.052). We found no differences between contact patients with and without transmission of ESBL-producing *E. coli* in regard to baseline characteristics; use of antimicrobial drugs; or exposure to index patients, except for contact time, which was longer for patients with transmission (Table). Exposure to an index patient for >5 days was associated with increased odds for transmission (odds ratio 10.18, 95% CI 1.28–80.91; p = 0.028) (Figure 2).

Conclusions

After contact precautions for ESBL-producing *E. coli* were discontinued at the 2 hospitals in this study, transmissions occurred in 2.6% of contact patients at UHB and in 8.8% of contact patients at FPH. Transmissions were associated with duration of hospitalization in the same room as an index patient. At UHB, the rate of transmissions was similar to that reported during the period before discontinuation of contact precaution measures (1.5%) (*2*). At other Swiss acute-care hospitals, ESBL-producing *E. coli* transmission has affected 4.5% of all contact patients (*3*), and transmission of all ESBL-producing *Enterobacteriaceae* has affected 2.8%, despite implementation of contact precautions (*4*). The proportion of contact patients with transmission at FPH (8.8%) compares well with the proportion reported from similar settings (6.5%) (*5*).

Our finding that the transmission rate at the acute-care hospital was similar before and after discontinuation

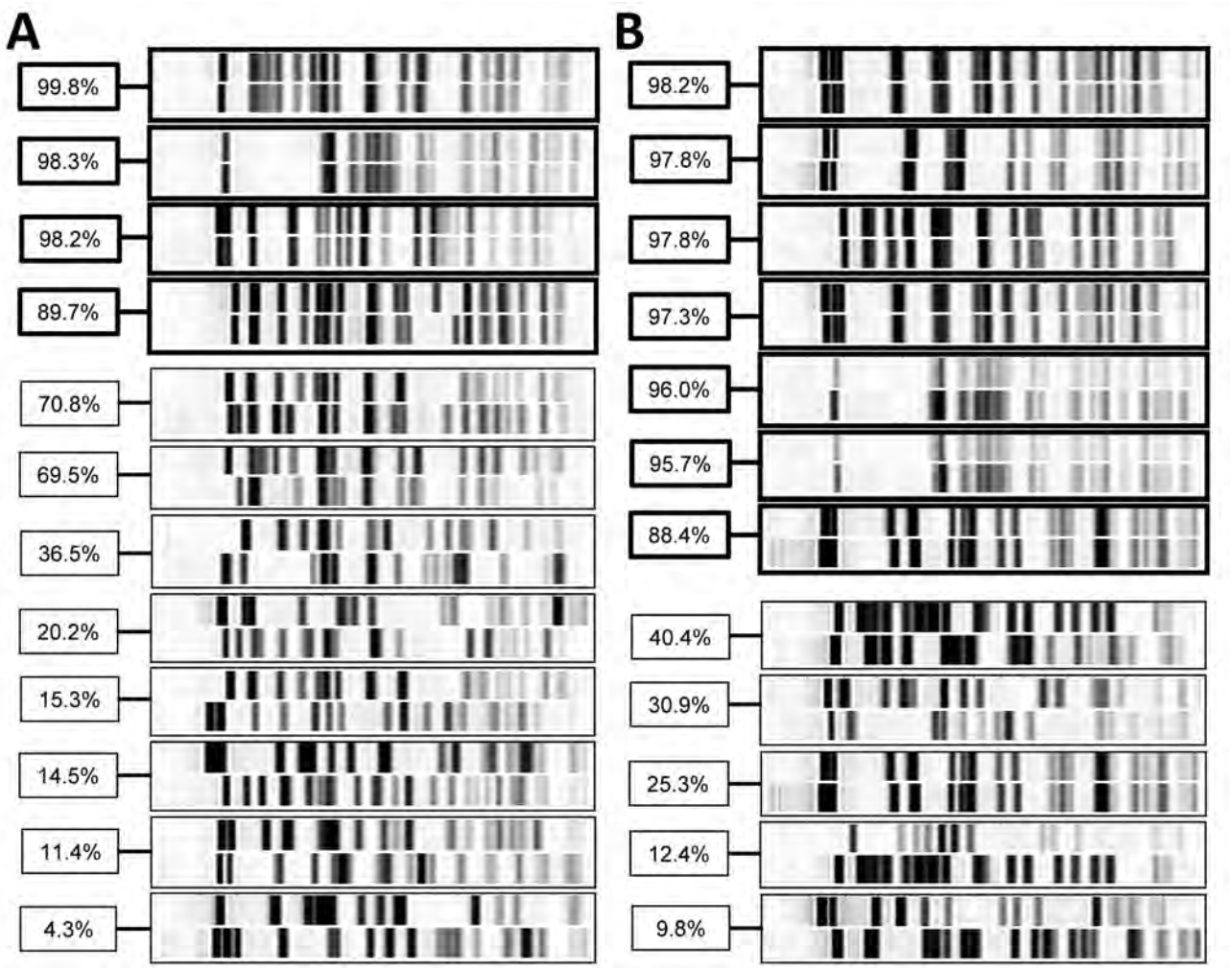

Figure 1. Pulsed-field gel electrophoresis results for *Escherichia coli* samples from A) index and B) contact patients who shared rooms for at least 24 hours in an acute-care hospital or a geriatric/rehabilitation center, Basel, Switzerland. Thick black outlining indicates results for patient pairs with extended-spectrum β-lactamase–producing *E. coli* transmission. FPH, Felix Platter Hospital; UHB, University Hospital Basel.

Table. Characteristics and exposures for hospitalized contact patients with and without transmission of ESBL-producing *Escherichia coli* from index patients, Basel, Switzerland*

Patient characteristics and exposures	Contact patients with transmission of ESBL-producing *E. coli*, n = 11†	Contact patients without transmission of ESBL-producing *E. coli*, n = 220†	p value
Contact patient characteristics			
Age, y, median (IQR)	81 (77–82)	75 (64–82)	0.153
Charlson Comorbidity Index, median (IQR)	2 (1–4)	2 (1–3)	0.399
Contact time, d, median (IQR)	13 (10–15)	8 (5–12)	**0.006**
Intensive care unit stay	0	54 (24.8)	0.122
Received any antimicrobial drug	4 (36.4)	93 (42.3)	0.765
Received systemic antimicrobial drugs with activity against ESBL *E. coli*	1 (9.1)	19 (8.6)	1.000
Index patient characteristics			
Age of index patient, y, median (IQR)	79 (64–87)	73 (62–80)	0.175
Charlson Comorbidity Index, median (IQR)	2 (1–3)	2 (1–3)	0.572
Infected with ESBL-producing *E. coli*	6 (54.6)	84 (38.2)	0.346
ESBL-producing *E. coli* infection			
Bloodstream	0	3 (1.4)‡	1.000
Urinary tract	5 (45.5)	68 (30.9)	0.330
Respiratory tract	1 (9.1)	10 (4.6)	0.422
Surgical site	0	6 (2.7)	1.000
Colonized with ESBL *E. coli*	5 (45.4)	136 (61.8)	0.346
Received systemic antimicrobial drugs with activity against ESBL *E. coli*	6 (54.6)	84 (38.2)	0.346

*Bold indicates significance. Contact patient exposures occurred through the sharing of a room for at least 24 hours with an ESBL-producing *E. coli*–infected or colonized index patient in an acute-care hospital or a geriatric/rehabilitation center. ESBL, extended-spectrum β-lactamase; IQR, interquartile range.
†Values are no. (%) patients except as indicated.
‡All patients with bloodstream infections had urinary tract infections.

of contact precaution measures may be explained by high adherence to standard precautions (*6*), especially hand hygiene, for which compliance exceeded 90% (*7*), and the mainly short-term hospitalizations (≤5 days). Thus, these findings may not be generalizable to other settings, especially when longer hospitalization is required, as is the case in geriatric/rehabilitation centers. Other factors may also have influenced transmission rates in our study, impeding generalizability of the findings to other countries. For example, the European Surveillance of Antimicrobial Consumption Network (http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/esac-net-database/Pages/database.aspx) reports that antimicrobial drug use in hospitals in Switzerland (1.9 defined daily doses/1,000 inhabitants/day) is lower than that in hospitals in other European countries (mean of 2.0 defined daily doses/1,000 inhabitants/day). Furthermore, the incidence of ESBL-producing *E. coli* may be lower in Switzerland than in other European countries (*8*), as suggested by a lower proportion (8.2%) of third-generation cephalosporin-resistant *E. coli* among invasive isolates in Switzerland as compared with those in other European countries (http://www.anresis.ch/index.php/anresisch-data-de.html).

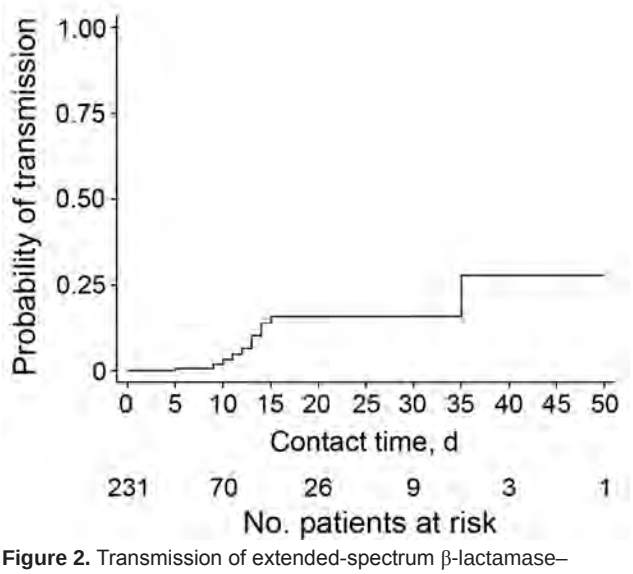

Figure 2. Transmission of extended-spectrum β-lactamase–producing *Escherichia coli* over contact time among index and contact patients who shared rooms for at least 24 hours in an acute-care hospital or a geriatric/rehabilitation center, Basel, Switzerland.

Person-to-person transmission may play a substantial role in sustaining the global ESBL epidemic. In nursing homes, ESBL-producing *E. coli* isolates from residents living in adjacent rooms were found to be closely genetically related (*9*), and high ESBL-producing *E. coli* transmission rates (23%) have been reported in households (*3*), supporting our results that sustained contact over longer periods may facilitate transmission. Furthermore, patients hospitalized in the FPH may require more care, resulting in increased contact between healthcare workers and patients, possibly facilitating transmission (*5*).

In our study, contacts were screened only once before discharge, long-term surveillance for acquisition was not performed, and preenrichment of rectal swab samples was not conducted, all of which may have led to an underestimation

of ESBL-producing *E. coli* cases. However, the circulation of ESBL-producing clones in the community may have resulted in an overestimation of transmission; before hospitalization, contact patients may have been colonized with strains in the community identical to those of index patients with whom they eventually shared a room. We acknowledge that our study lacks the robustness of a cluster-randomized trial to evaluate the effect of contact precautions on ESBL-producing *E. coli* transmission. However, we found that, when exposure times are short and adherence to standard precautions is high, the discontinuance of contact precautions for ESBL-producing *E. coli* in healthcare settings results in transmission rates similar to those observed when contact precautions are used.

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Whole-Genome Analysis of *Cryptococcus gattii*, Southeastern United States

Shawn R. Lockhart, Chandler C. Roe, David M. Engelthaler

Cryptococcus gattii is a recognized pathogenic fungus along the Pacific coast of the United States from California to Washington. Here we report that *C. gattii* may also be endemic to the southeastern United States and has probably been present there longer than in the Pacific Northwest.

The pathogenic fungus *Cryptococcus gattii*, recently designated as a separate species from *Cryptococcus neoformans*, is now recognized as a separate species complex (*1*–*3*). In the United States, *C. gattii* was first identified in culture collections in the late 1960s, when a substantial proportion of *C. neoformans* isolates from California were identified as serotypes B or C (now *C. gattii* serotypes A and B) (*4*,*5*). Since then, *C. gattii* has been identified not only in California but also in the Pacific Northwest (PNW) states of Washington and Oregon, where it is now considered endemic (*6*). Although non–travel-associated cases of infection, typically manifesting as pneumonia or meningitis, are occasionally reported in other areas of the United States (*7*,*8*), *C. gattii* has not been considered to be endemic to any other areas of the United States.

The Study

The Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) has been performing passive surveillance for *C. gattii* in the United States since 2009. Isolates were collected as part of routine surveillance for *C. gattii* or submitted to the Fungal Reference Laboratory at the CDC for identification. We identified all isolates to the species level as described (*8*) and used the consensus protocol for multilocus sequence typing (MLST) (*9*). In the course of this surveillance, several isolates were received from states in the southeast; genotypic analysis revealed that these isolates consisted of 2 molecular types, VGI and VGIII (*8*). Of >400 isolates received from throughout the United States, 42 were molecular type VGI. MLST analysis of VGI isolates showed a cluster of isolates with a single MLST sequence type originating in the southeastern states (Figure 1). Five isolates from Georgia and 1 each from Florida, Tennessee, and Michigan were indistinguishable by MLST (sequence type 162). A second isolate from Georgia differed from this group by 1 single-nucleotide polymorphism (SNP) in the intergenic spacer gene locus.

Because the population of VGI isolates from the southeastern states (VGI-SE) was only recently identified, it might be a newly emerging population similar to the independent emergences of VGIIa and VGIIc in the PNW (*6*). To further characterize this cluster, we used whole-genome sequence typing (WGST) of isolates from this cluster, other closely related isolates, and unrelated control isolates (Table 1; Figure 2). We sequenced isolates using Illumina HiSeq or MiSeq technology (Illumina Inc., San Diego, CA, USA), used GATK Unified Genotyper version 2.4 (Broad Institute, Cambridge, MA, USA) for SNP detection, and constructed maximum-parsimony SNP trees using PAUP* (Sinauer Associates, Inc., Sunderland, MA, USA), as previously described (*6*).

Although the overall topology of the tree we generated remained almost identical to that seen by MLST, we were able to separate each of the isolates within the cluster using WGST (Figure 2). A total of 41,024 SNPs were within the VGI-SE cluster, which had an average branch length of 4,558 SNP differences between any 2 isolates. The isolate from Georgia that differed in MLST by 1 SNP was placed well within the cluster by WGST. Outside the cluster, the nearest neighbor, an isolate collected from a resident of Oregon with a history of living near the southeastern United States in Texas (Table 1), differed by >29,000 SNPs. The other cluster of VGI isolates was separated from VGI-SE by 49,992 SNPs.

Previously, whole-genome sequencing was performed on isolates of *C. gattii* molecular type VGII exclusively from the PNW. Results showed that the VGII isolates consisted of 3 different highly clonal and recently emerged populations with only 107, 132, and 137 SNPs identified within the VGIIa, VGIIb, and VGIIc populations, respectively (*10*) (Table 2). This finding indicated very recent divergence within each of these 3 subtypes (*8*). The average branch length between any 2 isolates was <18 SNPs for the VGIIa and VGIIc populations and only slightly higher for the VGIIb population. On the basis of SNP diversity, the VGI-SE subtype is substantially older than the clonal VGII populations in the PNW and has likely been in the United States for a much longer time.

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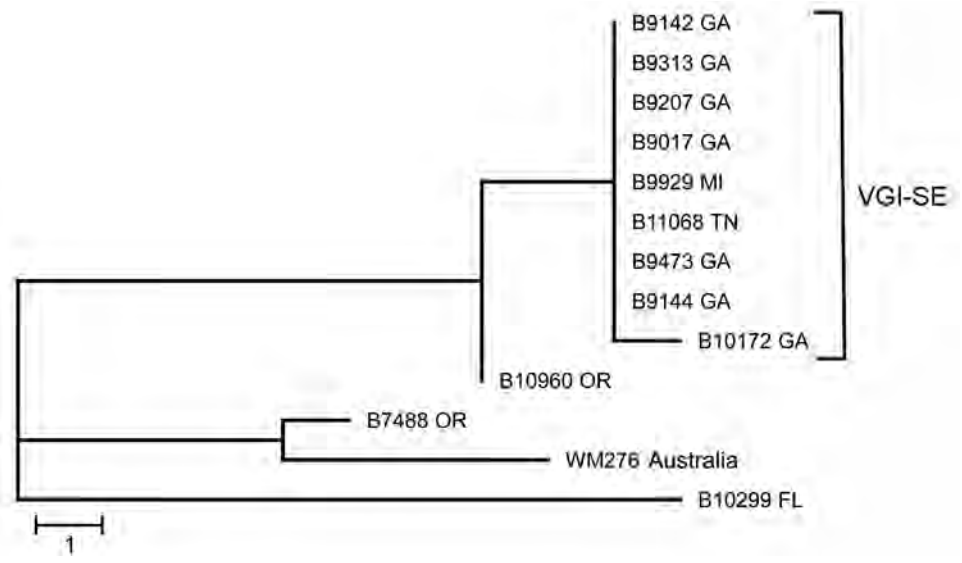

Figure 1. Maximum-parsimony tree of multilocus sequence typing analysis of VGI isolates of *Cryptococcus gattii* from the southeastern United States. In the predominant clade, 1 isolate was from Michigan; all remaining isolates were from the southeastern United States. Nearest neighbor isolates were included for comparison, and an environmental VGI isolate from Australia was used as an outgroup. VGI-SE, VGI southeastern clade. Scale bar indicates 1 single-nucleotide polymorphism.

Two of the isolates from Georgia that we analyzed differed by only 14 SNPs. These isolates, accessed from the same national reference laboratory, were submitted without clinical history; therefore, we cannot discern whether they represent the diversity of 2 isolates from 1 patient over time or whether they are part of a local clonal population.

All of the patients for whom disease manifestation was known had meningitis (Table 1). This finding differs notably from C. gattii VGII patients in the PNW, among whom a substantial proportion (59%) had a primary pulmonary infection (8). The average age of the patients with isolates in the VGI-SE cluster was 46 (range 18–70) years. For most patients, the source of infection was unknown, although 2 of the patients had been exposed to rotting wooden structures (1 demolished a wooden structure, 1 power-washed old houses).

Conclusions

Our data indicate that the clonal diversity of the *C. gattii* VGI-SE clade in the southeastern United States is an order of magnitude greater than that seen in isolates from the PNW. Gillece et al. hypothesized that the emergence in the PNW began in the mid-1990s in British Columbia, Canada, and spread to the United States in the early 2000s (*12*). Comparison of the average number of SNPs detected among the 3 VGII clonal groups in the PNW with the average number of SNPs detected between the VGI-SE isolates indicates that the VGI-SE isolates have been in place substantially longer. The limitation to this conclusion is that we do not know the rate of mutation for the VGI or VGII molecular types; however, there is no reason to believe that they would be substantially different. There may also be higher rate of recombination within the VGI-SE clade related to opposite-sex or same-gender sexual recombination. The isolates from this clade for which the mating type is known are all α, but that does not preclude the possibility that some mating type a isolates exist in the environment in the southeastern United States.

The first identified isolate of *C. gattii* in the United States was reported from West Virginia in 1924 (*11*), although the isolate was not recognized as *C. gattii* VGI

Table 1. Epidemiologic data for patients in cluster of *Cryptococcus gattii*, southeastern United States*

Isolate	State	Year	Age, y	Sex	Disease	Risk factors	Case-patient exposures	Outcome
B11068	TN	2015	62	F	Meningitis	ND	ND	ND
B9929	MI	2012	41	M	Meningitis	ND	ND	ND
B9473	GA	2012	45	M	Meningitis	Sarcoidosis	ND	Survived
B9313	GA	2011	70	M	Meningitis	COPD	Demolished an old shed	Died
B9207	GA	2011	39	M	Meningitis	None	Pressure washed houses	Died
B9144	GA	2011	ND	ND	ND	ND	ND	ND
B9142	GA	2011	ND	ND	ND	ND	ND	ND
B9017	FL	2011	ND	ND	ND	ND	ND	ND
B10172	GA	2013	48	M	Meningitis	HIV	ND	Survived
B10299	FL	2013	61	M	Meningitis	ND	ND	ND
B10960	OR	2014	33	M	Meningitis	None	Previously lived in Guatemala, Texas, and Missouri	Survived
B7488	OR	2009	18	M	Meningitis	HIV	None	Survived

*ND, no data.

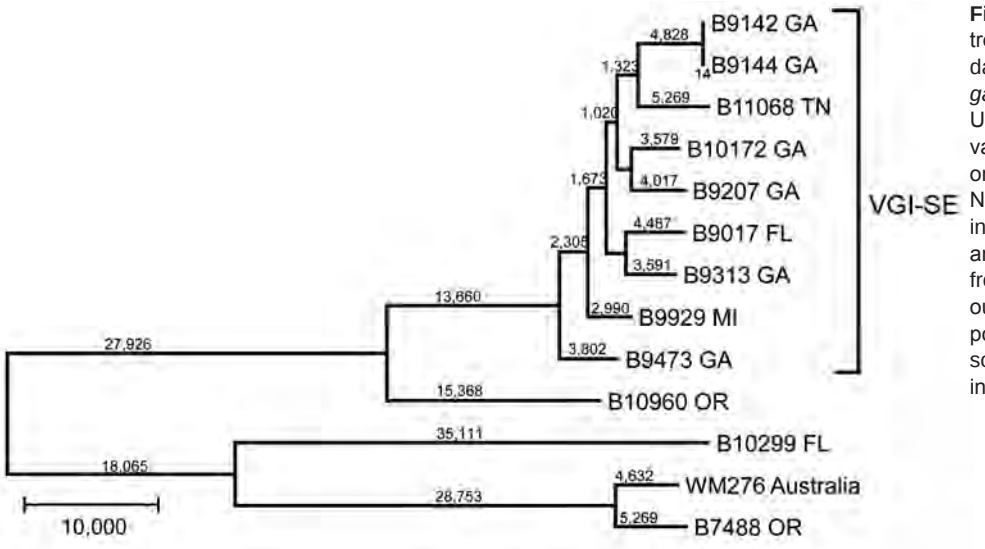

Figure 2. Maximum-parsimony tree of whole-genome sequence data of isolates of *Cryptococcus gattii* from the southeastern United States. All bootstrap values were 100%. Numbers on branches are SNPs. Nearest neighbor isolates were included for comparison, and an environmental VGI isolate from Australia was used as an outgroup. SNP, single-nucleotide polymorphism; VGI-SE, VGI southeastern clade. Scale bar indicates 10,000 SNPs.

until decades later (*12*). Two other historical reports of *C. gattii* isolates from the southeastern United States exist: the first, from 1968, describes clinical and environmental isolates from Savannah, Georgia; the other, from 1982, describes isolates from Alabama, Tennessee, and Louisiana from patients with no travel history to a *C. gattii*–endemic region (*13*,*14*). Although both of these reports use the previous name for *C. gattii*, *C. neoformans* serotypes B and C, their results clearly indicate that *C. gattii* has existed in the southeastern United States for >40 years, as opposed to the recent emergence in the PNW during the past 2 decades. A more recent report describes a *C. gattii* VGI isolate from an HIV-positive patient in North Carolina (*15*); those authors surmised that the isolate was related to travel to San Francisco, but that assumption may have to be revised.

