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Victor D. Brenner (1871–1924) International Congress on Tuberculosis Medal, 1908. Bronze, 1.4 × 1.2 in /35.5 × 30.9 mm. Photography by James Gathany

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On the Cover

Victor D. Brenner (1871–1924) International Congress on Tuberculosis Medal, 1908.

Bronze, 1.4 × 1.2 in/ 35.5 × 30.9 mm

Photography by James Gathany

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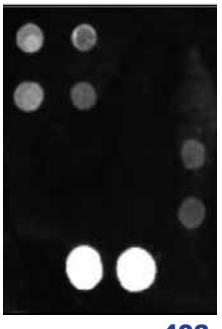

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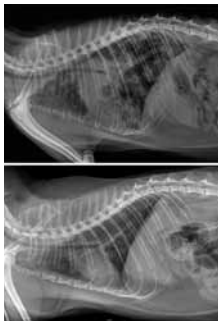

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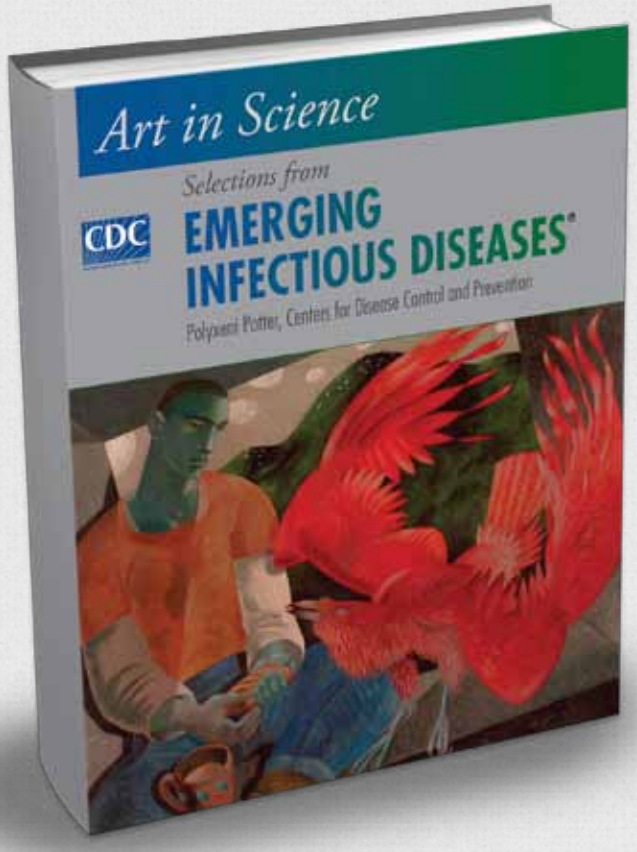

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Evaluation of the Benefits and Risks of Introducing Ebola Community Care Centers, Sierra Leone

Adam J. Kucharski, Anton Camacho, Francesco Checchi, Ron Waldman, Rebecca F. Grais, Jean-Clement Cabrol, Sylvie Briand, Marc Baguelin, Stefan Flasche, Sebastian Funk, W. John Edmunds

In some parts of western Africa, Ebola treatment centers (ETCs) have reached capacity. Unless capacity is rapidly scaled up, the chance to avoid a generalized Ebola epidemic will soon diminish. The World Health Organization and partners are considering additional Ebola patient care options, including community care centers (CCCs), small, lightly staffed units that could be used to isolate patients outside the home and get them into care sooner than otherwise possible. Using a transmission model, we evaluated the benefits and risks of introducing CCCs into Sierra Leone's Western Area, where most ETCs are at capacity. We found that use of CCCs could lead to a decline in cases, even if virus transmission occurs between CCC patients and the community. However, to prevent CCC amplification of the epidemic, the risk of Ebola virus–negative persons being exposed to virus within CCCs would have to be offset by a reduction in community transmission resulting from CCC use.