No current molecular clock for *C. gattii* indicates the time required to generate the level of diversity seen in the VGI isolates. However, our findings lend credence to the hypothesis that *C. gattii* has circulated in the southeastern United States long enough to be considered endemic.

Table 2. Comparison of SNP differences between *Cryptococcus gattii* isolates within the recently emerged VGII clades in the Pacific Northwest and the VGI-SE clade in the southeastern United States*

Genotype	No. isolates	Total no. SNPs	Average no. SNPs between isolates
VGIIa (*9*)	6	107	18
VGIIb (*9*)	4	132	33
VGIIc (*9*)	8	137	17
VGI-SE	9	41,024	4,558

*SNP, single-nucleotide polymorphism.

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Prevalence of Nontuberculous Mycobacterial Pulmonary Disease, Germany, 2009–2014

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We analyzed routine statutory health insurance claim data to determine prevalence of nontuberculous mycobacterial pulmonary disease in Germany. Documented prevalence rates of this nonnotifiable disease increased from 2.3 to 3.3 cases/100,000 population from 2009 to 2014. Prevalence showed a strong association with advanced age and chronic obstructive pulmonary disease.

Nontuberculous mycobacteria (NTM) are a biologically diverse group of microorganisms that may cause progressive NTM pulmonary disease (NTM-PD), particularly in persons who have risk factors such as advanced age or chronic airway diseases (*1*–*3*). However, population-based data on the epidemiology of NTM-PD are scarce, particularly in Europe. We used hospital discharge codes from the International Classification of Diseases, 10th revision (ICD-10), as a surrogate to demonstrate that NTM-PD prevalence is steadily increasing in Germany, as is the case in many other countries (*4*–*6*). However, we assumed that most patients with this chronic infection are managed in outpatient care. The objective of this population-based study was to estimate the annual overall prevalence rates of NTM-PD in Germany over a 6-year period and to analyze the distribution of age and sex, the site of healthcare provision, and concomitant conditions, using a representative sample of routine statutory health insurance claims data.

The Study

This study was based on the population of Germany of ≈81 million during 2009–2014, and a subgroup of ≈70 million persons (86%) covered by German statutory health insurance (Table 1) (*7*). Because this study was based on anonymous routine data, institutional review board approval and patient consent were not required. We obtained anonymous health claims data from the Health Risk Institute health services research database, which contains a subset of ≈7 million persons covered by statutory health insurance. This database has been validated and shows good overall accuracy in documenting the German population in terms of illness, death, and drug usage; in addition, the number of insured persons in the database remains high over time (*8*). We established data characteristics by adjustments for age and sex according to the distribution within the general population of Germany (*7*). Only persons who were continuously insured within the respective year were considered. Of those persons, we obtained a 5% sample representative of the German population for each year of the study period, consisting of ≈4 million persons per year (Table 1). From these samples, we extracted records that used the ICD-10 diagnosis code A31.0 (NTM-PD) for either a primary or a secondary diagnosis, and we analyzed data according to age, sex, the site of healthcare provision (Table 1), and the most common and relevant associated conditions, as represented by concomitantly recorded ICD-10 codes (Table 2).

We calculated rates, 95% CI, and differences between means using OpenEpi version 3.03a as previously described (*9*). Statistical significance was set to $p<0.05$. Accordingly, differences were considered insignificant if 95% CIs were overlapping. We obtained official census data from the German Federal Statistical Office (*7*).

During 2009–2014, we identified 85–126 case-patients with NTM-PD per year; distribution of sex was balanced (Table 1). Mean age was not significantly different between male and female patients except for in 2013, when the female patient ages were higher than those of the male patients ($p = 0.038$). Most (76%–87%) case-patients were treated in outpatient settings (Table 1). The most frequent concomitant diagnoses throughout the study period were chronic obstructive pulmonary disease and emphysema (COPD/emphysema [ICD-10 codes J43–J44]) in 62%–79% of the case-patients (Table 2).

During 2009–2014, the annual overall prevalence rate increased from 2.3 (95% CI 1.87–2.87) to 3.3 (95% CI 2.78–3.94) per 100,000 population (Figure 1), and the corresponding projected total number of case-patients with NTM-PD in Germany increased from 1,907 to 2,697. Overall, annual rates did not differ substantively between

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Table 1. Prevelance of nontuberculous mycobacterial pulmonary disease, stratified by age, sex, and year; Germany, 2009–2014*

Characteristic	2009	2010	2011	2012	2013	2014
Population of Germany, total, no.	81,802,257	81,751,602	80,327,900	80,523,746	80,767,463	81,197,537
Mean age, y	43.4	43.7	43.9	44.1	44.2	44.3
Sex ratio, F:M	1.04	1.04	1.05	1.05	1.04	1.04
No. persons insured by SHI (% Germany population)	70,011,718 (85.6)	69,803,236 (85.4)	69,637,277 (86.7)	69,704,323 (86.6)	69,861,165 (86.5)	70,289,808 (86.6)
No. persons in study sample (% Germany population)	3,646,060 (4.5)	3,708,501 (4.5)	3,985,981 (5.0)	3,982,716 (4.9)	3,977,676 (4.9)	3,793,331 (4.7)
Mean age, y	44.0	44.1	43.7	44.0	44.3	45.1
Sex ratio, F:M	1.03	1.03	1.04	1.04	1.04	1.04
No. case-patients with NTM-PD	85	96	106	106	104	126
M, no. (% case-patients with NTM-PD)	43 (50.6)	50 (52.1)	50 (47.2)	60 (56.6)	49 (47.1)	63 (50.0)
F, no. (% case-patients with NTM-PD)	42 (49.4)	46 (47.9)	56 (52.8)	46 (43.4)	55 (52.9)	63 (50.0)
Outpatient care, no. (% case-patients with NTM-PD)	74 (87.1)	79 (82.3)	80 (75.5)	88 (83.0)	85 (81.7)	104 (82.5)
Hospital care, no. (% case-patients with NTM-PD)	20 (23.5)	28 (29.2)	34 (32.1)	25 (23.6)	37 (35.6)	38 (30.2)
Outpatient and hospital care, no. (% Case-patients with NTM-PD)	9 (10.6)	11 (11.5)	8 (7.5)	7 (6.6)	18 (17.3)	16 (12.7)
Hospital but no outpatient care in the same year, no. (% case-patients who had hospital care)	11 (55.8)	17 (60.7)	26 (76.5)	18 (72.0)	19 (51.4)	22 (57.9)
Mean age of case-patients with NTM-PD, y (SD)	60.3 (20.0)	57.3 (22.2)	55.0 (23.8)	55.5 (25.4)	59.7 (22.6)	61.0 (20.4)
M	58.7 (20.1)†	55.3 (21.3)†	57.6 (20.0)†	55.2 (25.3)†	54.8 (24.6)‡	58.2 (19.3)†
F	62.1 (20.0)	59.4 (23.2)	52.7 (26.7)	56.0 (25.7)	64.1 (19.8)	63.7 (21.2)
Prevalence, rate/100,000 population, total (95% CI)	2.3 (1.87–2.87)	2.6 (2.11–3.15)	2.7 (2.19–3.20)	2.7 (2.19–3.21)	2.6 (2.15–3.16)	3.3 (2.78–3.94)
M	2.4 (1.76–3.20)	2.8 (2.06–3.59)	2.6 (1.92–3.34)	3.1 (2.36–3.92)	2.5 (1.88–3.29)	3.4 (2.63–4.31)
F	2.3 (1.65–3.03)	2.4 (1.81–3.22)	2.8 (2.10–3.56)	2.3 (1.68–3.0)	2.7 (2.07–3.51)	3.3 (2.53–4.14)
Projected total number of case-patients with NTM-PD in Germany	1,907	2,116	2,136	2,143	2,112	2,697

*SHI, statutory health insurance; NTM-PD, nontuberculous mycobacterial pulmonary disease.
†Mean age was not significantly different among male and female patients: 2009, p = 0.44; 2010, p = 0.37; 2011, p = 0.28; 2012, p = 0.87; 2014, p = 0.13.
‡p = 0.038 for mean age between male and female patients.

male and female patients (Table 1). We observed the highest prevalence rates among case-patients ≥50 years of age, in particular among those ≥80 years of age in 2014 (9.4 [95% CI 4.35–17.78] cases/100,000 population for men and 9.6 [95% CI 5.44–15.65] cases/100,000 population for women; Figure 2).

Conclusions

We found an increasing annual prevalence rate of NTM-PD in Germany during 2009–2014, from 2.3 to 3.3 cases/100,000 population. These rates are consistent with the average annual hospitalization rate of 0.9/100,000 population observed for 2005–2011 (*6*) but are considerably lower than the rate of 6.5/100,000 population that was recently estimated for Germany by an expert panel by using a 2-round Delphi consensus-building method (*10*). A study in the United States documented an overall prevalence of 5.5 cases/100,000 person-years and a prevalence rate of 27 and 57/100,000 population among case-patients ≥60 and ≥80 years of age, respectively, during 2004–2006 (*4*). This rate is ≈5 times higher than our findings of 6.5 and 9.5/100,000 population among the same age groups in Germany during 2014. In contrast to the US study, which found that NTM-PD was more frequent among women, we found no substantive differences concerning annual overall prevalence rates related to sex.

Several implications arise from our findings. Our analysis suggests that NTM-PD remains a rare disease in Germany, with a prevalence rate below the European cutoff of 5 cases/10,000 population (*11*) and less than half the prevalence of tuberculosis (≈2,700 versus 6,300 prevalent cases in 2014) (*12*). Furthermore, in agreement with recent data from the United States (*5*), we found that chronic airway diseases (e.g., COPD/emphysema and bronchiectasis) were present in most case-patients diagnosed with NTM-PD (≤79% and ≤18%, respectively). Hence, case-patients with these diseases, in particular those on long-term and high-dose inhaled corticosteroid therapy (*13*), should be considered for NTM-PD screening.

The data we collected indicate that most NTM-PD illness in Germany is managed in outpatient care (Table 1). In addition, our data show that a substantial proportion of case-patients had acute conditions (influenza and pneumonia, ≤34%) when concomitantly diagnosed with NTM-PD. We surmise that these case-patients are at risk for not being monitored for NTM-PD because >50% of them did not

Table 2. Most common and relevant comorbidities among case-patients with nontuberculous mycobacterial pulmonary disease, Germany, 2009–2014*

ICD-10 codes	Diagnosis	No. (%) patients					
		2009	2010	2011	2012	2013	2014
A31.0	NTM-PD	85	96	106	106	104	126
J43–44	COPD and emphysema	53 (62.4)	67 (69.8)	84 (79.2)	67 (63.2)	76 (73.1)	87 (69.0)
J40–42	Chronic or unspecified bronchitis	24 (28.2)	26 (27.1)	34 (32.1)	28 (26.4)	44 (42.3)	41 (32.5)
E10–11	Diabetes mellitus, type 1 and type 2	45 (52.9)	28 (29.2)	15 (14.2)	20 (18.9)	21 (20.2)	28 (22.2)
J09–18	Influenza and pneumonia	21 (24.7)	17 (17.7)	28 (26.4)	24 (22.6)	35 (33.7)	29 (23.0)
M80–81	Osteoporosis	18 (21.2)	20 (20.8)	27 (25.5)	23 (21.7)	23 (22.1)	26 (20.6)
J45	Asthma	18 (21.2)	25 (26.0)	31 (29.2)	22 (20.8)	23 (22.1)	34 (27.0)
J96	Respiratory failure	16 (18.8)	21 (21.9)	25 (23.6)	20 (18.9)	23 (22.1)	33 (26.2)
J20–22	Acute bronchitis and bronchiolitis	8 (9.4)	17 (17.7)	17 (16.0)	27 (25.5)	18 (17.3)	26 (20.6)
A15–19	Tuberculosis	12 (14.1)	14 (14.6)	16 (15.1)	21 (19.8)	25 (24.0)	17 (13.5)
F17	Tobacco use	13 (15.3)	17 (17.7)	17 (16.0)	16 (15.1)	14 (13.5)	16 (12.7)
K21	Gastro-esophageal reflux disease	13 (15.3)	15 (15.6)	18 (17.0)	14 (13.2)	17 (16.3)	20 (15.9)
J47	Bronchiectasis	6 (7.1)	9 (9.4)	9 (8.5)	7 (6.6)	16 (15.4)	23 (18.3)
C34	Lung cancer	3 (3.5)	4 (4.2)	7 (6.6)	9 (8.5)	5 (4.8)	13 (10.3)
M05–06	Rheumatoid arthritis	3 (3.5)	11 (11.5)	8 (7.5)	4 (3.8)	2 (1.9)	9 (7.1)
B20–24	Human immunodeficiency virus	1 (1.2)	2 (2.1)	5 (4.7)	4 (3.8)	5 (4.8)	5 (4.0)
D90	Immunosuppression	0	1 (1.0)	2 (1.9)	1 (0.9)	2 (1.9)	1 (0.8)
Z94	Transplant organ and tissue status	1 (1.2)	1 (1.0)	1 (0.9)	0	2 (1.9)	2 (1.6)
E84	Cystic fibrosis	0	1 (1.0)	0	0	2 (1.9)	2 (1.6)

*ICD-10, International Classification of Diseases, 10th revision; NTM-PD, nontuberculous mycobacterial pulmonary disease; COPD, chronic obstructive pulmonary disease.

return as outpatients with the diagnosis of NTM-PD in the same year (Table 1). If this is the case, this finding emphasizes the need for continuing medical education on NTM-PD to increase clinical awareness.

Our study has limitations. First, the annual overall numbers of samples from case-patients in this study in which NTM-PD was diagnosed were low. Second, our findings are based on ICD-10 diagnosis codes and therefore are likely to underestimate the overall prevalence of NTM-PD, in particular when considering the low percentage of case-patients receiving American Thoracic Society/Infectious Diseases Society of America guideline-compliant treatment for NTM-PD (*14*). ICD-10 codes are primarily intended for reimbursement purposes; they lack validation for NTM-PD within the healthcare system of Germany and have unknown sensitivity and specificity for the use of the ICD-10 diagnostic code for NTM-PD. Moreover, assignment of ICD-10 codes does not require compliance with the American Thoracic Society/Infectious Diseases Society of America diagnostic criteria for NTM-PD (*1*), and the codes do not provide details on isolated mycobacterial species. Last, we were unable to account for regional differences across Germany. The eastern parts of Germany are slightly underrepresented in the Health Risk Institute database (*8*); therefore, our findings may not fully apply to all regions, and they may not fully apply to cohorts outside the German statutory health insurance system.

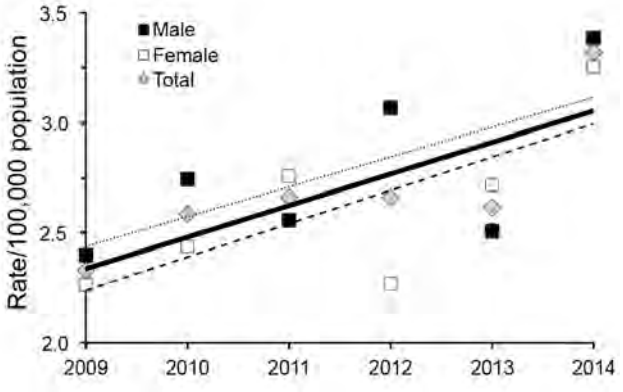

Figure 1. Trends in annual prevalence of nontuberculous mycobacterial pulmonary disease by sex and year, Germany, 2009–2014. Solid trend line indicates overall prevalence; dotted linear trend line, male prevelance; dashed linear trend line, female prevalence.

In conclusion, the annual prevalence rate of NTM-PD and, accordingly, the projected total number of cases in Germany increased during 2009–2014. Increased clinical awareness and additional reliable data on the epidemiology of NTM-PD are urgently needed, in particular with respect to COPD/emphysema. These goals could be met if NTM-PD were a notifiable disease or comprehensive disease-specific registries were established (*15*).

F.C. Ringshausen, R. Diel, and T. Welte are members of the German Center for Lung Research (DZL).

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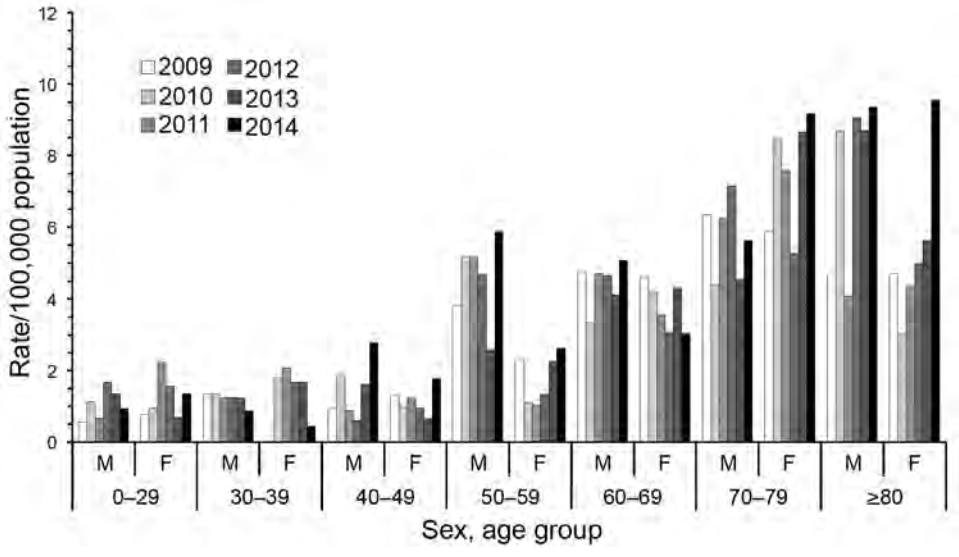

Figure 2. Prevalence rates of nontuberculous mycobacterial pulmonary disease, by age group, sex, and year, Germany, 2009–2014.

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Post-Ebola Measles Outbreak in Lola, Guinea, January–June 2015[1]

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During public health crises such as the recent outbreaks of Ebola virus disease in West Africa, breakdowns in public health systems can lead to epidemics of vaccine-preventable diseases. We report here on an outbreak of measles in the prefecture of Lola, Guinea, which started in January 2015.

In 2014 and 2015, Guinea reported 3,804 confirmed and suspected Ebola cases and 2,536 deaths (*1*). The consequences of Ebola include social instability, weakened food security, reduced vaccination coverage, and increased public mistrust of healthcare systems (*2*,*3*).

Lola, a prefecture in Guinea's forested area, borders Liberia and Côte d'Ivoire and consists of 9 subprefectures and ≈180,000 inhabitants. During September 2014–February 2015, 159 confirmed and probable Ebola cases in Lola were reported; 137 resulted in death. During this time, the World Health Organization (WHO) Regional Strategic Plan for Immunization 2014–2020 routine measles vaccination plan, which aims for 95% coverage by 2017 (*4*), was suspended in areas of Guinea where active Ebola virus transmission was reported (*5*). Furthermore, although supplementary immunization activities are essential to measles vaccination coverage in Guinea, a nationwide catch-up campaign scheduled for the second half of 2014 was interrupted because of the Ebola outbreak and did not reach Lola (*6*). The previous supplementary immunization activities took place in Guinea in 2012, aiming for 95% coverage among children 9–59 months of age (*7*). After that campaign, a cross-sectional survey estimated vaccination coverage to be 61% among this age group in the N'Zérékoré region, which includes Lola prefecture (*7*).

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Measles surveillance by the Direction Préfectorale de la Santé (DPS) in Lola is based on standard WHO case definitions (*8*). In January 2015, a total of 8 suspected measles cases were reported in Lola, with no subprefecture exceeding the WHO Regional Office for Africa definition of a suspected outbreak (5 suspected cases in 1 month) (*8*). In February 2015, another 20 suspected measles cases were reported in Lola; 12 were in the subprefecture of N'Zoo. A suspected outbreak was declared, and 6 blood specimens from N'Zoo were sent to the national measles reference laboratory in Conakry. Five samples were confirmed positive for measles (1 sample was inadequate for testing), thereby confirming the measles outbreak according to the WHO Regional Office for Africa definition (≥3 laboratory-confirmed measles cases in a health district in 1 month) (*8*). Thereafter, measles transmission intensified, and confirmation of measles cases was symptom-based. In total, DPS reported 702 measles cases in Lola during January 1–June 30, 2015 (Figure). No deaths were reported.

In response to the measles outbreak and in parallel to its Ebola efforts, the WHO Global Outbreak Alert and Response Network (GOARN) field team in Lola worked with DPS to strengthen epidemiologic surveillance, conduct field investigations, assist in social mobilization, and collect case information. Beginning in March 2015, active measles surveillance was reinforced through daily phone calls to regional health centers in each subprefecture of Lola to quickly identify new areas of transmission. In addition, the nongovernmental agency Alima offered free vitamin A supplements to measles patients (*9*), a valuable strategy for areas with poor nutritional levels and low herd immunity. Meanwhile, Guinea received a large shipment of vaccines by the end of March (*6*), but these did not arrive in Lola until mid-April. A vaccination campaign was launched on April 18, with a goal of 95% 1-dose coverage for all children in Lola 6 months to 9 years of age (53,720 children).

The measles case database constructed by the WHO GOARN field team covers every subprefecture in Lola and records cases identified during the early phase of the outbreak, during January 23, 2015–April 4, 2015. Although the case data are not exhaustive (n = 284), the database can be used to analyze transmission dynamics. The average and median age of case-patients was 2.8 years and 2.0 years,

[1]Preliminary results from this study were presented at the European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE); November 11–13, 2015; Stockholm, Sweden.