The current epidemic of Ebola virus disease in western Africa has resulted in thousands of cases during 2014 (*1*). To date, Ebola treatment centers (ETCs) have been used to isolate patients and provide clinical care. These facilities typically have large capacity (some have >100 beds) and function under high levels of infection control. However, in Sierra Leone, ETCs have reached capacity, and patients are being turned away (*1*). The reproduction number (defined as the average number of secondary cases generated by a typical infectious person) has been >1 in Sierra Leone, leading to growth in the number of cases reported each week (*2*–*4*). As a result, there is an urgent need to rapidly scale up treatment and isolation facilities. Delays in implementation will result in falling further behind the epidemic curve and in an even greater need for patient care facilities.

ETCs are complex facilities that require a substantial number of staff and time to set up; thus, the World Health Organization and other partners are looking at additional care options to supplement existing ETCs. One approach is the use of Ebola community care centers (CCCs), which would represent a possible change in operational approach (*5*–*7*). As envisioned in the World Health Organization approach, CCCs would be small units with 3–5 beds and would be staffed by a small group of health care workers. The main objective would be to isolate patients outside the home and, hence, reduce the movement and contacts of infectious persons within the community. CCCs are designed to engage the community and to increase the acceptance of isolation. Care for patients in CCCs would be provided primarily by a caregiver who would be given personal protective equipment (PPE) and basic patient care training. Patients would be free to leave the unit while awaiting test results. The specific utilization of CCCs would vary, depending on local context, and units would form part of a package of interventions, including monitoring of community contacts and burials within the community.

CCCs would be easier to set up than ETCs because they would be lightly staffed and could be made from local materials or even tents. Thus, CCCs have the potential to more rapidly begin treating patients. At present in Sierra Leone, the average time from symptom onset to hospitalization for Ebola virus disease patients is 4.6 days, which means patients remain in the community until the late stage of the disease (*4*). However, the use of CCCs has potential risks: the number of cases could be amplified if Ebola virus–negative patients in CCC assessment areas are exposed to infectious persons before admission, and virus could be transmitted between patients and caregivers or others in

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the community if virus containment within the CCC is not perfect. Given the urgent need for new operational solutions for Ebola patient care, it is critical to assess the conditions under which CCCs might exacerbate or mitigate the epidemic and to compare the scale-up of CCCs with the expansion of ETCs or home care.

We used an Ebola virus transmission model to evaluate the relative benefits and risks of introducing CCCs in a situation similar to that in Western Area, an administrative division of Sierra Leone. Western Area has exhibited consistent exponential growth in reported cases, and ETCs in the area are at capacity (*1*). Expert elicitation was used to estimate plausible values for key model parameters; these values were compared with simulation results to establish whether CCCs could be beneficial. We also estimated how many CCC beds, either alone or in combination with additional ETC beds, would be required to potentially turn over the epidemic (i.e., reduce the reproduction number below the critical threshold of 1).

Methods

Because precise medical and operational details of CCCs are still under discussion, we focused on the implications of CCC introduction under a set of general assumptions. We modeled Ebola transmission by using a modified susceptible-exposed-infectious-resolved framework (*8*–*10*). In the model, persons were initially susceptible to the virus; upon infection, patients moved into a latent state for an average of 9.4 days (*4*) and then became symptomatic and infectious for an average of 9.5 days (*4*) before the disease was resolved (through either recovery or death and burial) and the patient no longer contributed to transmission. The model accounted for changes in ETC capacity to date (details available at https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/).

First, we used the model to generate epidemiologic forecasts for Western Area and to establish a baseline scenario for the level of infection if no additional interventions were introduced. We fitted the model to the number of weekly reported Ebola virus disease cases in Western Area during August 16–November 31, 2014 (*1*). We estimated that in Western Area the basic reproduction number (defined as the average number of secondary cases generated by a typical infectious person in the absence of control measures) was 1.94 (95% credible interval [CrI] 1.86–1.98) and that there would be 1,060 exposed persons (95% CrI 800–1,420) and 650 symptomatic persons (95% CrI 460–910) in the community on December 1, 2014.