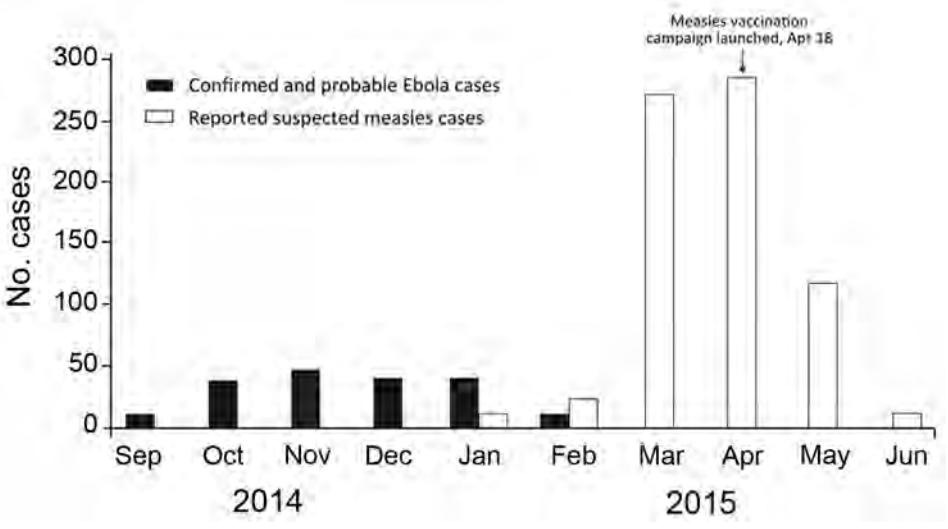

Figure. Ebola and measles cases per month in Lola, Guinea, September 2014–June 2015. Ebola data provided by the World Health Organization Global Outbreak Alert and Response Network field team in Lola. Measles data is monthly surveillance data reported by the Direction Préfectorale de la Santé, Lola.

respectively, substantially lower than the mean age of infection of 5.6 years in Africa (*10*). Of 284 case-patients, 263 (92.6%) were 0–5 years of age, 17 (6.0%) were 6–15 years of age, and 4 (1.4%) were >15 years of age. This low age distribution is indicative of a disruption in vaccination and also suggests that higher immunity existed among older age groups (*11*), related to either strong pre-Ebola vaccination coverage or prior exposure to measles. Of 281 cases with reported vaccination status, 267 (95%) were not vaccinated and 14 (5%) were vaccinated; health records did not differentiate between 1- and 2-dose coverage.

Persons in the subprefecture of N'Zoo in Lola benefited from active measles surveillance implemented by a well-organized local health team. N'Zoo was also the first subprefecture population in Lola to receive free vitamin A supplements (beginning March 2015). N'Zoo represents 8.7% of the population of Lola (15,559 inhabitants) but accounted for 30% of reported measles cases in Lola (212/702). Because of its efficiency of surveillance, N'Zoo may offer a better estimate of what occurred throughout the prefecture. Extrapolating the incidence rate from N'Zoo across all of Lola yields 2,450 cases. This figure should be treated with caution, however, because measles transmission dynamics cannot be assumed to have been consistent across Lola. Additionally, over-reporting during measles outbreaks, particularly in areas where active surveillance is implemented, is a possibility when cases are diagnosed principally on the basis of symptoms (*12*).

Discussion

Many barriers to an effective response to measles existed in Lola. Healthcare-seeking behaviors declined markedly during the Ebola epidemic (*2*), which likely contributed to probable underreporting of measles cases, a fact further substantiated by the lack of fatalities reported to DPS. Although there are wide variances in estimates of measles case-fatality rates, a large study has suggested an average case-fatality rate of 3.7% for Africa (*13*), which would correspond with 26 deaths from the 702 suspected cases reported in Lola during January–June 2015. The lack of reported fatalities may also be related to the effect of the Ebola epidemic on burial practices; any families notifying authorities about deaths would be required to conduct "safe and dignified burials," a protocol that had been met with resistance by many local groups (*2*).

Another barrier to response was the late initiation of the vaccination campaign. When the full shipment of vaccines arrived in Lola in mid-April, logistical planning was challenged by shortages of personnel, fuel for automobiles, and appropriate vehicles for traversing difficult terrain during the onset of the rainy season. The campaign is estimated to have reached 92% of the target population, but persons in some urban areas and villages were reluctant to receive vaccinations. The launch of the campaign coincided with reduced measles transmission (Figure), but further modeling research would be required to assess its effect on the course of the outbreak.

In the aftermath of the Ebola epidemic, discussions about strengthening global outbreak response capacities are ongoing (*14*,*15*). The front lines of disease surveillance and outbreak detection often occur in rural settings that are understaffed and underresourced. As Guinea transitions to a post-Ebola phase, the field presence of public health doctors may be reduced. This reduction would be unfortunate, because the technical and cultural expertise of the doctors from Guinea in Lola and other similar settings transcends Ebola and could be harnessed to support a wide range of public health activities.

Strengthened investments in local public health systems will be essential to ensure the population of Guinea

can recover from the Ebola epidemic and be better protected from future disease outbreaks. Aside from personnel, the public health infrastructure, including surveillance, information and communications technology, and temperature-controlled supply chains, particularly requires attention. Meanwhile, great efforts will also be needed to restore and enhance community trust in medicine and public health (*2*).

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Changes in Childhood Pneumonia Hospitalizations by Race and Sex Associated with Pneumococcal Conjugate Vaccines

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Introduction of pneumococcal conjugate vaccines in the childhood immunization schedule was associated with decreases in all-cause pneumonia hospitalizations among black and white children in Tennessee, USA. Although racial disparities that existed before introduction of these vaccines have been substantially reduced, rates remain higher in boys than in girls among young children.

Introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) into the childhood immunization schedule was associated with substantial reductions in invasive pneumococcal disease (IPD) and pneumonia in young children in the United States and elsewhere (*1*–*4*). In the United States specifically, black children traditionally had higher IPD and pneumonia rates than white children. However, this racial disparity in IPD was reduced after introduction of PCV7 (*5*).

After the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) in 2010, IPD and pneumonia hospitalizations among children <5 years of age were reduced further, and racial differences in IPD reached an historic low (*2*,*6*,*7*). However, the effect of PCVs on racial disparities for pneumonia, which is much more common than IPD, remains unclear. Furthermore, sex seems to persist as an independent risk factor for IPD after PCV7 introduction (*8*).

Identifying high-risk groups is essential for planning preventative strategies, but few studies have directly examined sex differences in pneumonia incidence since introduction of PCVs (*9*). Therefore, we examined changes in childhood pneumonia hospitalization rates between racial and sex groups since introduction of PCVs.

The Study

We used hospitalization data from all nonfederal hospitals in Tennessee for 1998–2013 in the Tennessee Hospital Discharge Data system. Pneumonia hospitalizations among Tennessee residents <18 years of age were defined by an International Classification of Diseases, Ninth Revision, Clinical Modification, coded diagnosis of pneumonia in any discharge diagnosis position. We also analyzed the rate of pneumonia hospitalizations with a more specific definition based on a primary discharge diagnosis of pneumonia or any diagnosis of pneumonia with a primary diagnosis of meningitis, septicemia, or empyema (*10*,*11*). These strategies for identification of pneumonia hospitalizations have been validated in other data systems (*10*,*11*).

Racial categories were black and white. Race-specific rates were not calculated for Hispanic and other racial-ethnic categories because of low numbers of hospitalizations (7.9% of total). Age groups were <2, 2–4, and 5–17 years of age. Four periods were defined that excluded years of vaccine introduction: pre-PCV7 (1998–1999), early PCV7 (2001–2005), late PCV7 (2006–2009), and post-PCV13 (2011–2013).

Annualized period rates were calculated for each study period by dividing the average annual pneumonia hospitalizations by the average annual Tennessee population estimate for all children combined and grouped by age, sex, and race (US Census Bureau and the National Center for Health Statistics Bridged Race Population Estimates). Rates were expressed as pneumonia hospitalizations/1,000 children annually. For comparisons, rate ratios and rate differences were calculated with 95% CIs to assess significance of the estimates at the 5% level. All analyses were performed by using STATA version 14 (StataCorp LP, College Station, TX, USA).

Pneumonia hospitalization rates decreased among children <18 years of age during the study period. Overall and age-specific pneumonia hospitalization rates include all race/ethnicity categories (black, white, Hispanic, other) because these estimates were not different from estimates that included only black and white children (Table). Most hospitalizations were for children <2 years of age in the pre-PCV7 period (49.0%) and for children 5–17 years of age in the post-PCV13 period (38.4%).

Overall, rates of pneumonia hospitalizations were higher among black children than among white children in the pre-PCV7 period for boys and girls (rate ratio [RR] 1.37, 95% CI 1.29–1.45 and RR 1.19, 95% CI 1.12–1.27, respectively). However, during the post-PCV13 period, black and white girls showed similar rates of pneumonia hospitalizations (RR 0.98, 95% CI 0.91–1.06). Rates for

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Table. Pre-PCV7 and post-PCV13 annualized pneumonia hospitalizations, by age, sex, and race, per 1,000 children, Tennessee, USA, 1998–2013*

Patient age, y, and race	Patient sex	Hospitalizations/1,000 children		Post-PCV13 vs. pre-PCV7	
		Pre-PCV7, 1998–1999, 11,059 hospitalizations	Post-PCV13, 2011–2013, 9,171 hospitalizations	Rate ratio (95% CI)	Rate difference (95% CI)†
All‡		4.0	2.0	0.51 (0.50–0.52)	−2.0 (−2.1 to −1.9)
<2‡		18.4	7.3	0.40 (0.38–0.41)	−11.1 (−11.6 to −10.6)
Black	M	27.1	8.5	0.31 (0.28–0.35)	−18.6 (−20.5 to −16.6)
Black	F	20.0	6.4	0.32 (0.28–0.37)	−13.6 (−15.3 to −11.9)
White	M	18.0	7.8	0.43 (0.40–0.46)	−10.2 (−11.1 to −9.3)
White	F	14.5	6.2	0.43 (0.40–0.47)	−8.2 (−9.0 to −7.4)
2–4‡		5.4	3.0	0.56 (0.52–0.59)	−2.4 (−2.6 to −2.1)
Black	M	6.6	3.4	0.50 (0.43–0.59)	−3.3 (−4.1 to −2.5)
Black	F	5.3	3.1	0.58 (0.49–0.70)	−2.2 (−3.0 to −1.5)
White	M	5.3	3.0	0.56 (0.51–0.62)	−2.3 (−2.7 to −1.9)
White	F	5.0	2.9	0.59 (0.53–0.65)	−2.0 (−2.4 to −1.6)
5–17‡		1.6	1.1	0.67 (0.63–0.70)	−0.5 (−0.6 to −0.5)
Black	M	2.0	1.1	0.57 (0.49–0.65)	−0.8 (−1.1 to −0.6)
Black	F	1.6	1.0	0.61 (0.53–0.71)	−0.6 (−0.8 to −0.4)
White	M	1.6	1.1	0.70 (0.65–0.75)	−0.5 (−0.6 to −0.4)
White	F	1.6	1.2	0.74 (0.68–0.80)	−0.4 (−0.5 to −0.3)

*PCV7, 7-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine.
†For hospitalizations per 1,000 children.
‡Includes all race/ethnicity categories (black, white, Hispanic, other). These estimates were not different from estimates including only black and white children.

black boys also decreased but were still higher than rates for white boys (RR 1.12, 95% CI 1.05–1.20) during the post-PCV13 period.

For children <2 years of age, pneumonia hospitalization rates decreased in each successive period for each of the 4 race/sex groups (Figure). Among children <2 years of age, black boys, black girls, and white boys showed higher pneumonia rates than white girls during the pre-PCV7 and PCV7 periods. In the post-PCV13 period, black and white girls showed similar rates, and rates for black and white boys remained higher than rates for white girls among children <2 years of age. Decreases for each of the 4 race/sex groups were also observed for children 2–4 and 5–17 years of age (Figure). However, no disparities were observed by race or sex in the post-PCV13 period for children 2–4 and 5–17 years of age (Figure).

When we compared post-PCV13 with pre-PCV7 for all age groups, we found that black boys had the greatest relative and absolute decreases for pneumonia hospitalization rates; the largest decreases were for children <2 years of age. We estimated that there were 18.6 fewer pneumonia hospitalizations per 1,000 black boys <2 years of age during the post-PCV13 period than during the pre-PCV7 period (Table). Similar trends were observed when we restricted our assessment to the subset of hospitalizations identified with the more specific definition of pneumonia (data not shown).

Conclusions

Rates of pneumonia hospitalizations decreased among black and white children in Tennessee after introduction of PCVs, in particular among children <2 years, with only minimal residual racial differences in the post-PCV13 period. In spite of these reductions, for children <2 years of age, rates of pneumonia hospitalizations for black and white boys were consistently higher than for girls. However, no differences were observed by race or sex in the post-PCV13 period for children 2–4 and 5–17 years of age.

An explanation for the persistent differences by sex in pneumonia incidence could be the proposed effect of sex hormones on the immune response or differences in behavior among young boys and girls (*9*,*12*). However, these observations require further scrutiny because few studies have examined the association between infection risk and sex-associated behavior or immune response in children (*9*,*12*).

Our study had several limitations. First, this was an ecologic study, and we cannot attribute changes in pneumonia hospitalization directly to PCVs. However, these designs are preferred for the evaluation of programs with high uptake and potential indirect benefits, such as PCV vaccination programs, because they avoid known selection issues that could affect comparisons of vaccinated and unvaccinated persons, and estimates of effect include those resulting from indirect vaccine effects. Second, the study was restricted to only hospitalizations, and additional studies conducted in the outpatient setting would be useful to complement these observations. However, previous studies have documented no compensatory increases in emergency department visits that could result from observed reductions in hospitalizations (*13*). Moreover, another study reported no changes in hospitalizations for fractures among children in Tennessee during 1998–2012 associated with introduction of PCVs, which should reduce concerns about

generalized changes in hospitalization practices in Tennessee (*2*). Third, other factors, such as socioeconomic characteristics or concurrent conditions, could contribute to observed differences between racial and sex groups. However, our study did not account for those factors. Fourth, because our study documents changes for only children in Tennessee, caution is warranted when extrapolating our findings to other settings.

Overall, these findings suggest that PCV7 and PCV13 have substantially reduced racial disparities in all-cause pneumonia between black and white children in Tennessee that existed before PCVs, especially among children <2 years of age. Although relative rate comparisons by sex for children <2 years of age after introduction of PCV13 indicated statistically significant differences, the absolute differences in rates were small and much lower than in the pre-PCV7 period.

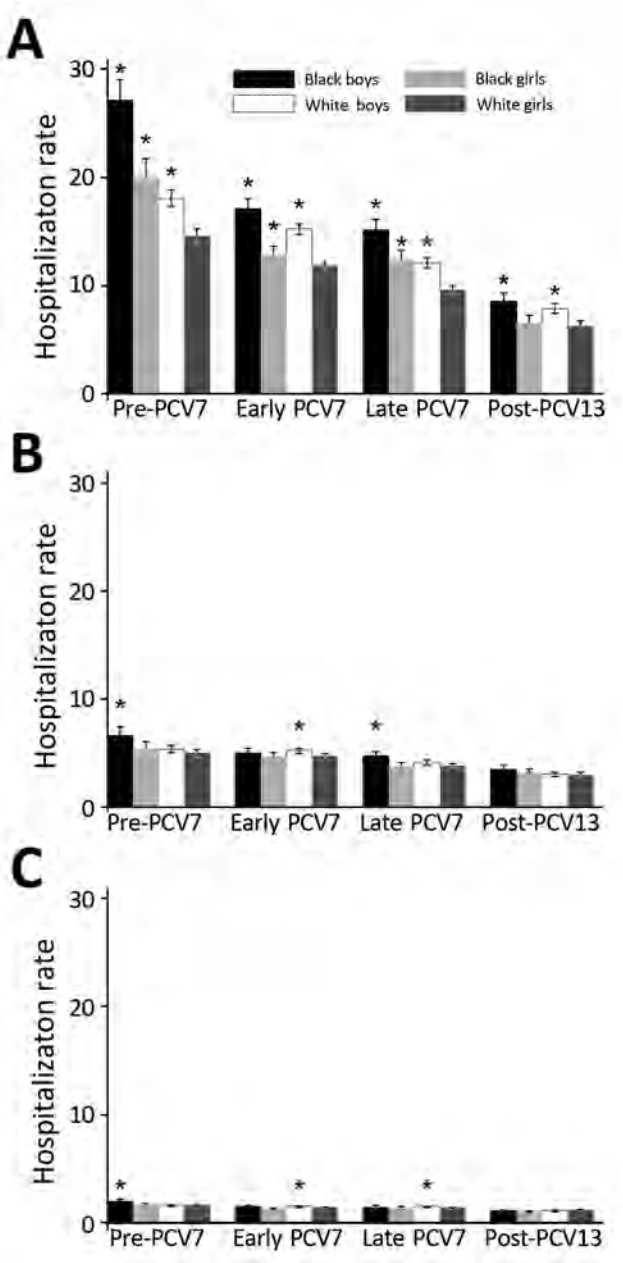

Figure. Annualized all-cause pneumonia hospitalization rates, by age group, race, and sex, per 1,000 children, Tennessee, USA, 1998–2013. A) <2 years of age; B) 2–4 years of age; C) 5–17 years of age. Asterisks indicate groups with a significantly higher rate than white girls within each period, determined on the basis of the 95% CI of the respective rate ratio (not shown). Error bars indicate 95% CIs of hospitalization rate. Periods were defined as follows: pre-PCV7, 1998–1999; early PCV7, 2001–2005; late PCV7, 2006–2009; and post-PCV13, 2011–2013.

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Antibody Response and Disease Severity in Healthcare Worker MERS Survivors

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We studied antibody response in 9 healthcare workers in Jeddah, Saudi Arabia, who survived Middle East respiratory syndrome, by using serial ELISA and indirect immunofluorescence assay testing. Among patients who had experienced severe pneumonia, antibody was detected for ≥18 months after infection. Antibody longevity was more variable in patients who had experienced milder disease.

A study evaluating the immune response in patients infected with severe acute respiratory syndrome coronavirus (SARS-CoV) showed antivirus antibodies in survivors can be detected by ELISA and immunofluorescence assay (IFA) for up to 24 months after infection (*1*). Another study revealed that SARS-CoV antibodies were not detectable at 6 years after infection (*2*). Antibody response to Middle East respiratory syndrome coronavirus (MERS-CoV) typically is detected in the second and third week after the onset of the infection (*3*–*5*), but little is known about the longevity of the response or whether the decrease in antibody response over time correlates with the severity of the initial infection. We conducted a longitudinal study of antibody response among a cohort of MERS survivors who had been treated at King Faisal Specialist Hospital and Research Center in Jeddah, Saudi Arabia (KFSHRC-J).

The Study

Our research proposal was approved by the KFSHRC-J institutional review board. Written informed consent was obtained from all study participants. During the Jeddah MERS outbreak in 2014, we tested specimens from 1,412 patients with suspected MERS-CoV infection by using a real-time reverse transcription PCR (rRT-PCR) assay. We identified 40 confirmed cases on the basis of rRT-PCR–positive specimens obtained by nasopharyngeal swab or bronchoalveolar lavage, as described previously (*6*; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/16-0010-Techapp1.pdf). For each patient, ≥2 specimens were analyzed and rRT-PCR was conducted twice. Eighteen of 40 cases were in healthcare workers (HCWs); 12 of these 18 HCWs were symptomatic. The 6 asymptomatic HCWs were identified through contact tracing during active hospital surveillance for MERS cases. The patient cohort for this study consisted of 9 HCWs who were MERS-CoV–positive on the basis of rRT-PCR results and who agreed to provide blood samples for serial serologic testing for MERS-CoV by ELISA and IFA.

Patients' medical records were reviewed for information on demographic characteristics, comorbidities, clinical presentation, intensive care unit admission, and outcome. Patients were classified into 4 categories according to their clinical presentation: asymptomatic, upper respiratory tract infection, pneumonia, or severe pneumonia. Patients with severe pneumonia were those who required intubation and ventilatory support and were treated in an intensive care unit. Serial ELISA and IFA testing was performed at 3, 10 and 18 months after illness onset (online Technical Appendix). Specimens were considered to represent previous infection only when ELISA and IFA test results both were positive. Microneutralization testing was not available in the KFSHRC-J laboratory.

Disease onset corresponded to the date of the first MERS-CoV–positive rRT-PCR result. Data were available for analysis from 9 patients who were MERS-CoV–positive and had serial MERS-CoV serologic testing at 3 and 10 months after illness onset. Patients with severe pneumonia who were MERS-CoV-antibody–positive at 10 months had follow-up testing at 18 months. Serum samples could not be obtained from patient 3, who was also MERS-CoV-antibody–positive at 10 months. All patients were initially healthy without underlying conditions except patient 2 (Table), who had hypothyroidism. Four of the 9 patients were women; 2 of them, patients 2 and 8, were 32 weeks and 20 weeks' pregnant, respectively, when they had MERS-CoV infection. Average patient age was 38 years (range 27–54 years).

Of the 9 patients, 2 had severe pneumonia, 3 had milder pneumonia not requiring intensive care, 1 had upper respiratory tract disease, and 3 remained asymptomatic. All patients recovered without sequelae. The 2 patients with severe pneumonia had the highest antibody titers detected

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Table. Antibody response in 9 confirmed survivors of MERS-CoV infection, by selected demographic and clinical characteristics, King Faisal Specialist Hospital and Research Center, Jeddah, Saudi Arabia, 2014*

Patient no.	Age, y/sex	Clinical presentation	Test results: PCR, C_t NPS	PCR, C_t BAL	Serology at 3 mo ELISA†	Serology at 3 mo IFA‡	Serology at 10 mo ELISA†	Serology at 10 mo IFA‡	Serology at 18 mo ELISA†	Serology at 18 mo IFA‡
1	49/M	Severe pneumonia	28	26	+ (3.17)	+	+ (2.99)	+	+ (3.3)	+
2	33/F	Severe pneumonia	31	26	+ (2.7)	+	+ (2.09)	+	+ (2.9)	+
3	54/F	Pneumonia	34	ND	+ (2.91)	+	+ (1.9)	+	ND	ND
4	40/M	Pneumonia	32	ND	+ (1.29)	+	– (0.65)	–	ND	ND
5	37/M	Pneumonia	35	ND	+ (3.2)	+	+ (1.2)	–	ND	ND
6	36/M	URTI	32	ND	– (0.07)	–	– (0.07)	–	ND	ND
7	27/F	Asymptomatic	33	ND	– (0.046)	–	– (0.04)	–	ND	ND
8	28/F	Asymptomatic	32	ND	– (0.12)	–	– (0.06)	–	ND	ND
9	35/M	Asymptomatic	33	ND	– (0.07)	–	– (0.04)	–	ND	ND

*+, positive; –, negative; BAL, broncholaveolar lavage; Ct, cycle threshold; IFA, indirect-immunofluorescence assay; MERS-CoV, Middle East respiratory syndrome coronavirus; ND, not done; NPS, nasopharyngeal swab; URTI, upper respiratory tract infection.
†ELISA for MERS-CoV S gene antibody; positive defined as a value >1.1, negative as <0.8, and borderline as between 0.8 and 1.1.
‡IFA for MERS-CoV IgG; endpoint titers not done.

among all patients and remained MERS-CoV-antibody–positive when tested at 18 months after illness onset. They also had prolonged viral shedding documented by persistent positive rRT-PCR results for 13 days (patient 1) and 12 days (patient 2); rRT-PCR analyses were negative after 2–5 days for patients 4–9. rRT-PCR was only repeated at day 13 for patient 3, and the result was negative. Three patients with pneumonia were MERS-CoV-antibody–positive at 3 months, but antibody was detected in only 1 of the 3 at 10 months (Table). All patients who had an upper respiratory tract function or remained asymptomatic had no detectable antibody response on the basis of ELISA and IFA results.

Conclusions

Our results indicate that the longevity of the MERS-CoV antibody response correlated with disease severity. Accordingly, 2 patients with severe MERS-associated pneumonia had a persistent antibody response detected for ≥18 months after infection, whereas patients with disease confined to the upper respiratory tract or who had no clinical signs had no detectable MERS-CoV antibody response. Two previous studies have described longitudinal analyses of MERS-CoV surface glycoprotein–specific antibody responses in recovered patients. In the first study, which described a MERS outbreak in Jordan (*7*), MERS-CoV antibodies, including neutralizing antibodies, were still detectable in 7 patients with pneumonia 13 months after infection; most of these patients had severe pneumonia. In the second study, Drosten et al. (*8*) demonstrated that MERS-CoV neutralizing antibodies were produced at low levels after mild or subclinical infection and were potentially short-lived.

The results of our study have implications for understanding the pathogenesis and the treatment of MERS. First, patients with mild or subclinical infections who had no detectable antibody response might be at risk for recurrent infection and would also not be detected in population-based studies, resulting in falsely low prevalence rates. Previous studies suggest that neutralizing antibodies were not sufficient to clear MERS-CoV, because neutralizing antibodies were detected in up to 50% of fatal MERS cases and antibody levels did not correlate well with virus load in the lungs (*3*). T-cell responses are critical for protection from subsequent challenge in animals experimentally infected with SARS-CoV (*9*). Although T cells have persisted up to 6 years in SARS survivors (*2*), whether these patients would have been protected if infected a second time with SARS-CoV is unknown. Additional studies will be required to assess the relative importance of T- and B-cell responses in MERS survivors. Second, we speculate that patients with low-level virus replication could provide a reservoir for infection of highly susceptible humans (i.e., those with underlying conditions). Such patients would be difficult to detect because they are only transiently positive for MERS-CoV antibody or might never mount an antibody response to MERS-CoV. Third, patients who recovered from severe pneumonia associated with MERS probably would be good candidates for providing MERS-CoV–specific convalescent-phase serum samples for use in treatment trials.

Our study is limited by the small number of patient numbers and the lack of neutralizing antibody testing. ELISA is highly sensitive but might cross-react with seasonal human coronavirus antibodies (*10*); it is useful as a screening test because it is 10-fold more sensitive than IFA. An IFA is required for confirmation (*11*), and use of a spike protein–specific IFA greatly diminishes the likelihood of cross-reactivity. Neutralization assays are considered definitive and must be performed whenever the results of ELISA and IFA are not conclusive (*12*). In the 9 patients reported here, ELISA and IFA results were consistent and conclusive. A limitation of this study is that serologic testing from single patients was not performed on the same day; therefore, only qualitative but not quantitative conclusions about changes with time after illness onset can be made.

In conclusion, our results indicate that MERS-CoV antibody persistence depends on disease severity. Further studies are required to determine the role of the

virus-specific T-cell response in MERS patients and determine whether patients with mild infections are at risk for reinfection and would therefore benefit from vaccination. Our data also show that potential donors of MERS-CoV convalescent-phase serum samples are limited to patients who recover from severe pneumonia.

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Epidemiology of Pulmonary Nontuberculous Mycobacterial Disease, Japan[1]

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To the Editor: Incidence of pulmonary nontuberculous mycobacterial disease (PNTMD) is reportedly increasing globally (*1*,*2*). Although such an increase is expected in Japan (*3*,*4*), the epidemiologic situation is unclear. The most recent survey, which used the 1997 American Thoracic Society diagnostic criteria, reported that the incidence rate for PNTMD in 2007 was 5.7 cases per 100,000 person-years (*5*). To update the data, we performed a nationwide hospital-based survey in Japan.

After a preliminary survey of 20 hospitals, we developed and disseminated questionnaires to all 884 hospitals in Japan that were certified by the Japanese Respiratory Society. The surveys asked about the number of newly diagnosed cases, from January through March 2014, of PNTMD, pulmonary *Mycobacterium avium* disease, *M. intracellulare* disease, or *M. avium* complex (MAC; the combination of the first 2 species listed); pulmonary *M. kansasii* disease; pulmonary *M. abscessus* disease; and tuberculosis (TB) for inpatients and outpatients. Hospital respondents returned the completed questionnaires by mail, fax, or Internet. To avoid potential reporting bias and misclassification, we counted only cases that met the 2007 American Thoracic Society/Infectious Diseases Society of America statements (*6*) and excluded cases diagnosed at other hospitals. Because the source population can be ascertained by using the epidemiologic data for TB as a reportable disease, to estimate the incidence rate of PNTMD, we used the ratio of TB to PNTMD cases. The PNTMD incidence rate was calculated as the national incidence rate of TB multiplied by the ratio of new PNTMD to TB cases reported by the responding hospitals (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/6/15-1086-Techapp1.pdf).

To clarify the chronologic changes in incidence, we followed the same method for comparing TB and PNTMD used in a prior epidemiologic study in Japan (*5*). We established methods for maximizing survey response rates and facilitating ease of completion by offering extensive support to survey recipients (online Technical Appendix Table 1).

We achieved a high response rate of 62.3% (551 hospitals), and in all regions the response rate exceeded 50% (online Technical Appendix Table 2). The numbers of newly diagnosed cases were 2,327 for TB and 2,652 for PNTMD. Because the incidence rate for TB was reported to be 12.9 cases per 100,000 person-years, that of PNTMD was estimated to be 14.7 cases per 100,000 person-years, which is ≈2.6 times the incidence rate reported in 2007 (Figure). By using the same method, we found the incidence of pulmonary MAC, *M. kansasii*, and *M. abscessus* disease to be 13.1, 0.6, and 0.5 cases per 100,000 person-years, respectively (online Technical Appendix Table 2). The ratio of pulmonary *M. avium* disease to MAC was higher in the northern and eastern parts of Japan, whereas the ratio of pulmonary *M. intracellulare* disease to MAC was higher in the southern and western parts of Japan (online Technical Appendix Figure 1).

From this survey, we observed that the incidence rate of PNTMD may exceed that of TB and that incidence rates of PNTMD in Japan may be among the highest worldwide (Figure). This finding implies that the prevalence of PNTMD as a chronic infection is estimated to be much higher than that of TB.

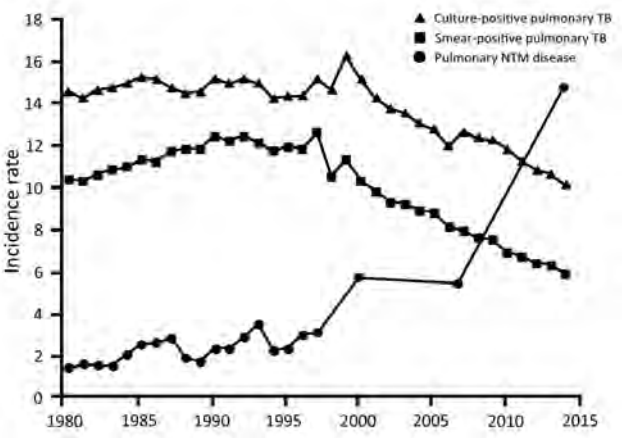

Figure. Incidence (no. cases/100,000 person-years) of pulmonary nontuberculous mycobacterial (NTM) disease, culture-positive tuberculosis (TB), and smear-positive TB in Japan during 1980–2014. The nationwide survey revealed that the incidence rate of pulmonary NTM disease exceeds that of TB. The epidemiologic survey before 1988 was conducted annually by the same research group; subsequently, another group performed the epidemiologic survey only in 2001 and 2007.

[1]Preliminary results from this study were presented at the American Thoracic Society 2015 International Conference; 2015 May 15–20; Denver, Colorado, USA (http://www.atsjournals.org/doi/abs/10.1164/ajrccm-conference.2015.191.1_MeetingAbstracts.A5268).

We assume that the high rates of PNTMD in Japan are consistent with data suggesting that Asians are particularly susceptible to PNTMD (*1*,*7*,*8*). Other factors contributing to the increase might be the simplified diagnosis according to the 2007 American Thoracic Society/Infectious Diseases Society of America statements, increased awareness by medical staff, population aging, and increased frequency of medical checkups with computed tomography of the chest.

Another finding was the characteristic gradient clustering of the ratios of *M. avium* and *M. intracellulare* (online Technical Appendix Figure 2). This finding supports the widely accepted belief that environmental factors strongly affect the epidemiology of PNTMD; therefore, the role of factors such as soil, humidity, temperature, and saturated vapor pressure should be seriously considered (*9*).

We also found dramatic increases in incidence of pulmonary *M. abscessus* disease and pulmonary MAC disease, whereas incidence of pulmonary *M. kansasii* disease was stable. Although we did not distinguish *M. massiliense* from *M. abscessus*, the incidence rate for pulmonary *M. abscessus* disease increased from 0.1 cases in 2001 to 0.5 cases per 100,000 person-years in 2014. This epidemiologic tendency should be monitored (*10*).

This study has several limitations. First, differing characteristics between the responding and nonresponding hospitals could cause bias. Second, we did not collect data outside of hospitals. Third, incomplete reporting could undermine the accuracy of our estimates (online Technical Appendix Tables 3, 4). Therefore, the epidemiologic data should be verified by using other approaches (online Technical Appendix Table 1).

The dramatic increase in incidence rates for PNTMD warrants its recognition as a major public health concern. Because the prevalence rates of this currently incurable lifelong chronic disease are estimated to be high, the effect on the community could be enormous. Further investigations are needed.

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Elevated Pertussis Reporting in Response to 2011–2012 Outbreak, New York City, New York, USA

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To the Editor: Pertussis is a highly communicable, acute bacterial respiratory infection caused by *Bordetella pertussis*. In the United States, the incidence of pertussis declined dramatically after pertussis-containing vaccine was introduced in the 1940s (*1*,*2*). However, a resurgence

of disease results in widespread outbreaks of pertussis nationally (*3*).

Beginning in August 2011, an outbreak of pertussis occurred in New York City (NYC), New York, USA. Reported pertussis incidence by month peaked in December 2011 (1.03 cases/100,000 persons) and remained above the baseline average monthly incidence of 0.11 cases/100,000 persons until February 2013. We hypothesized that provider awareness and altered practices after the start of the outbreak contributed to the sustained elevation in reported pertussis incidence.

To test this hypothesis, we surveyed NYC providers to assess their awareness of the outbreak, their consideration of pertussis in symptomatic patients, and the type and frequency of diagnostic testing ordered. The survey (available on request) was designed in FeedbackServer 5 (University of Massachusetts, Worcester, MA, USA; https://w3.umassmed.edu/fs/FeedbackServer/help/feedbackserver.htm) and consisted of 20 questions that required ≈5 minutes to complete by using a Web link. We distributed the survey in January 2013 to providers through 3 health department email lists: the NYC Health Alert Network, the Citywide Immunization Registry, and the Primary Care Information Project. The lists included ≈30,000 email addresses that were not mutually exclusive and that included nonmedical providers.

Through March 7, 2013, we received 1,316 responses; 887 (67%) were excluded from analyses for ≥1 reason: respondent did not complete all survey questions (74%); respondent did not practice in a hospital or outpatient facility (31%); respondent indicated that his or her primary facility was located outside NYC (6%); or response was a duplicate (<1%). Of the 429 (33%) responses included in our analyses, 69% of respondents served adults and 54% served children (23% served both adults and children); 38% practiced in a hospital, and 81% practiced in an outpatient setting (18% practiced in both hospital and outpatient settings).

Respondents were asked if and how they were aware of the pertussis outbreak; 84% reported previous awareness of the outbreak. The top reported sources contributing to respondents' outbreak awareness included health advisory alerts (local [80%], state [36%], and national [40%]); media reports (36%); and discussion with colleagues (29%).

In addition, respondents were asked how likely they were to consider pertussis infection in patients with prolonged cough before 2012 and currently. Reported consideration of pertussis before 2012 varied: 35% of respondents were likely or very likely to consider pertussis, 33% were somewhat likely to consider pertussis, 30% were unlikely to consider pertussis, and 3% did not know (unknown). However, 73% of respondents said that they were more likely to consider pertussis at the time of the survey than before 2012. The top reported sources contributing to increased consideration of pertussis mirrored those contributing to outbreak awareness.

Respondents were last asked to assess the type and frequency of diagnostic testing they used before and since 2012 (Table). Most (66%) respondents indicated that they did not perform diagnostic testing for pertussis before 2012. Among the 34% who tested for pertussis before 2012, the

Table. Provider responses to survey questions related to diagnostic testing for pertussis, New York City, New York, USA*

Questions and responses	No. (%)
Did respondent perform diagnostic tests for pertussis before 2012?	
Did not perform diagnostic tests	282 (66)
Performed ≥1 diagnostic test	147 (34)
Which types of diagnostic tests did respondent perform before 2012?†	
Bacterial culture	68 (46)
PCR	66 (45)
Serology	35 (24)
DFA	41 (28)
Did respondent change the types of diagnostic tests he/she performed since 2012?‡	
Yes	52 (12)
No	269 (63)
Unknown	108 (25)
Which types of diagnostic tests was respondent more likely to perform since 2012?§	
Bacterial culture	17 (33)
PCR	33 (63)
Serology	6 (12)
DFA	5 (10)
No test	3 (6)
Did respondent change the frequency of diagnostic tests he/she performed since 2012?	
More frequent testing	93 (22)
Less frequent testing	14 (3)
Frequency of testing unchanged	231 (54)
No response provided	1 (<1)

*N = 429 except as indicated. DFA, direct fluorescent antibody test.
†For 147 respondents who reported performing ≥1 diagnostic test before 2012. Response choices are not mutually exclusive.
‡For all respondents, including those who reported not performing diagnostic tests before 2012.
§For 52 respondents who reported a change in type of diagnostic test performed since 2012. Response choices are not mutually exclusive.

main diagnostic methods used were bacterial culture (46%) and PCR (45%). However, 12% of respondents indicated that they had changed the type of diagnostic test they used beginning in 2012; among these respondents, 33% were more likely to use pertussis culture and 63% were more likely to use PCR or to use culture and PCR. Of total respondents, 22% indicated that they ordered diagnostic tests more frequently since the beginning of 2012.

Our investigation has limitations. We could not determine a survey response rate because of extensive overlap of the email lists used, and we lacked access to the lists; the response rate is assumed to be very low. Respondents included in the analysis may not have been representative of the broader NYC provider community. In addition, respondents may not have uniformly interpreted the survey because of the subjective nature of some survey questions, and recall bias may have affected responses. Also, respondent awareness of the outbreak is likely overestimated because the email lists used for survey distribution were used during the outbreak to distribute health alerts. Despite these limitations, our investigation shows the value of Web-based surveys distributed by email to gather information rapidly from a large provider community in a cost-effective and practical manner.

This investigation indicates the importance of provider knowledge and practices for public health surveillance data. High awareness of an outbreak, increased clinical suspicion of pertussis, and increased frequency of diagnostic testing likely contributed to a sustained elevation in pertussis incidence. Advisory alerts and media reports were successful mechanisms for disseminating information to providers during the outbreak and likely altered provider behaviors that contributed to the increase in reported pertussis incidence. Previous reports have documented increased submission of disease notifications after media coverage of health concerns (*4*,*5*). Responses to our survey also highlight how pertussis incidence may be routinely underestimated because providers do not suspect the disease or test for it consistently.

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Hemophagocytic Lymphohistiocytosis and Progressive Disseminated Histoplasmosis

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To the Editor: Progressive disseminated histoplasmosis (PDH) of infancy occurs most commonly in previously healthy infants <1 year of age, typically after exposure to a large fungal inoculum (*1*). Even with treatment, the disease is fatal in ≈13% of cases (*1*). Common symptoms include fever, hepatosplenomegaly, lymphadenopathy, and failure to thrive. Laboratory abnormalities frequently include cytopenia and coagulopathy (*1*,*2*). Many clinical manifestations of PDH overlap with those of the hyperinflammatory condition hemophagocytic lymphohistiocytosis (HLH), and co-existence of HLH and histoplasmosis has been reported in adults. We report a case of simultaneous PDH and HLH in an infant.

A 6-month-old African American girl was brought for treatment to St. Jude Children's Research Hospital in April 2015 with a 1-month history of daily fever. Her history was notable only for a methicillin-resistant *Staphylococcus aureus* skin abscess diagnosed when she was 5 months old; it was drained, and she received oral clindamycin.

On initial evaluation, she had fever, lethargy, and hepatosplenomegaly. Laboratory testing showed pancytopenia and mild hepatitis, and abdominal ultrasound confirmed hepatosplenomegaly. After admission, respiratory distress

developed due to worsening hepatosplenomegaly. Additional laboratory testing revealed elevated ferritin (1,218 ng/mL; reference 10–100 ng/mL), low fibrinogen level (78 mg/dL; reference 185–443 mg/dL), elevated triglycerides (378 mg/dL; reference 0–149 mg/dL) and elevated soluble interleukin-2 receptor (21,530 pg/mL; reference <1,033 pg/mL). An HIV serologic test result was negative. Histologic examination of bone marrow showed many activated macrophages, including some with hemophagocytosis (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-1682-Techapp1.pdf). On the basis of clinical and laboratory findings, the patient received a diagnosis of HLH, and treatment was initiated with etoposide and dexamethasone.

Further evaluation of the bone marrow sample demonstrated fungal elements characteristic of *Histoplasma capsulatum* (online Technical Appendix). *Histoplasma* antigen assays on serum and urine samples were positive for *H. capsulatum* above the limit of quantification. *Histoplasma* complement-fixation antibody titers for both yeast and mycelial antigens were negative, but immunodiffusion antibody tests for H and M bands were positive. Cerebrospinal fluid was also positive for *Histoplasma* antigen. Results of magnetic resonance imaging of the patient's brain were normal for age.

Because the patient's condition met diagnostic criteria for both HLH and PDH, she was given etoposide, dexamethasone, and liposomal amphotericin B (LAmB), according to treatment guidelines for both conditions (*3*,*4*). Supportive care included transfusion of packed red blood cells, platelets, cryoprecipitate, and fresh frozen plasma. Her fever resolved after 10 days, and other symptoms resolved after 2 weeks. After 16 days of hospitalization, she was discharged to complete a 6-week course of LAmB with a plan to transition to oral itraconazole and complete 1 year of therapy, according to guidelines of the Infectious Diseases Society of America (*4*).

HLH is a rare disease, characterized by impaired function of natural killer and cytotoxic T-lymphocytes that results in an unchecked inflammatory response. If not treated promptly, HLH can progress rapidly to multiorgan failure and death (*3*). HLH is categorized into familial or secondary forms. Familial HLH is caused by mutations in ≥1 of the genes required for perforin-dependent lymphocyte cytotoxicity; secondary HLH occurs in a person who does not have genetic risk factors (*3*). Both forms can be triggered by infection or malignancy (*3*).

Diagnosis of HLH is based on clinical assessment (Table); no definitive diagnostic test exists (*6*). Initial treatment of HLH involves the use of corticosteroids, etoposide, or other drugs to block the hyperinflammatory response and specific therapy for the inciting infection if available (*7*). In some cases, in which a treatable inciting infection is identified, antimicrobial drug therapy alone might be sufficient. However, concurrent immunosuppressive therapy is usually recommended, especially for patients who are critically ill or whose condition is clinically deteriorating (*3*).

Table. Diagnostic criteria for HLH*

Criterion	HLH reference	Patient in this report
Fever	≥38.5°C	40°C†
Splenomegaly	Present	Present†
Cytopenia, 2 of 3 lineages		
ANC, cells/mm^3	<1,000	1,500
Hemoglobin, g/dL	<9	5.9†
Platelet count, × 10^3/μL	<100	11†
Low fibrinogen, mg/dL	<150	78†
or		
High triglycerides, mg/dL	>265 fasting	378†
Hemophagocytosis	Present	Present†
NK cell activity	Low or absent	Normal
Elevated ferritin, ng/mL	>500	317 (max 1,218)†
Soluble IL-2 receptor, pg/mL	>2,400	21,530†

*ANC, absolute neutrophil count; HLH, hemophagocytic lymphohistiocytosis; IL-2, interleukin-2; NK, natural killer.
†Criteria met by this patient. The HLH-2004 diagnostic criteria require either a molecular/genetic diagnosis consistent with HLH or fulfillment of 5 of the 8 criteria shown. Adapted from (*5*).

Many clinical manifestations of disseminated histoplasmosis, including prolonged fever, hepatosplenomegaly, pancytopenia, and coagulopathy, overlap with those of HLH. Because of the similarity in manifestations, differentiating these 2 conditions is challenging without specialized testing. Furthermore, laboratory tests specific for HLH, such as measuring soluble interleukin-2 receptor, have not been investigated in patients with isolated PDH, so whether they distinguish between the 2 conditions is unclear. To add further complexity, coexisting histoplasmosis and HLH has been described in several adult patients (*5*, *8*–*10*). However, whether HLH identification and adjunctive immunosuppressive therapy leads to improved outcomes in this situation is unknown.