To model the introduction of CCCs, we assumed that Ebola virus–susceptible persons could also become infected with other febrile diseases that have Ebola virus disease–like symptoms, which we assumed had symptoms that lasted an average of 7 days. Thus, 2 types of symptomatic persons were included in our simulation model: Ebola virus–positive and Ebola virus–negative patients (Figure 1; https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/). In the model, Ebola virus–positive and –negative patients took an average of 4.6 days (*4*) after the onset of symptoms before attending an ETC. The probability that a patient was admitted to an ETC depended on the number of currently available beds. Well-managed ETCs operate strict patient isolation, careful use of PPE, and safe burial procedures (*11*,*12*), so we assumed that no virus transmission occurred between Ebola virus–infected patients and community members once patients were admitted to an ETC. If suspected case-patients were admitted to ETCs and subsequently found to be negative for Ebola virus, they returned to the community; we assumed there was no risk of Ebola virus–negative patients becoming infected while waiting for test results.

We also included CCCs in the model. We assumed that for patients visiting local CCCs, the time between symptom onset and CCC visit was shorter than that for patients visiting the larger and more distant ETCs; in the main analysis, we assumed that the average time from symptom onset to CCC attendance was 3 days. If CCCs were full, then patients attended ETCs instead. If ETCs were full, patients remained in the community. We assumed there was a possibility for some transmission of virus from CCC patients to community members (either directly, through caregivers,

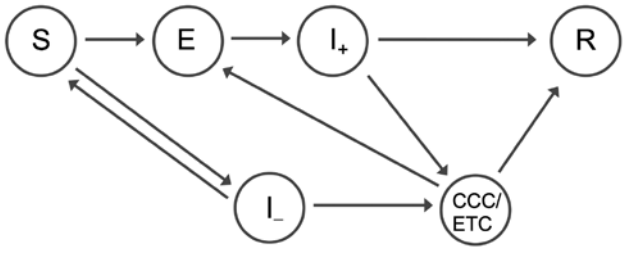

Figure 1. Structure of transmission model used to evaluate the benefits and risks of introducing CCCs into Western Area, Sierra Leone. Persons start off being susceptible to infection (S). Upon infection with Ebola virus, they enter an incubation period (E), and at symptom onset, they become infectious in the community (I_+). After this point, infected persons seek health care in CCCs or ETCs; if centers are full, the infectious persons remain in the community until the infection is resolved (R) (i.e., the patients have recovered from the disease or are dead and buried). Patients admitted to ETCs and CCCs also move into the resolved compartment (R). We also assume that Ebola virus–susceptible persons could also become infected with other febrile diseases that have Ebola virus disease–like symptoms (I_-). These Ebola virus–negative patients also seek health care; if centers are full, the patients return to the susceptible compartment (S) as symptoms wane. We assume the latent period is 9.4 days, the average time from symptom onset to CCC attendance is 3 days, and the average interval from symptom onset to ETC attendance is 4.6 days. CCCs, Ebola community care centers; ETCs, Ebola treatment centers.

or during burial); we did not assume any transmission of virus from ETC patients. There was also a chance that Ebola virus–negative patients would be exposed to Ebola virus while waiting for test results. We assumed that 50% of symptomatic patients who attended CCCs/ETCs were Ebola virus–positive; on the basis of the number of Ebola virus disease cases and noncases reported in Sierra Leone, this percentage is plausible (*1*,*13*,*14*).

Two other parameters, besides the shorter time between onset of symptoms and attendance at a center, make CCCs potentially different from ETCs in the model: 1) the probability that Ebola virus–negative patients would be exposed to Ebola virus while waiting for test results in CCCs and 2) the reduction in virus transmission from infectious patients to the community as a result of the patient being isolated in a CCC. If the CCC model had a 100% reduction in transmission and 0% chance that Ebola virus–negative patients would be exposed virus, it was equivalent to the ETC set-up in the model, except that there would be a reduced time from symptom onset to CCC attendance.

Results

We first considered the potential level of infection in the community during December 2014 based on our estimates for Western Area. With 259 ETC beds available (*1*,*14*–*16*), our model suggests that ETCs would be at capacity in mid-December and the number of cases would rise over the following weeks (Figure 2, panel A). We also considered the possibility that a proposed additional 500 ETC beds (*15*) would be introduced on December 15, 2014 (Figure 2, panel B). Our forecast suggested that the addition of these beds would cause the growth in number of cases to slow in the following weeks, but the change would not turn over the epidemic.