On the basis of our investigation of this infant with HLH and PDH, we recommend that all infants exhibiting HLH in *Histoplasma*-endemic regions be assessed for histoplasmosis. In addition, the similarity with the clinical features of PDH and the difficulty in diagnosis of HLH without specialized testing raise the question of whether a large number of infants with PDH would also meet the diagnostic criteria for HLH. If so, currently poor outcomes of PDH might be related to co-existing HLH with failure to control the inflammatory response, and the outcomes could be improved by diagnosis and simultaneous treatment of both conditions. Further research is needed to investigate this phenomenon.

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Novel Avian Influenza A(H5N8) Viruses in Migratory Birds, China, 2013–2014

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To the Editor: Novel highly pathogenic avian influenza (HPAI) A(H5N8) virus infections were first detected in poultry in eastern China in 2010 (*1*); the virus caused outbreaks in South Korea and Japan in 2014 (*2*) and reached Europe and North America by early 2015 (*3–6*). Phylogenetic analysis indicated that novel HPAI subtype H5N8 viruses might have originated in China and then circulated in East Asia countries and that the global geographic dissemination of this virus was strongly associated with the migration of wild birds (*7*). However, the role of migratory birds in the initial introduction and spread of novel H5N8 strains in China and other countries in the region is unclear. Shanghai, located at the Yangtze River estuary on the eastern coast of China, is a crucial stopover for migratory birds in East Asia. We report the presence of novel H5N8 strains from migratory birds sampled in Shanghai from October 2013 through December 2014.

A total of 26 novel H5N8 viruses were detected from migratory ducks and curlews captured and swabbed during their wintering period at the coastal wetlands of Shanghai. We collected 19 H5N8 viruses from 16 common teals (*Anas crecca*), 2 falcated ducks (*A. falcata*), and 1 spot-billed duck (*A. poecilorhyncha*) sampled in 2013 and 7 viruses from Eurasian curlews (*Numenius arquata*) sampled in 2014. Common teals were also found to be infected with subtype H5N1, detected by N1 gene fragments in 3 mixed-infection and 2 single-infection samples (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-1754-Techapp1.pdf). Sequences from this study were deposited in GenBank (accession nos. KT936635–KT936716).

Homology BLAST (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) searches showed that H5 and N8 genes of 18 influenza A(H5N8) viruses in ducks had ≥98% similarity to H5N8 isolates W24 and 6D18 detected in poultry in Zhejiang Province (*2*), adjacent to Shanghai. The H5 gene in A/common teal/Shanghai/1108–1/2013(mixed) (PD1108-1) was 96% related to low pathogenicity avian influenza (LPAI) subtype H5N1 isolated from a European teal sampled in Russia in 2011 (GenBank accession no. KF462362). Of the 7 viruses from curlews, H5 and N8 isolates were closely related to isolates H68 and H297 from wild ducks reported in South Korea in early 2014 (*8*). Matrix genes of all novel subtype H5N8 viruses were closely related (95%–99%) to isolates from China (S11090, W24), Japan (156), and South Korea (Gochang1, S005) (online Technical Appendix Table).

[1]These authors contributed equally to this article.

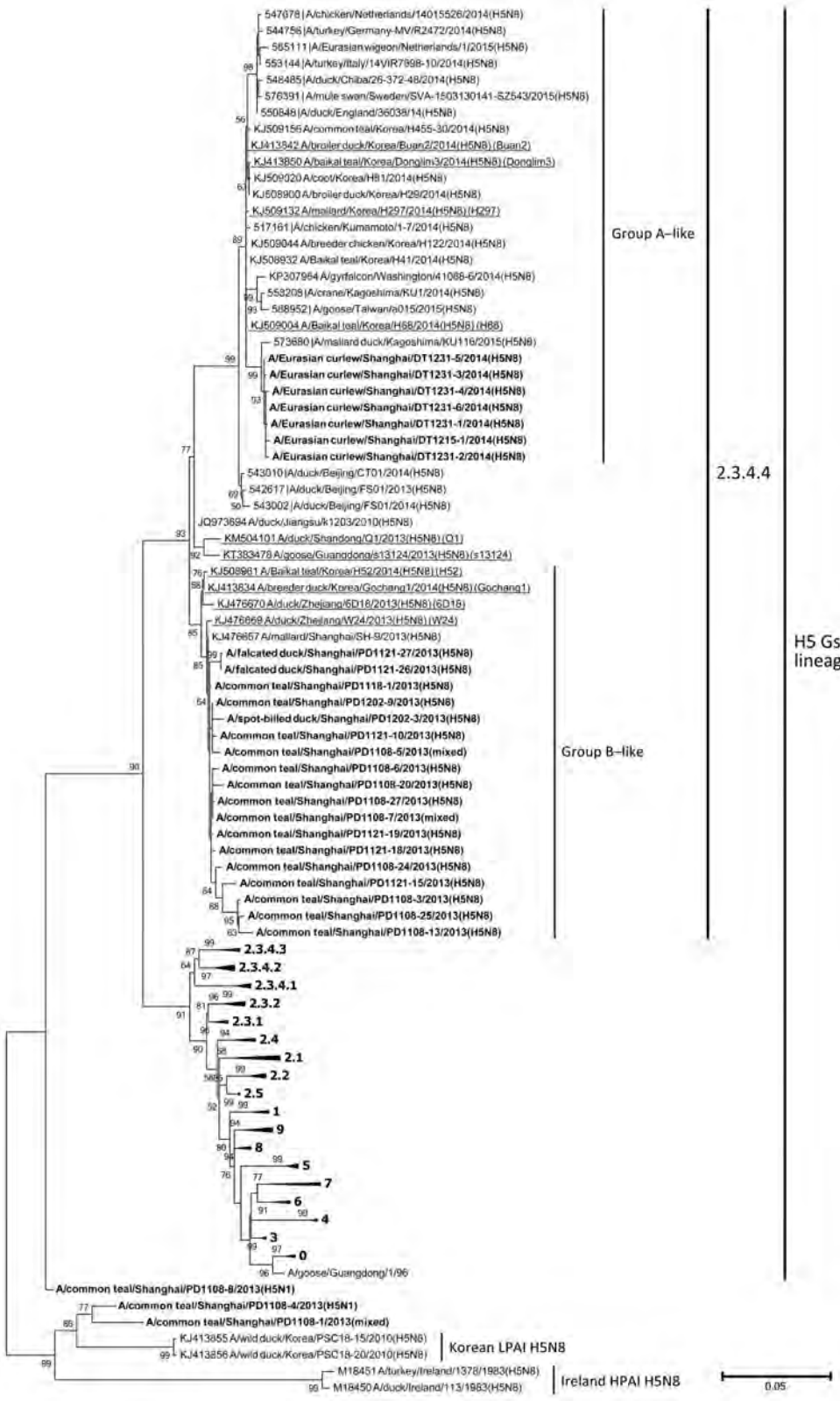

Figure. Phylogenetic tree of the hemagglutinin (HA) genes of influenza A subtype H5 viruses from wild birds of Shanghai, China, 2013–2014. Boldface indicates viruses from this study; representative isolates are underlined and referred to in abbreviated form in brackets. A total of 109 HA gene sequences (≥1,594 nt) were used for tree reconstruction. Representative strains and clades are recommended by WHO/OIE/FAO H5N1 Evolution Working Group and were retrieved from Influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/select.cgi) and GISAID's EpiFluTM Database (http://platform.gisaid.org/epi3/frontend). The phylogenetic tree was constructed by using the maximum likelihood method based on the general time reversible model with bootstrap analysis (100 replicates), by MEGA version 6 (http://www.megasoftware.net/). Bootstrap values ≥50% are shown. Scale bar indicates nucleotide substitutions per site. LPAI, low pathogenicity avian influenza. HPAI, highly pathogenic avian influenza.

Phylogenetic analysis of HPAI H5 descendants of A/goose/Guangdong/1/1996(H5N1) showed that clade 2.3.4.4 (*10*) H5N8 viruses fall into 2 distinct groups, closely related to group A (Buan2-like) and group B (Gochang1-like) (*8*). The hemagglutinin (HA) genes of the 18 subtype H5N8 viruses from ducks in 2013 shared a protease cleavage site motif of REKRRKR/GLF and the sequence cluster with H5N8 viruses from eastern China poultry (W24, 6D18) (*2*) and Korean group B isolates (Gochang1, H52) (*8*,*9*) to form group B. The HA genes from all 7 H5N8 isolates from curlews in 2014 had a protease cleavage site motif of RERRRKR/GLF and the sequence cluster, along with Korean group A (Buan2-like) (*8*,*9*), European (*3*,*4*), and North American (*7*) H5N8 lineage viruses to form group A. The HA from PD1108-1 had a cleavage site motif (RE–TR/GLF) characteristic of LPAI HA, and its sequence clustered with the Eurasian LPAI H5 lineage (Figure).

According to the sampling dates, the identification of the 18 group B H5N8 isolates from Shanghai was the earliest detection of HPAI H5N8 virus in wild birds in East Asia, before the first reported outbreak in South Korea in January 2014. Although poultry isolates from China obtained during the same period were phylogenetically clustered with group A (Figure), no group A viruses were detected in wild birds during the 2013–2014 wintering season in China. Notably, 2 of the group A Chinese poultry isolates (Q1 and s13124) have the HA cleavage site motifs of group B. Their topologically basal positions in group A (Figure) implied the connection between the 2 groups. Eurasian curlews are widely distributed in the Northern Hemisphere, including Europe, Siberia, Japan, the Korean Peninsula, and China (http://ibc.lynxeds.com/species/eurasian-curlew-numenius-arquata). Populations wintering in Shanghai have overlapped migratory routes and habitat distribution with duck species in East Asia (Shanghai Chongming Dongtan National Nature Reserve, unpub. data), which suggested possible transmission routes through overlapped habitats in their northern territory (breeding areas) or close contacts among these species. These data support the theory that asymptomatic migratory birds may have played a role in geographic dissemination of HPAI subtype H5N8 and facilitation of viral evolution and reassortment. Moreover, that HPAI subtypes H5N1 and H5N8 co-infected and co-circulated in migratory ducks suggests that rapid and active mutation and reassortment of H5 subtypes may take place in these hosts. Therefore, to monitor and then control the epidemics of H5 subtype viruses, it is urgent that more intensive surveillance be carried out in poultry and wild birds and that information be promptly shared among countries.

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Interferon-γ Autoantibodies as Predisposing Factor for Nontuberculous Mycobacterial Infection

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To the Editor: Recent advances in the understanding of antimycobacterial immune response have led to descriptions of predisposing conditions to dissemination of nontuberculous mycobacteria (NTM) infection. The interferon-gamma (IFN-γ)/interleukin-12 (IL-12) axis is a critical pathway for intracellular killing of mycobacteria (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/6/15-1860-Techapp.pdf) (*1*).We report a case of disseminated NTM infection in a woman from Laos who showed IFN-γ autoantibodies. We also conducted a literature review to review similar cases.

A previously healthy 50-year-old woman from Laos was referred to Hospices Civils de Lyon, France, for fever and generalized lymphadenopathies; pathological examination revealed a nonnecrotizing granuloma. Culture yielded *Mycobacterium fortuitum*. After a 3-month azithromycin/ciprofloxacin regimen, symptoms resolved. A few months later, a computed tomography scan showed persistence of enlarged mediastinal lymph nodes; biopsy disclosed nonspecific sinus histiocytosis. Mycobacterial cultures were negative. One month later, the patient reported intense dorsal spine pain. She was diagnosed with multifocal vertebral osteomyelitis, and tissue specimens tested positive for *M. intracellulare*. Azithromycin/ciprofloxacin was prescribed again. At 6 months, lesions worsened, and epidural abscess led to spinal cord compression, requiring decompressive laminectomy. Postoperative cultures were positive for *M. intracellulare*. Treatment was changed to azithromycin, rifabutin, ethambutol, and amikacin for 2 weeks, then rifabutin and moxifloxacin, which led to clinical improvement.

The patient experienced 2 episodes of thoracic herpes zoster concomitantly to each episode of illness. The recurrent NTM infection led us to investigate her immune status. Results of standard immunologic tests (leukocyte, serum level of immunoglobulin isotypes, and T-, B-, and NK0cell counts) and in vitro T-cell proliferation were normal; HIV testing was negative. Given its critical role in *Mycobacteria* clearance by host cells, we used IFN-γ whole-blood activation to investigate further. IFN-γ plasma level was undetectable, excluding complete forms of IFN-γ R1/R2. IL-12 p40 subunit plasmatic level was decreased, but the IL-12 receptor was present and functional. In addition, both BCG and IL-12 stimulations failed to trigger IFN-γ production. Ultimately, the detection of IFN-γ autoantibodies in high levels explained these abnormalities. Antimycobacterial treatment was continued for 2 years, then changed to azithromycin suppressive therapy; IFN-γ autoantibodies remained positive at the time of this report, 2 years after azithromycin initiation.

We reviewed the literature and found 63 other cases of IFN-γ autoantibody-related NTM infection with sufficient detail to provide clinically relevant material for optimizing disease management (Table; online Technical Appendix). These cases overwhelmingly occurred among Asian populations (92.2%) (*2*). Disease incidence may be underestimated, as suggested by large studies describing Asian patients with disseminated NTM infection without evidence of impaired immunity but for whom the IL-12/IFN-γ axis was not investigated (*3*). This acquired autoantibodies–mediated immunodeficiency is more frequent among women, in whom the disease typically manifests in the second half of adult life (median 48 [IQR 44.8–60.0] years of age). Reported female:male sex ratios are 23/36 in Asian-born (n = 59) and 4/1 in non-Asian (n = 5) case-patients, respectively. Mechanism of the disease is briefly discussed in the online Technical Appendix.

M. avium complex predominated in the literature review, accounting for 40.4% of cases, followed by *M. abscessus* (31.6%) and *M. fortuitum* (12.3%). Infections were mostly multifocal, affecting lymph nodes (n = 51, 79.7%), osteoarticular tissue (n = 37, 57.8%), lungs (n = 30, 46.9%), and skin and/or soft tissues (n = 24, 37.5%). Aside from NTM infections, other opportunistic infections were reported in 39 (75.0%) patients, mostly *Herpesviridae* reactivations (44.2%) and *Salmonella* infections (25.0%) (*3*–*6*).

Specific treatment for IFN-γ autoantibodies–associated NTM infection is not codified and required prolonged, multiple-drug regimens. The median treatment duration for the studies we reviewed was 31 (IQR 22.8–60.0) months. In some studies, clinicians used IFN-γ administration (5 patients, 1 of whom was cured), but treatment likely was invalidated by the autoimmune-driven inhibitory

Table. Summary of clinically relevant characteristics of 64 case-patients with IFN-γ autoantibody-related nontuberculous mycobacterial infection found in literature review*

Characteristics	Value
Demographics	
Female sex	40 (62.5)
Median age, y (IQR)	48.0 (44.8–60.0)
Asian origin	59 (92.2)
Other autoimmune diseases	7/27 (25.9)
Etiologic agents	
Mycobaterium avium	23 (40.4)
M. abscessus	18 (31.6)
M. fortuitum	7 (12.3)
M. tuberculosis	*6 (10.5)*
Other opportunistic infections	39/52 (75.0)
Herpesviridae reactivations	23/52 (44.2)
Salmonella spp.	13/52 (25.0)
Median duration of intensive-phase therapy, mo (IQR)	31.0 (22.8–60.0)
≥6	31/31 (100)
≥12	29/31 (93.5)
≥18	27/31 (87.1)
≥24	23/31 (74.2)
Long-term antimicrobial suppressive therapy	3/30 (10.0)
Immunomodulatory therapies	10/30 (33.3)
Rituximab	6/30 (20.0)
IFN-γ	5/30 (16.7)
Intravenous immunoglobulins	2/30 (6.7)
Plasmapheresis	2/30 (6.7)
Cyclophosphamid	1/30 (3.3)
Outcome	
Cure	21/62 (33.9)
Improvement	6/62 (9.7)
Relapse/persistence	29/62 (46.8)
Death	6/62 (9.7)

*Values are no. (%) except as indicated. Only articles with sufficient detail were analyzed. A complete listing of articles reviewed from the literature is provided in the online Technical Appendix Table (http://wwwnc.cdc.gov/EID/article/22/6/15-1860-Techapp1.pdf). IQR, interquartile range; IFN-γ, interferon gamma.
†Denominators indicate number of case-patients for which category of data was available.

activity (*5*,*2*,*7*). Other strategies included intravenous immunoglobulin (n = 2), plasmapheresis (n = 2), and cyclophosphamide (n = 1) (*7*–*9*). The use of rituximab, a chimeric anti-CD20 monoclonal antibody targeting B-cells, has been recently associated with clinical response and decrease in IFN-γ autoantibody levels as well as neutralizing ability (*6*,*7*).

Final outcome was available for 56 patients who completed the intensive treatment phase; 21 (37.5%) were declared cured. Six (10.7%) patients died, and 29 (51.8%) had persistent or relapsing infections. At the time of this report, additional patients were still being treated and showed improvement of symptoms. Despite this high rate of failure, long-term antimicrobial drug suppressive therapy has rarely been proposed as a causal factor. The origin of the case we report was related to the use of azithromycin suppressive therapy, similarly to disseminated NTM disease prophylaxis in HIV-infected patients before the era of highly active antiretroviral therapies (*10*), assuming the risk/benefit balance including the possibility of NTM macrolide-resistant strain selection.

IFN-γ autoantibodies are evidence of acquired immunodeficiency that should be considered in cases of unexplained disseminated NTM infections in Asian-born persons. Use of immunomodulation strategies is still debated, and long-term suppressive treatment should be considered for persisting high levels of neutralizing antibodies.

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Loss of 89K Pathogenicity Island in Epidemic *Streptococcus suis*, China

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To the Editor: *Streptococcus suis* serotype 2 (SS2) is a previously neglected, newly emerging human pathogen that causes occupational and opportunistic infections (*1*,*2*). Outbreaks of fatal human SS2 infections in China, featuring streptococcal toxic shock syndrome, in 2005 seriously challenged global public health (*3*–*5*). The epidemic strain is unusual in that it contains a unique 89-kb (89K) pathogenicity island (PAI) (*3*,*6*,*7*). We observed the loss of genes from the 89K PAI in sporadic cases in southern China in 2007, implying the dynamic evolution of this PAI (*8*). Therefore, 89K PAI might be able to be used to monitor prevalent strains of *S. suis* in China (*8*).

We report 10 recurrent cases of human *S. suis* infections during 2008–2015 in southern China. Most of the hospitalized patients were male workers in close contact with pigs, pork products, or both. These patients typically exhibited clinical syndromes of meningitis, including headache, coma, vomiting, and fever. The bacterial strains acquired from humans were as follows: 2 isolates in 2008 (Stre08001 and Stre08002), 2 in 2009 (Stre09001 and Stre09002), 2 in 2011 (Stre11001 and Stre11002), 3 in 2013 (Stre13002, Stre13003, and Stre13004), and 1 in 2015 (Stre15001). Microbial and molecular assays proved that these clinical isolates were *S. suis* (online Technical Appendix Figures 1, 2, http://wwwnc.cdc.gov/EID/article/22/6/15-2010-Techapp1.pdf). Multiplex PCR–based molecular determination (*16S rDNA*, *mrp*, *epf*, and *cps-2j*) suggested that all strains except Stre13002 were SS2 (online Technical Appendix Figure 2) (*3*). To determine whether these clinical isolates derived from the same Chinese epidemic clone 05ZYH33, we sequenced an array of virulence factor–encoding genes, as well as the *16S rDNA* genes. Phylogenetic trees indicated that all 10 clinical strains are classified into the same subclade as that of the strain 05ZYH33 (online Technical Appendix Figure 2).

Subsequent analyses by pulsed-field gel electrophoresis revealed that genotypes are diverse among these clinical strains, which can be roughly divided into 6 groups (online Technical Appendix Figure 3). Given that the Sao surface antigen protein possesses 3 allelic variants (Sao-L [670 aa], Sao-M [580 aa], and Sao-S [489/490 aa]) (9), we thus assayed it with these clinical strains. Unexpectedly, we found 2 more new allelic variants, referred to as Sao-L1 (640 aa) and Sao-L2 (611 aa). Except for the strain BM407, which is a Chinese epidemic SS2 encoding Sao-L1, a version 30 residues shorter than Sao-L, 8 of the 10 clinical *S. suis* isolates consistently had the same new form of Sao protein, Sao-L2 (611 aa) (online Technical Appendix Figure 4).

Because 89K PAI is in dynamic evolution, determining whether it remains present in clinical strains is of interest. As previously designed (online Technical Appendix Table 1), a specific pair of boundary primers (1/6) was applied for PCR-based detection of the 89K PAI (Figure, panel A, http://wwwnc.cdc.gov/EID/article/22/6/15-2010-F1.htm). In principle, the PCR-positive result suggests the absence of 89K PAI, whereas the PCR-negative result indicates the presence of 89K PAI (*6*,*8*). Unlike the epidemic strain 05ZYH33 that has the 89K PAI, 9 of the 10 clinical strains examined (Stre08001, Stre08002, Stre09001, Stre09002, Stre11001, Stre11002, Stre13002, Stre13003, and Stre13004) were unexpectedly found to be PCR positive for the unique 1/6 DNA fragment with expected size of ≈1.5 kb (Figure, panel B). This finding indicates that the 89K PAI is lost in these 9 clinical strains. We saw similar scenarios in the subsequent PCRs for other inner genes/DNA fragments (943 and 944 [*10*]; 1/2, 3/4, and 5/6 [*8*]) inside of 89K PAI (Figure, panel C). Further DNA sequencing of the 1/6 PCR product showed that it matches well with the 2 boundary regions neighboring the 89K PAI, validating the loss of 89K PAI in these 9 clinical isolates (Figure, panel D). In contrast, the strain Stre15001 behaved similarly to that of the 05ZYH33 containing the 89K PAI, in that both are PCR positive for the 4 amplicons

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of 1/2, 5/6, 943, and 944 but PCR negative for the 1/6 amplicon (Figure, panels B–D). The only minor difference between strains Stre15001 and 05ZYH33 lay in the 3/4 amplicon (Figure, panel C). Clearly the 3/4 DNA fragment is present in the 89K PAI from strain 05ZYH33 but not in the counterpart of the strain Stre15001 (Figure, panel C); that is, strain Stre15001 carries a variant of 89K PAI lacking (at least part of, if not all) the 3/4 DNA fragment. In terms of 89K PAI (and pulsed-field gel electrophoresis/Sao protein), we propose that a heterogeneous SS2 population is circulating in China. Also, we observe that the differentiation of bacterial virulence is related to the clinical strains using the infection model of Balb/c mice (online Technical Appendix Figure 5).