To assess what reduction in transmission and in risk of Ebola virus–negative patient exposure to virus would be required for 500 CCC beds to be beneficial, we varied 2 key parameters and, after 30 days, compared model outputs with those for the baseline scenario (Figure 3, panel A). If there is a high probability that Ebola virus–negative patients will be exposed but only a small reduction in transmission, CCCs could act as incubators and generate more cases than the baseline scenario with 259 ETC beds only.

The CCC approach has not been fully tested in the field, so we conducted an elicitation of 6 expert opinions to obtain estimates for the median and interquartile range (IQR) for reduction in transmission as a result of patients being in CCCs and for the probability of exposing Ebola virus–negative patients to infectious patients (details at https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/). The distribution for the group opinion for reduction in transmission while in a CCC had a median of 63% (IQR 41%–81%). The distribution for the probability of exposure had a median of 0.09 (IQR 0.01–0.36). When compared with model results, these estimates were within the region of parameter space in which CCCs would be beneficial (see Figure 3 at https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/).

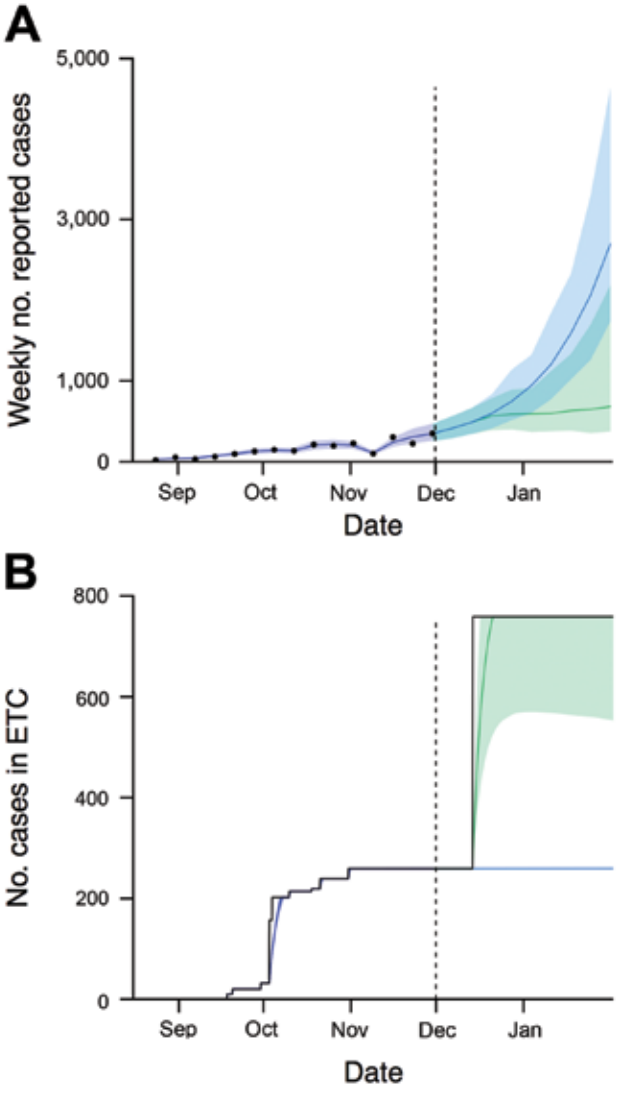

Figure 2. Model fits and forecasts used to evaluate the benefits and risks of introducing Ebola community care centers into Western Area, Sierra Leone. A) Reported cases over time. Black points show reported incidence data. B) No. patients in ETC beds. Blue lines to the left of the dashed vertical divides show the median estimate; blues line to the right of the dashed vertical divides show forecast with no change in number of ETC beds; green lines show forecast if 500 ETC beds are introduced on December 15, 2014. Shaded areas represent 95% credible interval, which reflects uncertainty about reporting and model parameters; darker shading indicates overlap between 2 forecasts. Estimates were scaled depending on the number of daily situation reports issued by the Sierra Leone Ministry of Health and Sanitation each week (see https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/). ETC, Ebola treatment center.

To confirm that 63% was a plausible value for reduction in transmission, we used the following theoretical argument. In the model, the basic reproduction number, R_0, was near 2, the time from onset to outcome was 9.5 days on average, and patients took an average of 3 days after onset of symptoms to attend CCCs. If infected persons did not enter an available CCC and instead remained in the community for the next 6.5 days, they would generate an average of 1.4 secondary cases (because $2 \times 6.5/9.5 = 1.4$). Even if Ebola patients had a 50% probability of infecting their sole caregiver, it meant they would, on average, generate 0.5 secondary cases while in a CCC. The relative reduction in cases as a result of being in a CCC would therefore be $(1.4 - 0.5)/1.4 = 64\%$. If each case-patient generated an average of 0.25 cases while in a CCC, the expected reduction would be ≈80%.

To elucidate the potential benefits and risks of CCC introduction, we considered 2 specific examples. If CCCs reduced virus transmission from Ebola virus–infected patients to the community by 75% once the patient was admitted and if Ebola virus–negative patients have a 25% probability of exposure while waiting for test results, then the introduction of 500 CCC beds would slow virus transmission (Figure 3, panel B). However, if CCCs only reduced transmission by 25% and Ebola virus–negative patients have a 50% probability of exposure to Ebola virus, the introduction of 500 CCC beds could lead to a rise in the number of cases within the community (Figure 3, panel B).

We also assessed how many CCC beds would be required to stop the exponential increase in cases and turn over the epidemic (i.e., reduce the reproduction number of the infection, R, to <1). A larger number of beds would be required if the reduction in transmission was smaller (Figure 4, panel A). The requirement was also larger if Ebola virus–negative patients were more likely to be exposed to virus, patients took longer to attend CCCs, or there were more Ebola virus–negative patients (see figures 4 and 5 at https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/). The large number of infected persons on December 1, 2014, meant that the number of cases still rose in the model (Figure 2), suggesting additional interventions would be required to control the epidemic. Therefore we assessed a combination of the 2 health care approaches, with additional ETC beds, CCC beds, or both introduced on December 15, 2014 (Figure 4, panel B). Because CCCs reduce the time from symptom onset to attendance at a health care center, our results suggest it would be possible to turn over the epidemic in Western Area with a sufficient number of CCC beds, either as a standalone strategy or in combination with additional ETCs.