In summary, the loss of 89K PAI might highlight the emergence of an epidemic SS2 population. This population appears to have genetic heterogeneity that is undergoing evolution in an adaption to some selection pressure from the environment, host restriction, or both.

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Next-Generation Sequencing of *Mycobacterium tuberculosis*

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To the Editor: Next-generation sequencing (NGS) technology is becoming more affordable and is increasingly being widely used for high-resolution molecular epidemiology of tuberculosis. Using an example of the emerging multidrug-resistant strain of *Mycobacterium tuberculosis*, we showed the value of informed understanding when in silico prediction from NGS data achieved with available bioinformatics tools is placed within the context of the existing genotyping framework.

Spoligotyping is a classical method of *M. tuberculosis* genotyping, and the SITVIT_WEB database contains data on 7,105 spoligotype patterns of 58,180 isolates from 153 countries (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE). Spoligotyping targets a variation of the DR/CRISPR locus, whose evolution in *M. tuberculosis* occurs through deletion of single or multiple spacers. By

virtue of the orientation of the associated *cas* genes, the locus is situated on the minus strand, whereas its spacers are numbered within the locus, not the genome. Spoligotype international type (SIT) 266 (Figure, panel A) is an epidemiologically significant genotype. It constitutes a substantial proportion of the population structure of *M. tuberculosis* in Belarus, a post-Soviet state in Eastern Europe (*1*,*2*), and has been described sporadically in the neighboring provinces in northwestern and central Russia (*2*–*5*) and in Latvia (*6*). More important, it is multidrug resistant (MDR) (and most likely extensively drug resistant). In a recent Belarus study, SIT266 was found in 25 of 163 strains; all 25 were MDR (*1*). This situation contrasts clearly with its apparently parental type SIT264, which differs from SIT266 in a single spacer 8 (Figure, panel A). SIT264 is more widespread across Eastern Europe but at very low prevalence and is not associated with multidrug resistance (*3*,*6*,*7*). On the basis of 24 mycobacterial interspersed repetitive unit variable number tandem repeats clustering and robust phylogenetic single-nucleotide polymorphisms, SIT264 and SIT266 isolates are assigned to the Latin American–Mediterranean lineage of *M. tuberculosis* (*2*).

We recovered 2 MDR *M. tuberculosis* isolates of SIT266 from pulmonary tuberculosis patients from northwestern Russia in 2014. Bacterial DNA was subjected to macroarray-based spoligotyping (*8*) and whole-genome sequencing on the MiSeq platform (Illumina, San Diego, CA, USA). *M. tuberculosis* NGS data were deposited in the National Center for Biotechnology Information Sequence Read Archive (project no. PRJNA305488).

The short sequencing reads were subjected to analysis by using the SpoTyping program (https://github.com/xiaeryu/SpoTyping) (*9*) and the TGS-TB online tool (https://gph.niid.go.jp/tgs-tb/) (*10*) to deduce their spoligoprofile. We used the TGS-TB tool to map the IS*6110* insertion sites and detect drug resistance mutations. The reads also were mapped to the genome of reference strain H37Rv (GenBank accession no. NC_00962.3) by using the Geneious 9.0 package (Biomatters Ltd, Auckland, New Zealand). We obtained 1,294,895 and 816,693 paired reads for strains 4542 and 8279, respectively, and mapped them to the reference. Mean read length was 300 bp, and the average genome coverage was 72.

Strains 4542 and 8279 were phenotypically MDR and harbored mutations associated with resistance to all 5 first line-drugs. The macroarray hybridization spoligotyping assigned both strains to spoligotype SIT266. However, by in silico typing, their spoligotype was predicted to be SIT264 (Figure, panel A). To reconcile these findings, we hypothesized that this discrepancy resulted from an IS*6110* asymmetrically inserted in the direct repeat unit adjacent to the spacer 8 in a SIT266 isolate. This insertion would disrupt a target sequence for biotin-labeled DRa primer, thus preventing spacer 8 from amplification. Indeed, in both SIT266 isolates, the in silico analysis identified a forward IS*6110* insertion that was mapped to position 3122916 in H37Rv genome (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/6/15-2051-Techapp1.pdf). This location correlates with the location of spacer 8 in this same genome from positions 3122954 to 3122918. Thus, IS*6110* precedes spacer 8 in the genome of isolate with spoligotype SIT266, or follows it, within the DR locus (Figure, panel B; online Technical Appendix Figure).

An immediate excellent contribution of NGS with regard to tuberculosis treatment and control is its capacity to rapidly screen for multiple gene targets linked to the development

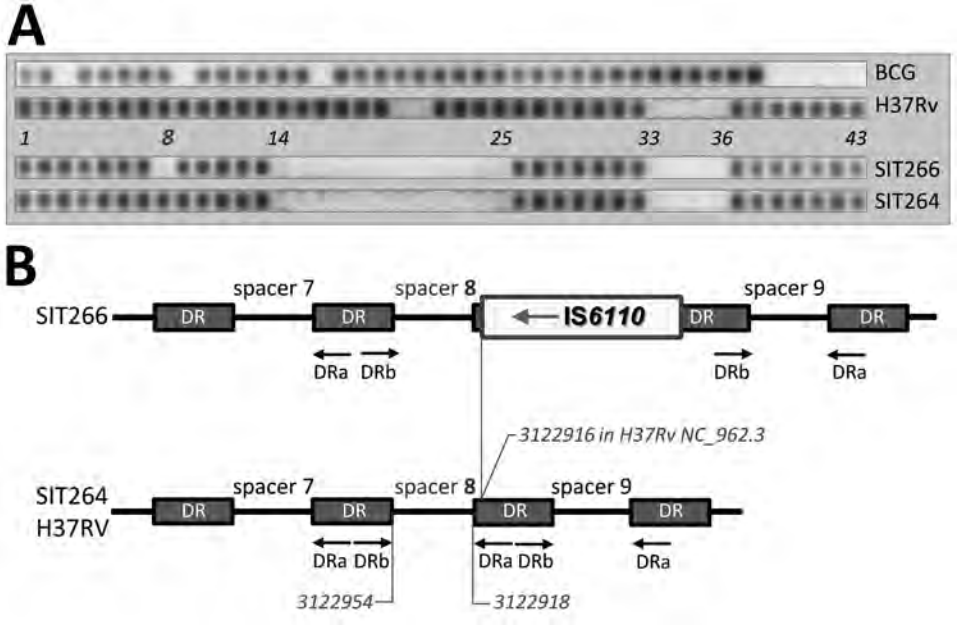

Figure. A) Spoligotyping hybridization profiles of H37Rv and BCG reference strains, *Mycobacterium tuberculosis* SIT266 (2 strains in this study) and SIT264 (previously published strain in [*3*]). SIT, spoligotype international type, designated according to SITVIT_WEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE). B) Schematic view of the DR/CRISPR locus (region of spacers 7–9) in spoligotypes SIT264 and SIT266 and reference strain H37Rv, inferred from next-generation sequencing data. Reverse DRa primer is biotin-labeled. IS*6110* is not to scale.

of drug resistance. However, knowledge of strain genotype is no less clinically and epidemiologically relevant. A superspreading strain might be marked with other pathobiologically important features. In the case presented here (indeed emerging and MDR), the NGS-based in silico spoligotyping would confuse the MDR/extensively drug resistant SIT266 with "less dangerous" SIT264. To be precise, the revealed discrepancy is not inherent to the NGS technology itself. Although the general limitation of the use of short sequencing reads to infer repetitive genome regions is known, it did not pose a problem in our study. However, both bioinformatics tools predicted the spoligoprofile solely from the presence or absence of spacer sequences and did not take into account a "hiding" effect exerted by a putative IS*6110* insertion on adjacent spacer under classical spoligotyping.

In conclusion, we suggest that an accurate NGS-based prediction requires an integrative approach to all relevant information obtained by in silico analysis of a given genome locus. In particular, not only presence of CRISPR spacers but also presence and location of potentially interfering IS*6110* insertion(s) should be considered for correct NGS-based assignment to internationally recognized spoligotypes.

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MERS-CoV Infection of Alpaca in a Region Where MERS-CoV is Endemic

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To the Editor: Accumulating evidence indicates that dromedaries (*Camelus dromedarius*) are a reservoir for zoonotic transmission of Middle East respiratory syndrome coronavirus (MERS-CoV). Although numerous studies have looked at other livestock in the Middle East region, evidence for MERS-CoV infection has only been found in dromedaries (*1*). Extensive and continuous circulation of MERS-CoV occurs in the Al Shahaniya region of Qatar, most likely because of the presence of an international camel racing track and numerous barns holding camels (*2*,*3*). In April 2015, we investigated the MERS-CoV infection status of 15 healthy alpacas (*Vicugna pacos*) in a herd of 20 animals and 10 healthy dromedaries in a herd of 25

[1]These authors contributed equally to this article.

animals at a farm in this region (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/15-2113-Techapp1.pdf).

The herds were located at a distance of ≈200 m from each other within the barn complex and were cared for by the same animal workers, who lived in a common house between the herds at the complex. Both the alpacas and camels were kept as hobby animals.

Serum samples were collected from all 25 animals. Nasal swabs were collected from all camels, whereas nasal, rectal, and oral swab specimens were collected only from a subset of the alpacas (online Technical Appendix) because of logistical constraints. The serum samples were tested for IgG antibodies reactive with the S1 antigens of MERS-CoV and severe acute respiratory syndrome coronavirus (SARS-CoV), and titers were calculated as described previously (*4*,*5*). MERS-CoV reactivity was confirmed by using a 90% plaque-reduction neutralization test ($PRNT_{90}$) (*3*). Swab specimens were analyzed for MERS-CoV RNA by a screening PCR targeting the upE gene (*6*). MERS-CoV–specific antibodies were detected in all alpacas and all but 1 camel by protein microarray; reciprocal titers ranged from 49 to 773 for the alpacas and were ≥1,280 for the camels (Figure, panel A). $PRNT_{90}$ testing confirmed the presence of MERS-CoV–specific antibodies; reciprocal neutralizing titers ranged from 80 to 320 for the alpacas and from 80 to ≥2560 for 9 camels (Figure, panel B). All swab specimens were negative by PCR (online Technical Appendix). None of the serum samples were reactive to SARS-CoV S1. The microarray was also conducted for bovine CoV and human CoV-229E antigens, which were used as a proxy for the serologically closely related dromedary betacoronavirus-1 HKU23 and 229E-related camelid alphacoronaviruses, respectively (*7*). Positive binding was detected for both antigens in alpaca and dromedary (data not shown).

Our observations prove the susceptibility of alpacas for natural MERS-CoV infection and lay the foundation for future studies to determine the potential of alpacas as another livestock reservoir for MERS-CoV. The alpacas in this study were the only alpacas in Qatar at the time and were located in a region where MERS-CoV is endemic. In a previous study, by using the same microarray technology, we found no evidence for MERS-CoV infection in alpacas from regions where MERS-CoV is not endemic (*4*). Although a study by Eckerle et al. demonstrated the potential of MERS-CoV to infect alpaca kidney cells in vitro (*8*) and alignment of mammalian DPP4 indicate that the 14 residues interacting with the MERS-CoV receptor binding domain of alpaca DPP4 are identical to that of dromedary DPP4 (online Technical Appendix), the in vivo susceptibility of alpacas remained to be determined.

The observed natural susceptibility of alpacas to MERS-CoV infection potentiates a broadening of the geographic range of MERS-CoV circulation to areas with large populations of alpacas. Alpacas are New World camelids, and the worldwide population of alpacas is estimated at 3 million animals, with ≈94% living in the high Andean regions of South America (Peru, Bolivia, Chile and Argentina), of which most are in Peru (constituting ≈88% of the world alpaca population) (http://lib.icimod.org/record/23682). Alpacas are increasingly being kept outside

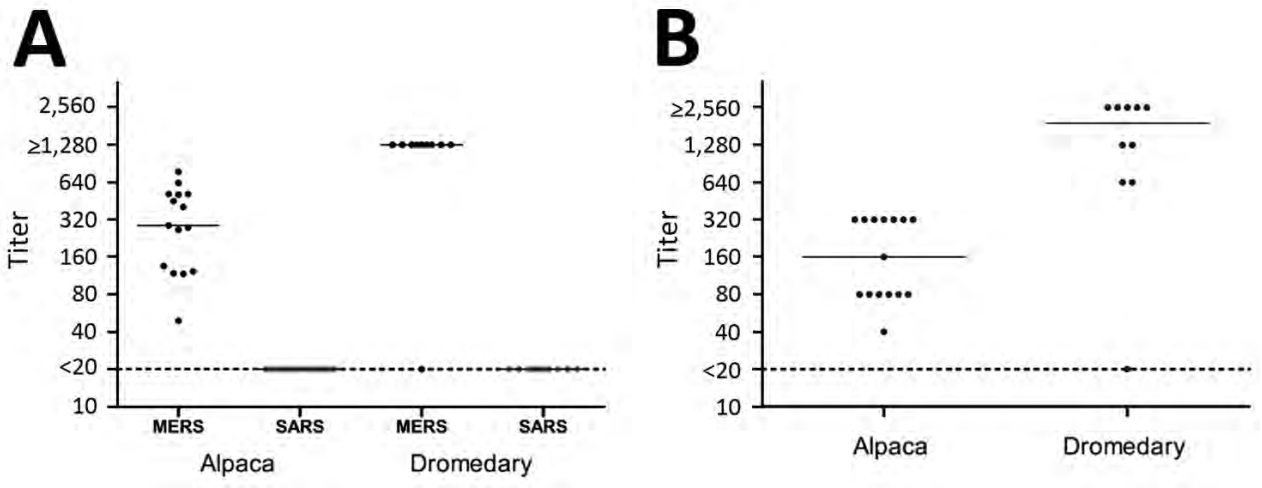

Figure. Column scatterplots of MERS-CoV reactivity of serum samples from alpaca (n = 15) and dromedaries (n = 10) in the Al Shahaniya region of Qatar, April 2015. A) Plot of alpaca and dromedary serum titers of antibodies specific for S1 antigens of 2 coronaviruses as determined by protein microarray. Titers were defined as the interpolated serum concentration that provoked a response half-way on a concentration-response curve between the minimum and maximum signal and were calculated from the inflection point of a 4-step dilution series (1:20 to 1:1,280) as described previously (*5*). B) Plot of alpaca and dromedary serum titers of MERS-CoV neutralizing antibodies as determined by $PRNT_{90}$. The highest serum dilution neutralizing 90% of plaque formation is depicted. For both panels, solid lines indicate median, and dotted lines indicate detection limit. MERS, Middle East respiratory syndrome; CoV, coronavirus; $PRNT_{90}$, 90% plaque-reduction neutralization test; SARS, severe acute respiratory syndrome.

South America, mainly for their fleece, with estimated numbers in 2014 reaching 230,000 in the United States (http://lib.icimod.org/record/23682), 35,000 in the United Kingdom (http://www.bas-uk.com), and 150,000 in Australia (http://www.alpaca.asn.au). Although MERS-CoV has not been found in camelids other than dromedaries outside the Arabian Peninsula so far (*9*), our observations raise the question of whether other camelids could become infected if MERS-CoV were introduced to regions with large populations of alpacas and possibly other closely related camelids of the genera *Lama*, *Vicugna*, and *Camelus*.

Because the date of infection of the alpacas and camels in this study is not known, we cannot speculate on the level of susceptibility of alpacas versus dromedaries based on the observed differences in antibody titers, which were lower in alpacas. It remains to be determined whether alpacas, in parallel with dromedaries, will actually shed MERS-CoV and are capable of independent maintenance of the virus in their population. Differences in susceptibility to viral pathogens between New and Old World camelids have been observed before (*10*). Therefore, understanding the risk requires further assessment of the reservoir competence of alpacas for MERS-CoV (e.g., through experimental infections) and an assessment of MERS-CoV–related viruses present in alpacas and other camelids in different parts of the world.

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Cryptococcus gattii VGIIb-like Variant in White-Tailed Deer, Nova Scotia, Canada

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To the Editor: *Cryptococcus gattii* is a fungal pathogen that is emerging in the Pacific Northwest of North America. In Nova Scotia, Canada, previously not recognized as a *C. gattii*–endemic area, a variant strain similar to VGIIb caused cryptococcosis with nasopulmonary, lymph node and central nervous system involvement in a free-ranging, yearling white-tailed deer (*Odocoileus virginianus*). The deer was found in the village of Greenwood (latitude 44.9717246; longitude –64.9341295) on July 14, 2014. The deer exhibited behavioral and neurologic abnormalities, including

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loss of fear of humans, ataxia, circling, high-stepping gait, torticollis, and a fixed stare. Additional clinical signs were ptyalism with frothing from the mouth and dyspnea with gurgling respiration. The animal was euthanized, and the head, lungs, heart, gastrointestinal tract, liver, and kidneys were submitted for pathologic examination.

Gross examination revealed multifocal, soft, round, expansile, pale tan masses of variable sizes that had replaced or effaced the normal architecture of the tracheobronchial lymph nodes and pulmonary parenchyma. The center of the largest lymph node mass was necrotic and filled with viscous yellow material. Similar yellow gelatinous material obliterated the right ethmoturbinates rostral to the cribriform plate. In the brain, cerebellar coning was prominent. Several small, pitted lesions with dark rims were noted in the neuropil of the thalami, superior colliculi, and hippocampus. Gross lesions were absent in the liver, kidney, and gastrointestinal tract (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/6/16-0081-Techapp1.pdf).

Microscopically, the nasal cavity, lung, tracheobronchial lymph node, and brain lesions were similar, consisting of variably sized, cystic spaces supported by various thicknesses of well-differentiated fibrovascular septa or remaining normal parenchyma. The cystic spaces, immediately adjacent tissues, meninges, and ependyma contained variable numbers of yeast associated with a granulomatous inflammatory response or a pleocellular population of lymphocytes, plasma cells, macrophages, and neutrophils (Figure). The yeast were round to oval, 15–33 μm in total diameter, with poorly staining central portions (5–12 μm in diameter) surrounded by a pale acidophilic or basophilic capsule (5–21 μm thick), which stained positively with a mucicarmine stain. Some yeast were dematiaceous, and Fontana-Masson staining was consistent with presence of melanin. Very rarely, narrow-based budding was observed in the yeast. All aforementioned morphologic characteristics, staining affinities, and lesion distributions are consistent with an infection with fungi in the genus *Cryptococcus* (*1*,*2*).

One species of *Cryptococcus* was isolated from a tracheobronchial lymph node aspirate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting of the isolate (Biotyper RTC software; Bruker Daltonics Ltd, Bremen, Germany) yielded 8 diagnostic signals consistent with *C. gattii* VGIIb and VGIIc; therefore, the isolate was further classified by multilocus sequence typing based on 7 genetic loci, following the International Society for Human and Animal Mycology consensus multilocus sequence typing scheme for the *C. neoformans*/*C. gattii* species complex (*3*). Further discrimination based on allele congruence with established *C. gattii* VGII genotypes (*4*) classified the isolate as being most similar to genotype *C. gattii* VGIIb (CAP59 allele no. 2, GPD1 allele no. 6, LAC1 allele no. 4, PLB1 allele no. 2, URA5 allele no. 2, IGS1 allele no. 10). However, because of a slight difference in the SOD1 allele (99.5% similarity with allele no. 15), this strain is considered to be a unique variant strain, most similar to that of the VGIIb genotype. Whole-genotyping studies have provided evidence of multiple distinct introductions of the VGIIb genotype to North America (*5*). Because of the observed difference in the SOD1 allele, the VGIIb-like variant strain may represent a fourth introduction or a different VGII genotype altogether.

The white-tailed deer represents a new host species for *C. gattii* in North America. Because white-tailed deer are nonmigratory, generally exhibiting only minor seasonal

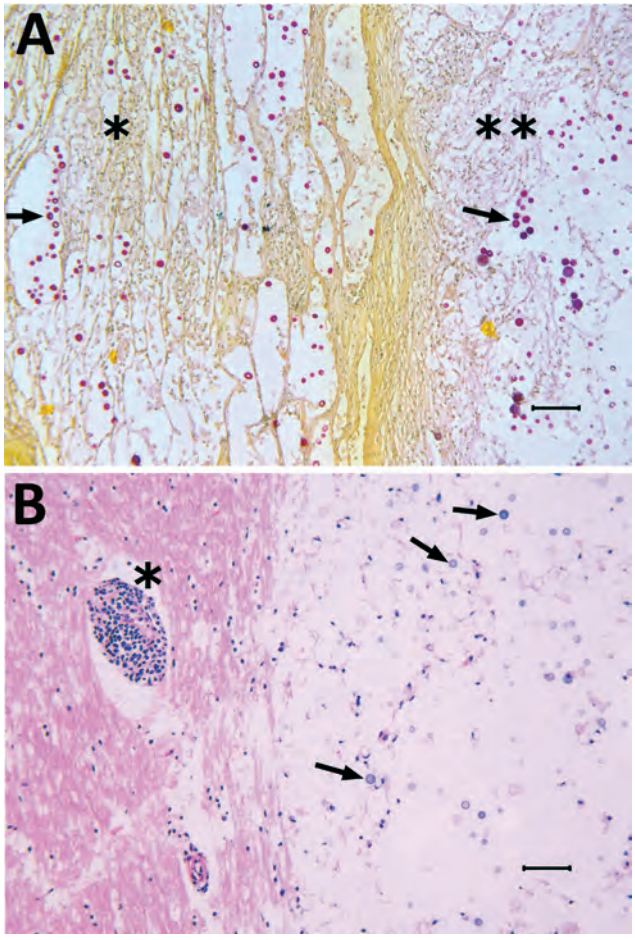

Figure. Tissue from white-tailed deer *(Odocoileus virginianus*), showing microscopic lesions caused by a unique *Cryptococcus gattii* VGIIb-like variant strain most similar to that of the VGIIb genotype; etiology was confirmed by molecular sequencing. A) Photomicrograph of lung lesions with intralesional *C. gattii* (arrows indicate examples of individual yeast) in a mass (**) and in adjacent compressed alveolar spaces (*). Mucicarmine stain. Scale bar indicates 100 μm. B) Photomicrograph of a brainstem lesion with intralesional *C. gattii* (arrows indicate examples of individual yeast) and an adjacent blood vessel with a perivascular infiltrate of inflammatory cells (*). Hematoxylin and eosin stain. Scale bar indicates 50 μm.

movements (*6*), this infection was considered to be autochthonous, indicating endemicity of the *C. gattii* VGIIb-like variant in Nova Scotia and highlighting the value of nonmigratory animals as sentinels for emerging diseases (*7*). Incidence for this disease is highest in the Pacific Northwest, where the primary agents are *C. gattii* VGII genotypes (*2*,*4*). A pertinent literature review and consultation with regional public and veterinary health authorities determined that Québec was the most eastern province in Canada where crytococcosis associated with *C. gattii* VGII has caused clinical disease that was not potentially travel related in humans (Phillippe Dufresne, pers. comm.). In eastern North America, the *C. gattii* VGIIb genotype is reported to have caused disseminated cryptococcosis in a human in Florida, USA (*8*,*9*). Because *C. gattii* is potentially pervasive in the environment, the Nova Scotia Department of Health has alerted provincial infectious disease specialists and the provincial public health laboratory to ensure availability of the diagnostic capacity to test for the fungus.