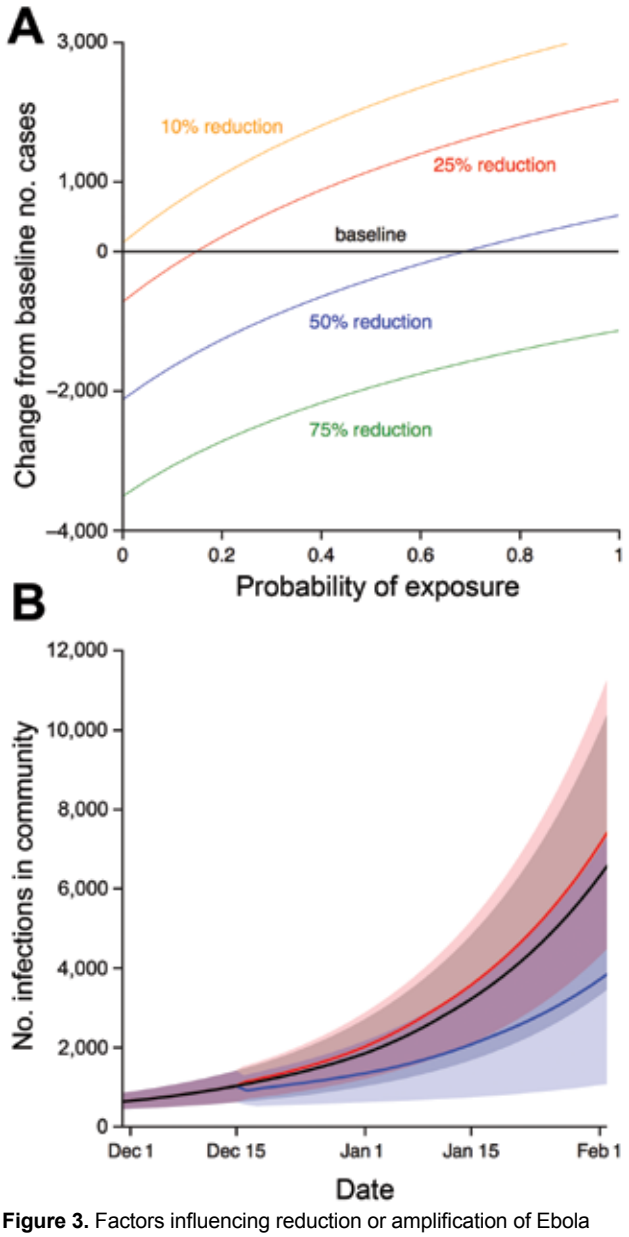

Figure 3. Factors influencing reduction or amplification of Ebola virus infection in the community if 500 CCC beds were introduced in Western Area, Sierra Leone, on December 15, 2014. A) Change in infection compared with baseline scenario (259 Ebola treatment center beds) between December 1, 2014, and February 1, 2015, for a range of values for reduction in transmission and probability of exposure to virus. Median parameter estimates for Western Area were used (Table). B) Change in infection over time. Black line, baseline scenario. Blue line, 500 CCC beds with transmission reduced by 75% (blue line in A), and Ebola virus–negative patients have 25% probability of exposure to virus. Red line, 500 CCC beds with transmission reduced by 25% (red line in A), and Ebola virus–negative patients have 50% probability of exposure to virus. Shaded areas show 95% bootstrapped credible intervals generated from 1,000 simulations with parameters sampled from posterior estimates. We assumed that time from symptom onset to CCC attendance was 3 days and that 50% of symptomatic patients were Ebola virus–positive. CCC, Ebola community care center.

Discussion

We used a transmission model to evaluate the potential effects of the introduction of Ebola CCCs in Western Area,

Table. Parameters used in a transmission model for evaluating the benefits and risks of introducing CCCs into Western Area, Sierra Leone*

Parameter	Value	Source
Mean time from symptom onset to outcome		
Ebola virus–positive patients	9.5 d	(*4*)
Ebola virus–negative patients	7.0 d	Assumed
Mean time from symptom onset to admission		
To ETC	4.6 d	(*4*)
To CCC	3.0 d	Assumed
Mean time from exposure to symptom onset (latent period)	9.4 d	(*4*)
Proportion of patients with Ebola-like symptoms in Western Area who are Ebola-positive	50.0%	(*1*)
Population of Western Area	1.4 million	(*17*)
Probability that an Ebola virus–negative patient seeking care in CCC will be exposed to Ebola virus	Varies†	NA
Reduction in transmission from infected patients to the community as a result of being in CCC	Varies†	NA
Basic reproduction no. (95% CrI)‡	1.94 (1.86–1.98)	Estimated
No. infectious persons on August 16, 2014 (95% CrI)§	51 (39.0–57.0)	Estimated
Proportion of cases in Western Area reported in Sierra Leone Ministry of Health situation reports (95% CrI)	0.42 (0.33–0.46)	Estimated
Variability in accuracy of reports, define as standard deviation of proportion of cases reported (95% CrI)	0.014 (0.010–0.024)	Estimated

*CCC, Ebola community care center; CrI, credible interval; ETC, Ebola treatment center; NA, not applicable.
†In the analysis, the full range of possible values for these parameters is tested.
‡Basic reproduction number refers to the average number of secondary cases generated by a typical infectious patient at the start of an epidemic
§This parameter represents the initial no. of infectious patients at the start of the model simulation. Additional information is available at https://drive.google.com/file/d/0B_BzCqSK1DZaYnRoeWtHOTU2TVk/.

Sierra Leone. Our results show that CCCs could reduce the number of Ebola virus disease cases in the community if 1) the probability for Ebola virus–negative patients being exposed to the virus is low and 2) there is reduction in virus transmission as a result of infected patients being in CCCs. The introduction of CCCs could potentially turn over the epidemic (i.e., reduce the reproduction number, R, below the critical threshold of 1) if the time from symptom onset to CCC attendance is ≤3 days. Assuming that CCCs open in mid-December, ensuring epidemic turnover would require a large number of CCC beds (potentially at least 500 for Western Area). In addition to reducing the time from symptom onset to attendance at a treatment facility, a large number of CCCs would have the added benefit of reducing the time from symptom onset to admission because infected patients would not have to wait for ETC beds to become available.