The *C. gattii* VGIIb genotype causes substantial, life-threatening disease in otherwise healthy hosts (*2*), and a unique VGIIb-like variant is endemic to Atlantic Canada. Therefore, continued surveillance by physicians and veterinarians in the region is warranted.

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The cost of the diagnostic testing for this case was covered by the scanning wildlife health surveillance program of the Canadian Wildlife Health Cooperative.

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Zika Virus in a Traveler Returning to China from Caracas, Venezuela, February 2016

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To the Editor: Zika virus, a member of the *Flaviviridae* family, is primarily transmitted through *Aedes* spp. mosquitoes, and evidence of vertical, sexual, and blood

[1]These authors contributed equally to this article.

transmission of Zika virus has been reported (*1*–*3*). The virus has spread rapidly across Latin America and the Caribbean since the end of 2014 and has been linked to an increase in neurologic disorders and neonatal malformations in these areas (*4*). Zika virus has the potential to spread internationally through the carriage of goods and travelers (*5*). Traveler volume between China and areas with autochthonous transmission of Zika virus is increasing; in 2015, China received ≈84,000 travelers who had departed from international airports in Brazil (*5*). The *Ae. aegypti* mosquito, the competent vector for Zika virus, is found in areas of Hainan, Guangdong, and Yunnan provinces on the mainland of China, where the known distribution is limited to areas below 22° latitude. However, *Ae. albopictus* mosquitoes are widely distributed, extending from the southern reaches to the northern and western parts of China, with north fringes from Shenyang in Liaoning Province, through Tianshui and Longnan in Gansu Province, to Motuo in Tibet (*6*). Surveillance of Zika virus infection among Chinese travelers has been enhanced since January 2016. We report the clinical and laboratory findings for a case Zika virus infection imported from Venezuela.

A previously healthy 34-year-old Chinese man was admitted to the Hospital of Ganxian on February 6, 2016. He had worked in Caracas, Venezuela, during January 1–February 2 and had onset of fever (38.0°C), headache, and

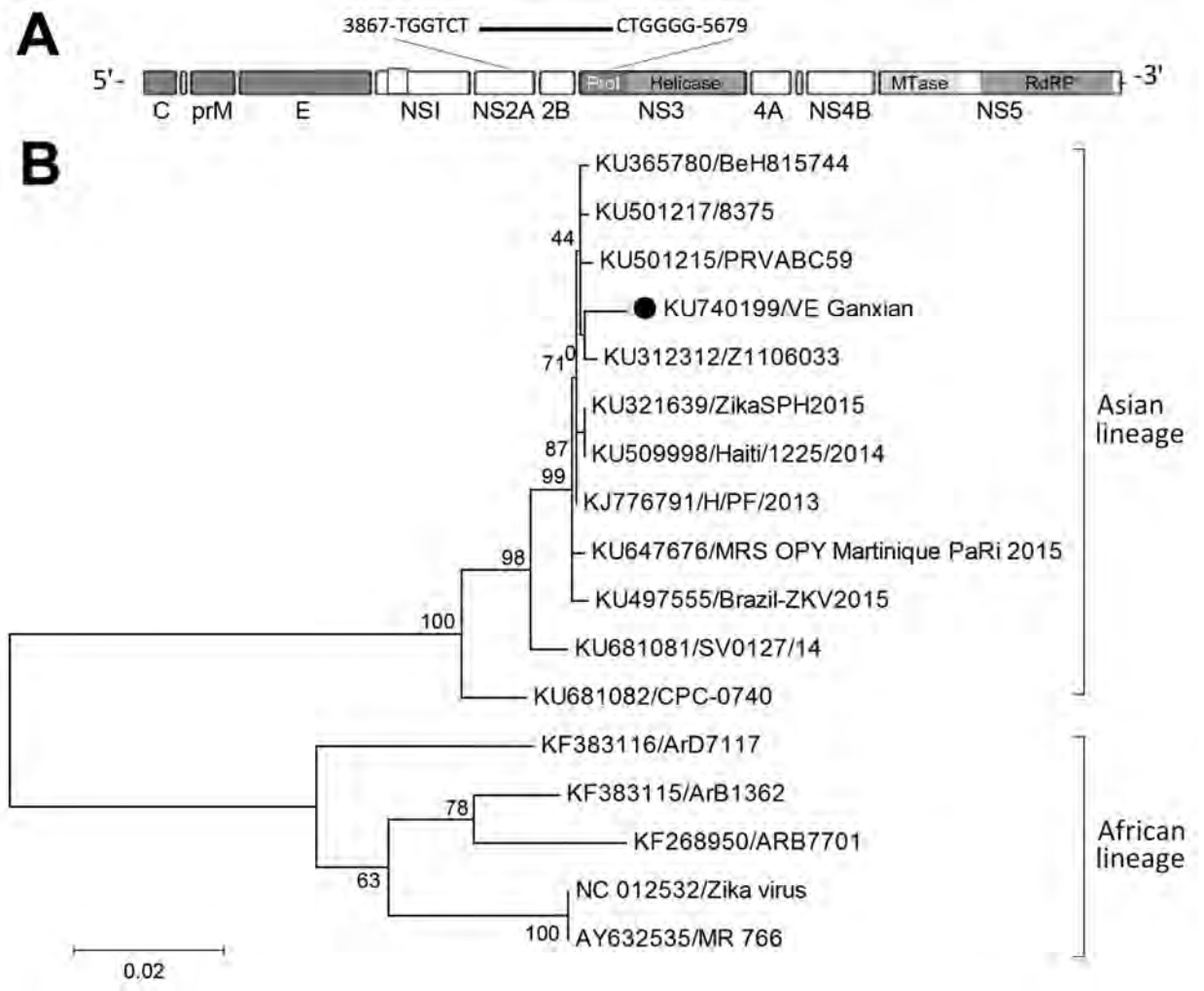

Figure. Phylogenetic analysis of partial sequences of Zika virus for an imported case of Zika virus infection in a traveler returning to China from Caracas, Venezuela, February 2016, compared with selected other strains from GenBank. A) Schematic diagram showing the contiguous sequence, obtained from de novo assembly and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), targeted at the 3′ terminus of nonstructural protein 2B and the 5′ terminus of nonstructural protein 3 genes (figure not drawn to scale). B) Maximum-likelihood phylogenetic tree inferred based on the Tamura-Nei model (*8*). The partial sequence of VE_Ganxian (black dot) obtained in this study was analyzed against 11 reference strains of Asian lineage and 5 reference strains of African lineage. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA version 7.0 (http://www.megasoftware.net). GenBank accession numbers are given. Scale bar indicates number of substitutions per site. C, capsid; E, envelope; MTase, methyltransferase; PrM, premembrane; RdRP, RNA-dependent RNA polymerase.

dizziness on January 28. He subsequently had rash, chills, retro-orbital pain, and mild diarrhea on February 2, the day on which he departed from Caracas and traveled to Jiangxi Province via Paris and Hong Kong, arriving in Shenzhen, China, on February 5. At the time of hospital admission, the patient had fever (36.7°C), headache, conjunctivae, rash on his back and face, retro-orbital pain, and mild diarrhea. General clinical examination was unremarkable. Results of a complete blood cell count and liver function tests were within reference ranges.

Serum samples were collected at day 9 and day 10 after symptom onset, and urine samples were collected once a day from day 10 through day 14. In the 2 serum samples, no dengue virus (DENV) or chikungunya virus (CHIKV) IgM or IgG were detected by a Panbio IgM and IgG capture ELISA for DENV (Panbio, Queensland, Australia) or by an indirect immunofluorescence assay slide test kit for CHIKV (EUROIMMUN AG, Lübeck, Germany). Serum and urine samples were negative for DENV nonstructural protein 1 (NS1) antigen on an NS1-ELISA test kit (Wantai Bio-Pharm, Beijing, China). To detect virus RNA in samples, in-house–designed probe and primers specific to DENV and CHIKV were used (*7*). The PCR for Zika virus was targeted to the NS1 gene. RNA was extracted from 140 μL of serum or urine by using the QIAamp Viral RNA Mini Kit (QIAGE, Hilden, Germany). Amplification reactions were performed by using the AgPath-ID One-Step RT-PCR Kit (Ambion, Carlsbad, CA, USA). A standard curve with serial dilutions of known concentrations of in vitro–transcribed RNA from a reference plasmid was used to estimate viral load in samples. Test results for DENV and CHIKV were negative. However, the serum sample collected at day 9 was positive for Zika virus RNA (viral load 1.4×10^4 copies/mL), and Zika virus RNA was detected from urine samples collected on days 10, 11, and 12 (viral loads 8.6×10^4, 4.5×10^4, and 1.2×10^4 copies/mL, respectively).

Next-generation genomic sequencing of the Zika virus genome was conducted by using the MiSeq platform (Illumina, Hayward, CA, USA) on serum and urine samples. A 1,813-bp of partial genome sequences (strain VE_Ganxian, GenBank accession no. KU740199) was obtained from urine and was used for comparing with selected other strains from GenBank. Phylogenetic analysis showed that the virus was of Asian lineage (Figure). Pairwise genetic distance calculation indicated that the sequence was most closely related to other viruses reported from French Polynesia in 2013 (strain H/PF/2013), the Caribbean in 2014 (strain Haiti/1225/2014), and Latin America in 2015 (strain ZikaSPH2015), having a 99.4% similarity in sequence.

The clinical findings for the patient were similar to those previously reported among Zika virus–infected patients (*9*), although no arthralgia was apparent. Viral RNA remained detectable for 9 days after symptom onset in serum and for an additional 3 days in urine. We did not test this patient's semen and thus cannot comment on risk for onward sexual transmission; however, the patient was told about the risks for sexual transmission of Zika virus and was advised to adopt safer sexual practices or to abstain from sexual activity for at least 1 month after recovery. In February, the mosquito density is low in Jiangxi Province (*10*), suggesting that this imported case is unlikely to cause mosquitoborne transmission. However, with the onset of summer and increased density of *Aedes* mosquitos, the risk for onward transmission of travel-associated Zika virus should not be overlooked.

Acknowledgments

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Pericarditis Caused by Hyperinvasive Strain of *Neisseria meningitidis*, Sardinia, Italy, 2015

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To the Editor: Invasive meningococcal disease is usually defined by the occurrence of meningitis or septicemia. Pericarditis might occur during the course of invasive infection. This clinical picture, defined as disseminated meningococcal disease with pericarditis (*1*) or secondary meningococcal pericarditis, was reported in 1918 (*2*). In 1939, primary or isolated meningococcal pericarditis (*1*,*3*) was described. In this form of pericarditis, pericardial or blood cultures are positive for *Neisseria meningitidis* but there is no meningeal involvement or clinical meningococcemia (*4*).

Since its description, several cases of primary meningococcal pericarditis have been reported (*5*). Although its pathogenesis remains largely undefined, it has been hypothesized that the onset of primary pericarditis occurs after a transient bacteremia or as a consequence of involvement of the lower respiratory tract (*4*). Blaser et al. reported that serogroup C meningococci are usually associated with this disease, especially in adults. However, serogroups B, W, and Y have also been identified (*4*). We report a case-patient with primary meningococcal pericarditis caused by a serogroup C strain of *N. meningitidis*.

The patient was a 32-year-old man who lived in Sardinia, Italy. He had no predisposing factors, such as immunodeficiency or other chronic disorders. Disease onset occurred on August 29, 2015. Clinical manifestations were fever (temperature 38°C), hypotension, epigastralgia, arthralgia, asthenia, chest pain, and reduced vesicular murmur. The left ventricle was widely hypokinetic, and a light ST increase was observed. A blood culture was positive for *N. meningitidis.*

The patient was given piperacillin/tazobactam (4.5 g 3×/d) and metronidazole (500 mg 3×/d) for 4 days. After 4 days, treatment with ceftriaxone (2 g 2×/d) for 4 days was started. Because of persistent fever (38.8°C), levofloxacin (500 mg 2×/d) for 23 days was also started on day 7. On day 10, ceftriaxone was replaced with piperacillin/tazobactam (4.5 g 4×/d) for 21 days. A major bilateral pleural effusion was detected on the left side. On day 11, the fever had resolved. The outcome was favorable for this patient.

Drug resistance of the strain was determined by using the MIC Test Strip Method (Liofilchem, Abruzzi, Italy). Breakpoints used were those recommended by the European Committee on Antimicrobial Susceptibility Testing version 5.0 (http://www.eucast.org/). The strain was susceptible to cefotaxime, ceftriaxone, ciprofloxacin, penicillin G, and rifampin. Serogroup was determined by using slide agglutination with commercial antisera (Remel Europe, Ltd., Dartford, UK) and confirmed by PCR (*6*).

Whole-genome sequencing was conducted to obtain molecular data and enable comparison with other meningococci of the same serogroup that were isolated in Italy. Multilocus sequence typing (MLST) and typing of *porA* and *fetA* genes and Bexsero (meningococcal group B vaccine) antigen genes (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm431374.htm) were conducted as described (http://neisseria.org/). Whole-genome sequence was analyzed by using the BIGSdb Genome Comparator Tool (http://pubmlst.org/neisseria/). Genomes of meningococci belonging to the same finetype were compared by using the core genome MLST (cgMLST) approach.

The *N. meningitidis* strain of serogroup C was susceptible to all antimicrobial drugs tested. Although serogroup C was associated with 53 (41%) of 132 invasive meningococcal disease cases in Italy in 2015 (http://www.iss.it/binary/mabi/cont/Report_MBI_20151223_v4.pdf), this serogroup has not been detected in Sardinia since 2010.

Molecular analyses showed that the strain belonged to the hypervirulent clonal complex (cc) 11, sequence type (ST) 11. The complete finetype was C:5–1,10–8:F3–6:ST-11(cc11). This finetype has been reported in the United

States and several countries in Europe (*7*), including Italy, and is responsible for several disease outbreaks. In Italy, this finetype represents 61% (70/115) of all serogroup C strains collected during 2012–2015. Two outbreaks caused by this strain were reported in Italy in 2007 (*8*) and in 2012 (*9*). The *N. meningitidis* factor H binding protein and heparin binding protein alleles were 1.13 and 20, respectively. The *N. meningitidis* adhesin A variant had an insertion sequence that disrupted this gene, as described for ET-15 meningococci (*10*). On the basis of results of cgMLST, the strain was determined to be related to strains responsible for an outbreak in Italy in 2015.

In summary, we report a case of meningococcal pericarditis caused by a strain of *N. meningitidis*. This strain belongs to hyperinvasive clonal complex cc11 and was identified as C:P1.5–1,10–8:F3–6:ST-11(cc11), an emerging strain in Italy and worldwide. Timely diagnosis and complete molecular characterization of this strain, which causes a rare form of invasive disease (*4*), is needed for appropriate management of patients with this disease.

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Ecologic Study of Meningococcal B Vaccine and *Neisseria gonorrhoeae* Infection, Norway

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To the Editor: Gonorrhea is a sexually transmitted disease that can cause pelvic inflammatory disease, ectopic pregnancy, and salpingitis in women and infertility in men and women. Rates vary; incidence is 12.5 cases/100,000 population in Europe (*1*) and ≈6,000 cases/100,000 population in parts of sub-Saharan Africa (*2*). Recurrent infection is common, antimicrobial drug resistance is growing, and no licensed vaccine is available to protect against gonorrhea infection. Components of some meningococcal B (MenB) vaccines could provide protection against the causative bacterium, *Neisseria gonorrhoeae* (M. Pizza, pers. comm.), because the meningococcus bacterium is of the same *Neisseria* genus and the 2 bacteria share key protein antigens, such as the outer membrane vesicle (OMV). Ecologic evidence from Cuba supports a decline in gonococcus

[1]Now a GlaxoSmithKline company.

infection after a nationwide OMV vaccine campaign in the 1980s (*3*). In Norway, a trial of another OMV MenB vaccine (MenBvac, Norwegian Institute of Public Health, Oslo, Norway) was conducted among teenagers during 1988–1992. We retrospectively examined associations between MenB vaccine coverage during 1988–1992 and national gonorrhea rates for persons ≥16 years of age during 1993–2008 in Norway.

Age, year of birth, and year of diagnosis for anonymized, laboratory-confirmed gonorrhea patients were available from the Norwegian Surveillance System for Communicable Diseases from 1993 onwards. We collected aggregate vaccination coverage by MenB vaccine from the electronic trial register for 1988–1992. Only children enrolled in secondary schools and ≈13–15 years of age were offered the vaccine. This group accounted for 63% of the 148,589 children resident in Norway during the trial period and born during 1973–1976. Using annual population estimates, we derived gonorrhea notification rates and compared 3 cohorts: the vaccinated cohort (VC) born during 1973–1976; the unvaccinated cohort born during 1965–1972 (pre-VC); and the unvaccinated cohort born after 1976 (post-VC). Using Poisson log-linear regression adjusted for year of diagnosis, we calculated incidence rate ratios (IRRs; number of new diagnoses of gonorrhea per 100,000 population) for VC and post-VC and compared them with IRRs for pre-VC (the reference group).

During 1993–2008, a total of 2,601 cases of gonorrhea were reported. To avoid case ascertainment bias, cases reported from 2009 onwards were excluded because >50% were diagnosed by PCR. In men, notification rates fell from 27.3/100,000 population in 1993 to 12.3 in 1995 but increased to 22.9 in 2008 (IRR 1.02, 95% CI 1.00–1.03, p = 0.001). The recent increase is largely attributable to transmission between men who have sex with men, a group accounting for 12% of cases among men in 1993 but 40% in 2008. Among women, incidence rates dropped from 20.7/100,000 population in 1993 to 3.1 in 1999 and have remained stable since 2008, when the rate was 4.1.

We hypothesized that an immunogenic effect of MenB vaccination on gonorrhea notification rates might be most expected among persons in their early 20s in the mid-1990s, a group at risk for gonorrhea infection during years closest to the vaccination campaign. A small but significant reduction occurred in crude incidence rates for women 20–24 years of age in the VC (IRR 0.58, 95% CI 0.42–0.8); however, this reduction was not significant after incidence rates were adjusted for year of diagnosis (IRR 0.72, 95% CI 0.51–1.02). No change in incidence occurred for other age groups or birth cohorts among women (Figure). Reduced

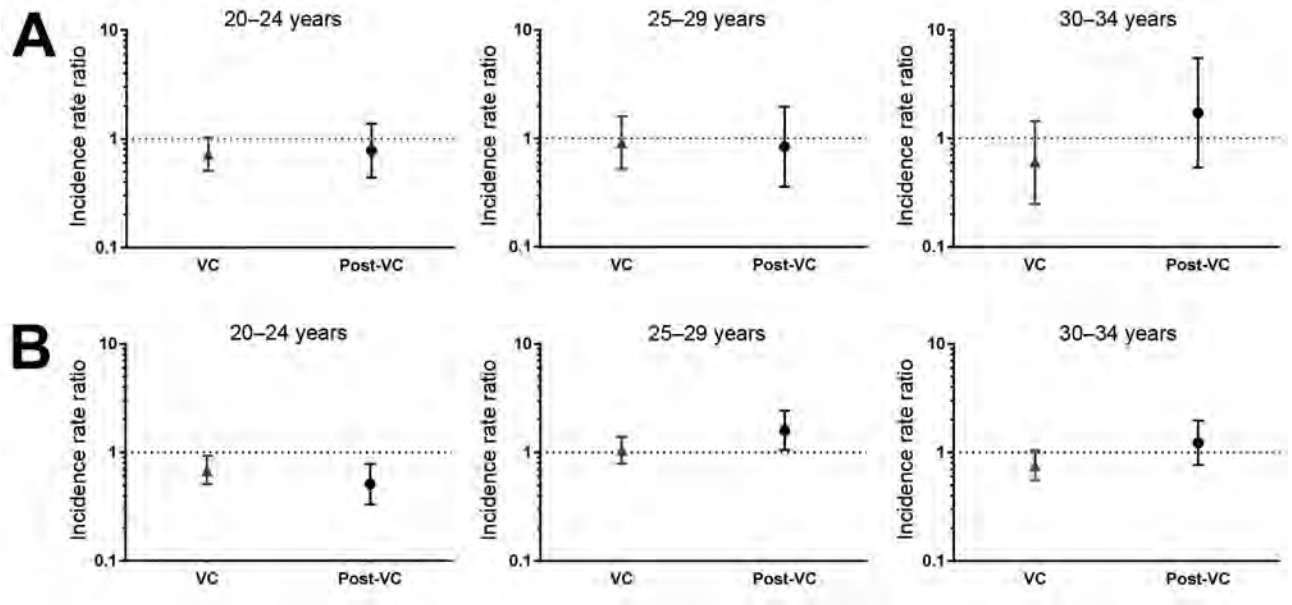

Figure. Incidence rate ratios (IRRs) of cases of gonorrhea by age group and birth cohort, adjusted for year of diagnosis in A) women and B) men, Norway. IRRs were calculated by using Poisson log-linear regression and adjusted for year of diagnosis. IRRs indicate incidence of gonorrhea in the vaccinated cohort (VC), (i.e., persons born during 1973–1976, 63% of whom were vaccinated), represented by triangles, and in the post-VC (i.e., those born after 1976), represented by circles, relative to the reference birth cohort, the pre-VC (i.e., persons born during 1965–1972), represented by the horizontal dotted line. Also shown are 95% CIs. Differences in the IRR were considered statistically significant if the CI did not include 1. A) Among women, the adjusted IRR in the VC 20–24 years was of borderline significance: 0.72, 95% CI 0.51–1.02. No effect was seen in other age groups or birth cohorts. B) Among men, a reduced adjusted incidence was evident for those 20–24 years of age in the VC (IRR 0.68, 95% CI 0.51–0.93) and persisted among post-VC who were 20–24 years of age (IRR 0.51, 95% CI 0.33–0.78). IRR increased for men 25–29 years of age in the post-VC (IRR 1.6, 95% CI 1.06-2.43).

incidence also occurred for men 20–24 years of age in the VC (adjusted IRR 0.68, 95% CI 0.51–0.93) and persisted in the post-VC (adjusted IRR 0.51, 95% CI 0.33–0.78); the IRR increased for men 25–29 years of age in the post-VC (adjusted IRR 1.6, 95% CI 1.06-2.43). (Figure).