Our analysis does have limitations. One of those limitations is that we used an illustrative scenario for Western Area based on current epidemiologic reports. Given uncertainty about the influence of factors such as changes in behavior (*18*), we focused our analysis on short-term forecasts and estimation of the number of beds required to turn over the epidemic. However, the epidemiologic landscape is changing rapidly, and the situation might have been different by late December/January, which would influence our specific estimates for bed requirements. In addition, transmission dynamics may vary by district, which would influence the precise number of beds required in different areas. Our results should therefore be viewed as qualitative rather than quantitative. In addition, the reduction in transmission as a result of patients being in CCCs will, in reality, depend on several factors, including patient movements, PPE effectiveness, infection control in the facility, and burial procedures (*12*), and these factors will likely differ between settings. Because it was not possible to establish the contribution of each factor to disease transmission without detailed data on the source of infection (*8*), we used a single parameter to capture the reduction in transmission as a result of a patient being in a CCC. Given the uncertainty about the precise magnitude of this reduction, we assessed the effect of CCCs under the full range of potential reductions in transmission, from no change to full containment, and conducted an elicitation of expert opinions to identify plausible parameter ranges.

Furthermore, we assumed that infectiousness does not vary over the course of Ebola virus infection. However, if patients are most infectiousness during the final stages of infection (*19*,*20*), then CCCs and ETCs would provide an even greater reduction in transmission because they would isolate patients when they are most infectious. In addition, it has been shown that it is not possible to reliably estimate multiple routes of transmission for Ebola virus from a single incidence curve (*8*); thus, we chose to model community transmission by using a single parameter, rather than attempting to estimate the contribution from living infected persons and from funerals. In the model, we also assumed that all patients seek health care. If in reality some do not, this will have the effect of increasing the average time from symptom onset to admission in a care center. A crucial point is that if patients on average spent more than half of their infectious periods in the community, then expansion of bed capacity alone would not be enough to turn over the epidemic in regions where the reproduction number is near 2.

In summary, CCCs may offer a rapid, high-coverage complement to ETCs and, thus, hold considerable potential for bringing about a sizeable shift in the epidemic pattern in Sierra Leone. The UK government is therefore supporting such a combined intervention in the Sierra Leone (*7*). However, the CCC approach is little tested in the field and could be harmful if infection control in CCCs is worse than that in the community or if Ebola virus–negative patients have a high risk of exposure to virus. Settings with limited triage, such as primary health care facilities, may also expose Ebola virus–negative patients to the virus and could therefore also have the potential to amplify the Ebola epidemic. Given the potential benefits and risks of introducing CCCs, real-time evaluation of their effectiveness must be carried out as they are implemented. In particular, to confirm the usefulness of CCCs as an epidemic control strategy, estimates must be determined for the reduction in virus transmission as a result of infected patients being isolated in CCCs and for the probability of Ebola virus–negative patients being exposed to virus in CCCs.