Overall, rates of gonorrhea dropped among men and women after the vaccination campaign; however, rates had already been in decline since the mid-1970s (*4*). A limited age-specific vaccine effect occurred among men and women 20–24 years of age. No vaccine effect was found among women in other age groups or birth cohorts. Among men 20–24 years of age, the persistent decline occurring among the post-VC could be explained by a herd effect, but rates subsequently increased for men 25–29 years of age. Different effects for men and women may be explained in part by changing transmission patterns and sexual behavior occurring among men who have sex with men and by inadequate adjustment of the underlying gonorrhea trend in men; however, differences by sex are difficult to interpret.

Among study limitations, our ecologic study design could not distinguish between long-term trends or behavioral factors and vaccine effects. For example, in the early 1990s, condom use increased in Norway, especially among persons in their early 20s (*5*), possibly in response to the evolving HIV epidemic (*6*,*7*). In addition, moderate population vaccine coverage in the VC (i.e., 63%) and a time lag between the vaccination program (1988–1992) and the start of surveillance (1993) may have diluted tangible vaccine effects. For further examination of cross-immunity of MenB vaccines with gonococci, vaccine effectiveness studies in regions where OMV vaccine was used (e.g. New Zealand) and evaluation of new protein-based vaccines are warranted.

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Neisseria gonorrhoeae Resistant to Ceftriaxone and Cefixime, Argentina

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To the Editor: Antimicrobial resistance in *Neisseria gonorrhoeae* is increasing globally. In recent years, gonococcal strains with resistance to the extended-spectrum cephalosporin (ESC) ceftriaxone have been reported from many countries (*1*). In South America, 7 ceftriaxone-resistant strains (MICs >0.25 μg/mL) were reported from Brazil in 2007; however, these isolates have not been characterized (*2*). Emergence of cephalosporin-resistant gonorrhea would substantially limit treatment options and represent a major public health concern. We report an *N. gonorrhoeae* isolate in Argentina that was resistant to ceftriaxone and cefixime.

In September 2014, a 19-year-old heterosexual man with no underlying disease was admitted to a hospital emergency department in Rio Negro, Argentina. Physical examination showed a purulent urethral discharge. He reported having had unprotected insertive vaginal sex with multiple partners in the past few months. The patient denied recent travel outside Argentina. He had a history of gonococcal urethritis (March 2014), which was treated with a single dose (500 mg) of ceftriaxone; a gonococcal isolate obtained at that time was not sent to a reference laboratory as part of the Argentinian Gonococcal Antimicrobial Susceptibility Surveillance Program for determination of the antimicrobial susceptibility profile.

At the time of admission, we obtained and cultured a urethral swab specimen and identified an isolate as *N. gonorrhoeae* by using conventional methods (*3*). The isolate was sent to a reference laboratory, and its identity was confirmed by using the Phadebact GC Monoclonal Test (MKL

Diagnostic AB, Sollentuna, Sweden) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany). The patient was given a single dose (500 mg) of ceftriaxone. Five days later, the patient returned for clinical evaluation, and symptoms had resolved. Culture for test of cure was not performed.

We determined MICs of the isolate for penicillin, cefixime, ceftriaxone, tetracycline, ciprofloxacin, and azithromycin by using the agar dilution method according to the standard of the Clinical and Laboratory Standards Institute (*4*). Results were interpreted in accordance with breakpoints of this standard, except for azithromycin, for which we applied breakpoints of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/).

This isolate was resistant to ceftriaxone and cefixime (MIC 0.5 μg/mL), penicillin (8 μg/mL), tetracycline (8 μg/mL), and azithromycin (1 μg/mL). However, the isolate was susceptible to ciprofloxacin (0.03 μg/mL). We obtained a negative result for β-lactamase by using the chromogenic nitrocefin disk assay (Becton Dickinson, Franklin Lakes, NJ, USA).

Resistance determinants involved in ceftriaxone resistance were amplified by PCR and sequenced as reported (*5*,*6*). We than analyzed full-length *penA* and *pilQ* gene sequences and other genetic determinants, including *mtrR*, *penB*, and *ponA* genes. We purified and sequenced PCR products by using an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and performed molecular epidemiologic characterization by using *N. gonorrhoeae* multiantigen sequence typing (http://www.ng-mast.net).

Sequence analysis of the *penA* gene, which encodes penicillin-binding protein 2 (PBP2), identified a nonmosaic PBP2 IX allele. Analysis of the *mtrR* gene and its promoter identified a single nucleotide (A) deletion in the inverted repeat of the promoter region, and a single amino acid substitution at position H105 (H→Y). Amino acid substitutions in the *porB1b* gene were found at positions G120 (G→K) and A121 (A→D). The *ponA* gene, which encodes PBP1, had an amino acid substitution at position L421 (L→P), and full-length PilQ amino acid sequence had sequence type VI. The isolate was assigned to serogroup PorB1b (WII/III), and *N. gonorrhoeae* multiantigen sequence typing showed that it had novel sequence type ST13064 (*porB*-7592 and *tbpB*-33).

Mosaic PBP2 alleles have been strongly associated with decreased susceptibility or resistance to ESCs (*7*). However, the isolate we obtained had a nonmosaic PBP IX allele, which contains the P551L substitution that has been associated with increased MICs for ESCs (*8*). Association of the nonmosaic PBP IX allele, most likely with *mtrR*, *penB*, and *ponA* gene mutations, might be involved in resistance to ESC (*9*). Although Whiley et al. (*6*) did not report an association between sequence type VI and resistance to ESCs, the contribution of this sequence type to ESC resistance requires additional studies.

Isolates with decreased susceptibility and resistance to ESC have now emerged in Argentina (*10*), increasing from 1.1% in 2011 to 5.6% in 2014 (Argentinian Gonococcal Antimicrobial Susceptibility Surveillance Program, unpub. data). Treatment failures and isolates with reduced susceptibilities and resistance to ESC have been reported (*9*). However, in Argentina, syndromic management of gonorrhea has resulted in suboptimal diagnosis and lack of specimens to culture to distinguish between treatment failure and reinfection. Syndromic management represents a major problem that not only compromises surveillance program but also increases selective pressure and facilitates development of drug resistance. Accordingly, surveillance should be strengthened to support detection and verification of asymptomatic infections and treatment failures, identify communities at high risk, and trace sexual contacts. These efforts should be used in public health responses to mitigate emergence and spread of ESC-resistant gonococci.

This study was conducted as part of the reference work of the Argentinian National Reference Laboratory and the Gonococcal Antimicrobial Surveillance Susceptibility Program.

This study was supported by the National Administration of Laboratories and Institute of Health Dr. Carlos G. Malbrán, Argentina Ministry of Health.

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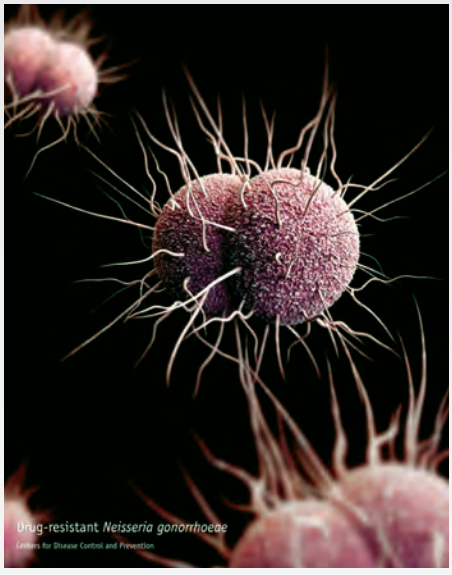
etymologia

Neisseria

A gram-negative, non-motile diplococcal bacterium, *Neisseria* is named after Albert Ludwig Sigesmund Neisser, a German physician who discovered *Neisseria gonorrhoeae* in 1879.

Gonorrhea comes from the Greek *gonos*, meaning "seed," and rhoe, "flow. The disease caused by this bacterium was known as "gonorrhea" because early physicians incorrectly thought the purulent discharge was semen. As early as 1719, gonorrhea was referred to as "the clap," although theories for why it was called this vary. It may refer to the old French term *clapier*, "brothel," a place where the disease spread easily. Another theory refers to preantibiotic days when the infection was treated by slapping the penis against a board, or clapping it between two boards to force out infected discharge.

N. gonorrhoeae is 1 of only 2 *Neisseria* species that is pathogenic to humans. The second, *N. meningitidis*, causes outbreaks of meningitis and septicemia. It was isolated by Anton Weichselbaum in 1887 and designated as *Diplococcus intracellularis meningitidis*.



A 3-dimensional computer-generated image of drug-resistant *Neisseria gonorrhoeae* diplococcal bacteria. Source: Public Health Image Library.

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Microbial Biofilms, Second Edition

Mahmoud Ghannoum, Matthew Parsek, Marvin Whitely, Pranab K. Mukherjee, editors

ASM Press, Washington, DC, USA, 2015; ISBN (print): 978-1555817459, ISBN (ebook): 9781555817466; Pages: 404; Price: US$160.00

Microbial Biofilms provides an overview of the formation, structure/architecture, cell-to-cell interactions, and dispersal of fungal and bacterial biofilms. The target audience is biofilm researchers, but this second edition of the book should also be useful for healthcare practitioners seeking a better understanding of microbial biofilms in healthcare delivery.

The number of published papers pertaining to microbial biofilms in healthcare and public health has continued to grow since the publication of the first edition of Microbial Biofilms in 2004. For example, a search using PubMed for 2004–2016 identified 488 publications when using the search terms "biofilm and healthcare-associated infection" and 7,303 publications using the search terms "biofilm and public health," compared to 73 and 1,459 publications, respectively, for 1992–2003. The current edition addresses this level of interest; several of the contributions in this book specifically focus on the role of biofilms in disease processes (Chapters 6, 7, 8, 14, and 19) or biofilm susceptibility to antimicrobial agents (Chapters 2 and 13).

Chapter 1 provides a balanced comparison of static and continuous flow methods for growing biofilms that should be beneficial for researchers investigating biofilm development or dispersion, and for applied studies evaluating new treatment strategies for biofilm prevention and control. This beginning can enable those new to the field to evaluate the benefits and drawbacks of different biofilm testing methods for specific applications. Chapter 1 also provides a helpful but brief discussion of the use of "omic" approaches (i.e., genomic, metagenomic, transcriptomic, and proteomic) in the study and characterization of biofilms.

Protocols to evaluate biofilm control strategies in vivo are needed, since in vitro methods may not predict performance under the more robust conditions provided in an animal model (*1*). Published animal model protocols for evaluating biofilm control are few. Chapter 3 provides several animal models for the evaluation of fungal biofilms (primarily *Candida* spp.), including vascular catheter, urinary catheter, and subcutaneous implant model systems. However, animal model systems for the characterization of bacterial biofilms are not described.

Several recent papers have used culture-independent methods to characterize biofilms on indwelling medical devices (*2*–*4*). With the exception of a brief discussion in Chapter 1, very little information is provided on the benefits and drawbacks of culture-independent methods to characterize clinically relevant biofilm communities.

Chapter 13 provides an excellent overview of antimicrobial tolerance in biofilms, with a good summary of the factors that can influence susceptibility. I found particularly helpful the use of tolerance factors to compare reduced susceptibility of different biofilm-associated organisms toward biocides, antiseptics, and antibiotic drugs. Tolerance factors were plotted as a function of antimicrobial agent molecular weight, substratum material, and biofilm density, providing the reader a method for quickly visualizing these patterns for a wide range of organisms. This information can be very helpful when developing experimental approaches to evaluate biofilm control strategies.

In summary, Microbial Biofilms is a useful compendium suitable for students and a practical guide for researchers investigating new biofilm treatment strategies. The emphasis on the role of biofilms in the pathogenesis of various microbial diseases, as well as discussions of biofilm tolerance and antimicrobial resistance should also be helpful and interesting to anyone working in the field of healthcare delivery.

Rodney M. Donlan

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Katsushika Hokusai (1760–1849). *Yejiri Station, Province of Suruga,* 1832. Woodblock color print, 9 9/16 x 14 5/16 in. Brooklyn Museum, Gift of Frederic B. Pratt, 42.74

Perspective and Surprise in the Floating World

Byron Breedlove and Jared Friedberg

Katsushika Hokusai, one of the most prolific Japanese artists of the late Edo period (1615–1868), has been described as "cocky, quarrelsome, restless, aggressive, and sensational." He obsessed with his art to the extent that he would move frequently rather than clean or repair his cluttered houses. During an artistic career that spanned 7 decades, he signed his works with more than 30 different names—typically in tandem with a shift in his artistic style and technique. Though this practice was common in Japan during his lifetime, Hokusai changed his name more often than any other major Japanese artist.

During his mid-70s, he used as his signature "Gakyo Rojin Manji," "The Art-Crazy Old Man." His humorous, astute self-characterization is supported by the fact that he left more than 30,000 varied works of art, including woodblock prints, travel and book illustrations, manga, and silk paintings. Hokusai is one of the outstanding figures and innovators of the *Ukiyo-e* or "pictures of the floating world" (that is, scenes of everyday life) school of printmaking. He is credited with expanding the range of subjects from traditional *Ukiyo-e* depictions of courtesans and kabuki actors to encompass landscapes and to include people from various social levels.

Though the exact date of its publication is unknown, Hokusai's defining work, "Thirty-Six Views of Mount Fuji," was created from 1826 through 1833. Mt. Fuji, which is tied firmly into the cultural, spiritual, and social identity of Japan, had long served as inspiration for Hokusai and his art. In response to brisk sales of the initial set of 36, the artist expanded this popular collection to comprise

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46 woodblock prints. Art scholar Sarah Thompson notes that the universal appeal of Hokusai's works relates to his use of Western style perspectives and to "their strong internal structure. Many prints in the Fuji series explicitly contrast the strikingly triangular shape of the mountain with triangles, rectangles, trapezoids, or curved shapes formed by other elements of the design."

Included among the original series of 36 views is this month's cover image "Yejiri Station, Province of Suruga." In this gently humorous image, Hokusai captures the reactions of several travelers on a serpentine path through the rustling marsh grasses, caught off guard by a sudden squall. Some stoop and clutch their hats and clothes as others fruitlessly reach toward their papers and hats blowing skyward, mixing with the leaves stripped from the bending trees. The British Museum offers this description: "The silhouette of Mt. Fuji is drawn with a single line, providing a backdrop for the figures and trees battling the wind in the foreground. Bending their bodies and clutching at scarves and hats, all turn their faces away from us—as if we were the source of the blast that carries off the tissues that had been tucked into the woman's kimono."

The element of surprise is central to Hokusai's visual narrative. The Honolulu Museum of Art notes that Hokusai "focused on an instant of drama caused by a gust of wind. Fuji stands white and unshaken, affected neither by the wind nor the human drama."

Like the unseen wind in Hokusai's print, severe respiratory disease outbreaks can be sudden, disruptive, and chaotic. Emerging or reemerging pathogens may trigger serious, widespread respiratory disease outbreaks—including adenovirus bronchitis, influenza, Legionnaires' disease, Middle East respiratory syndrome, multidrug-resistant tuberculosis, and severe acute respiratory syndrome (SARS).

To gain perspective on the nature of such threats, determining the etiology and understanding the clinical and epidemiologic characteristics of an outbreak is crucial. Surveillance systems—which are a basic component in the routine counting of disease cases—enable public health professionals to detect and investigate unexpected increases in these cases and prompt measures to control outbreaks, including those of emerging and reemerging respiratory disease. In a sense, surveillance systems embody the character of Mt. Fuji—which provides Hokusai's travelers in "Yejiri Station, Province of Suruga" with a reference point during a storm—as they provide a constant, stable approach for outbreak investigations.

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- *Tropheryma whipplei* as a Cause of Epidemic Fever, Senegal, 2010–2012
- African Swine Fever Epidemic, Poland, 2014–2015
- Restaurant Cooking Trends and Increased Risk for *Campylobacter* Infection
- Red Fox (*Vulpes vulpes*) as a Sentinel for *Blastomyces dermatitidis*, Ontario, Canada
- Vesicular Disease in 9-Week-Old Pigs Experimentally Infected with Senecavirus A
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- Travel-Associated Rabies in Pets and Residual Rabies Risk, Western Europe
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- Outbreak of *Vibrio parahaemolyticus* ST-120, Peru, 2009
- Spread of H5 Clade 2.3.4.4 Highly Pathogenic Avian Influenza Virus Subgroups and Generation of Novel Reassortant Viruses, USA
- Post-Booster Antibodies as Source of Diphtheria Antitoxin
- Major Persistent 5′ Terminally Deleted Coxsackievirus B3 Populations in Endomyocardial Tissues of an Idiopathic Dilated Cardiomyopathy Patient
- Natural Norovirus Infections in Rhesus Macaques, Louisiana, USA

Complete list of articles in the July issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

June 16–20, 2016
American Society for Microbiology
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http://www.asmmicrobe.org/

July 18–22, 2016
21st International AIDS Conference
Durban, South Africa
http://www.aids2016.org/

August 24–28, 2016
Options IX for the Control of Influenza
Chicago, IL, USA
http://2016.isirv.org/

October 29–November 2, 2016
American Public Health Association
Denver, Colorado, USA
https://www.apha.org/events-and-meetings/annual/past-and-future-annual-meetings

November 4–7, 2016
IMED
International Meeting on Emerging Diseases and Surveillance
Vienna, Austria
http://imed.isid.org/

November 13–17, 2016
ASTMH
American Society of Tropical Medicine and Hygiene
Atlanta, GA, USA
https://www.astmh.org/annual-meeting?utm_source=ASTMH%2DInformz&utm_medium=email&utm_campaign=default

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African Society for Laboratory Medicine
Cape Town, South Africa
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Article Title

Human Infection with Influenza A(H7N9) Virus during 3 Major Epidemic Waves, China, 2013–2015

CME Questions

1. You are consulting with a regional Chinese Department of Health regarding influenza outbreaks. According to the surveillance study by Wu and colleagues, which of the following statements about epidemiologic features including changes in laboratory-confirmed human infections with influenza A(H7N9) virus infection across 3 epidemic waves in 2013–2015 in mainland China is correct?

A. Laboratory-confirmed H7N9 cases in waves 2 and 3 vs wave 1 were older
B. Laboratory-confirmed H7N9 cases in waves 2 and 3 vs wave 1 were more likely to be reported from large cities
C. Peak incidence during the first wave of infections was in the summer of 2013
D. A second epidemic of infections occurred in the winter of 2013–2014, and a third epidemic occurred in the winter of 2014–2015

2. According to the surveillance study by Wu and colleagues, which of the following statements about changes in hospitalized cases of H7N9 across 3 epidemic waves in 2013–2015 in mainland China is correct?

A. In waves 2 and 3, the risk for death among hospitalized patients was higher in Jiangxi and Fujian than in provinces in eastern and southern China
B. After adjustment for age and underlying medical conditions, hospitalized patients with confirmed H7N9 in waves 2 and 3 had a lower risk for death than those in wave 1
C. Time to admission and laboratory confirmation among hospitalized patients were earlier in the second and third waves than in wave 1
D. Higher risk for death among hospitalized patients in the second and third waves was fully explained by differences in age, underlying medical conditions, and urban or rural residence

3. According to the surveillance study by Wu and colleagues, which of the following statements about possible reasons for the observed changes in laboratory-confirmed and hospitalized cases of H7N9 across 3 epidemic waves in 2013–2015 in mainland China is correct?

A. Increased risk for death in waves 2 and 3 was definitely caused by changing pathogenesis associated with genetic clades of H7N9 virus appearing in later epidemic waves
B. Increased risk for death in waves 2 and 3 was definitely caused by differences in clinical management in different provinces
C. Case ascertainment bias could not be ruled out
D. Increased risk for death in waves 2 and 3 was definitely caused by increased population contact with live poultry

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

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Article Title

Infectious Disease Risk Associated with Contaminated Propofol Anesthesia, 1989–2014

CME Questions

1. You are evaluating your hospital's safety protocols after sepsis related to the administration of propofol developed in a 70-year-old woman. According to the current study, what should you consider in general regarding infections related to propofol?

A. Nearly all cases of infections related to propofol occur in low-income countries
B. The rate of infections related to propofol appears to be grossly overreported
C. Outbreaks of infections related to propofol generally last 1 day or less
D. There are 144 reported cases of infections related to propofol, including 10 fatal cases

2. Which of the following procedures has been most commonly associated with propofol-related infections?

A. Total hip arthroplasty
B. Endoscopy
C. Major cardiac surgery
D. Intubation in the intensive care unit

3. Which of the following delivery devices has been most often implicated in outbreaks of propofol-related infections?

A. Vials
B. Intravenous stopcock dead space
C. Intravenous tubing
D. Syringes or microdroppers

4. What else should you consider regarding outbreaks of propofol-related infections?

A. Most cases of infections are reported in the intensive care unit
B. Adding edetate disodium to propofol essentially eliminates the risk for infection
C. Specific protocols for handling propofol have failed to curb the risk for contamination
D. Bacterial, fungal, and viral infections have been reported

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

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Summary of Authors' Instructions

Author's Instructions. For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

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. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

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. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and 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. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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.

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. Include biographical sketch.

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. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

DEPARTMENT OF
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Katsushika Hokusai (1760–1849). *Yejiri Station, Province of Suruga*, 1832. Woodblock color print, 9 9/16 x 14 5/16 in. Brooklyn Museum, Gift of Frederic B. Pratt, 42.74

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