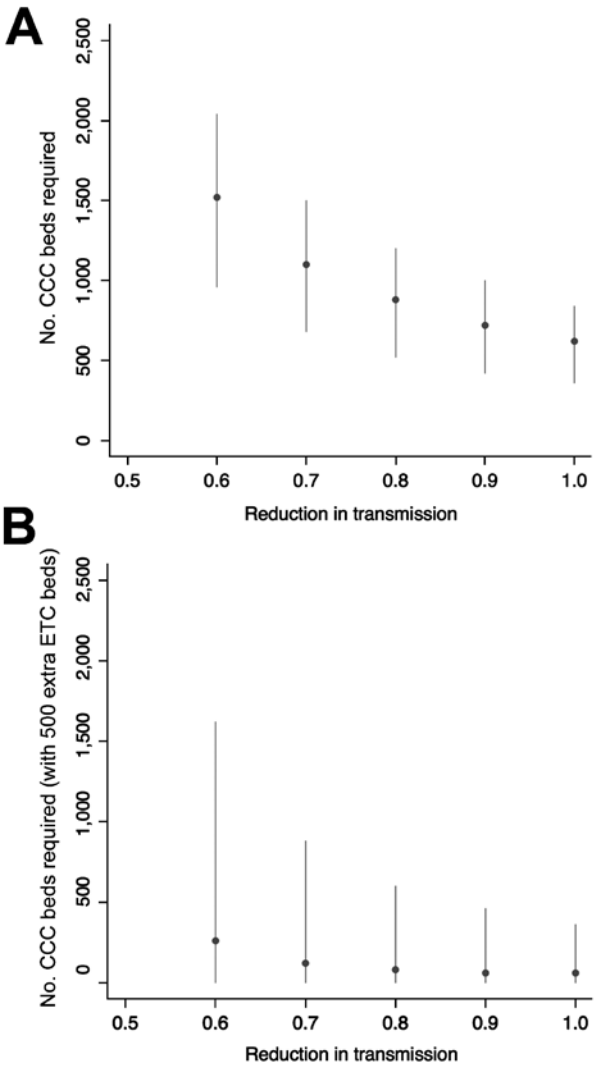

Figure 4. Estimated number of CCC beds required to control Ebola virus epidemic in Western Area, Sierra Leone. A) Number of CCC beds required to turn over the outbreak (i.e., reduce the reproduction number, *R*, to <1). When transmission is reduced by only 50%, no amount of CCC beds can stop the growth in cases. We assume there is a 10% probability that Ebola virus–negative patients are exposed to virus. Lines show bootstrapped 95% credible intervals generated from 1,000 simulations with parameters sampled from posterior estimates; points show median estimates. B) Number of CCC beds required to turn over the epidemic when an additional 500 Ebola treatment center beds are also introduced on December 15, 2014. CCC, Ebola community care center.

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Nanomicroarray and Multiplex Next-Generation Sequencing for Simultaneous Identification and Characterization of Influenza Viruses

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Conventional methods for detection and discrimination of influenza viruses are time consuming and labor intensive. We developed a diagnostic platform for simultaneous identification and characterization of influenza viruses that uses a combination of nanomicroarray for screening and multiplex next-generation sequencing (NGS) assays for laboratory confirmation. The nanomicroarray was developed to target hemagglutinin, neuraminidase, and matrix genes to identify influenza A and B viruses. PCR amplicons synthesized by using an adapted universal primer for all 8 gene segments of 9 influenza A subtypes were detected in the nanomicroarray and confirmed by the NGS assays. This platform can simultaneously detect and differentiate multiple influenza A subtypes in a single sample. Use of these methods as part of a new diagnostic algorithm for detection and confirmation of influenza infections may provide ongoing public health benefits by assisting with future epidemiologic studies and improving preparedness for potential influenza pandemics.

Influenza A virus consists of 8 negative, single-stranded RNA segments encoding 11 proteins: polymerase basic 1 and 2 (PB1 and PB2); polymerase acidic (PA); hemagglutinin (HA); nucleoprotein (NP); neuraminidase (NA); matrix (M1/2); and nonstructural (NS1/2). Influenza A viruses are classified into 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11), determined on the basis of the antigenic differences in the surface glycoproteins HA and NA (*1*–*4*). All known HA subtypes of influenza A virus are found in aquatic birds, and some, including H1, H2, H3, H5, H7, and H9, have been reported to infect humans (*1*,*5*–*7*). Direct transmission of avian influenza A virus subtypes H5N1, H7N2, H7N3, H7N7, H9N2, and H10N7 from domestic poultry to humans has been reported (*8*–*13*).

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In early 2009, a novel swine-origin virus, designated influenza A(H1N1)pdm09 (pH1N1), emerged in Mexico and spread rapidly around the world, causing a global influenza pandemic (*14*,*15*). This virus was generated by multiple reassortment events over 10 years (*16*,*17*) and continued to circulate in humans after the initial pandemic period, replacing the previously circulating seasonal H1N1 viruses. Influenza A(H3N2) variant virus (H3N2v) isolated from humans in the United States in 2011 was also generated through reassortment originating from swine, avian, and human viruses, including the M gene from pH1N1 virus (*18*,*19*). More recently, a novel avian-origin influenza A(H7N9) virus capable of poultry-to-human transmission was identified in China (*7;* http://www.who.int/influenza/human_animal_interface/influenza_h7n9/140225_H7N9RA_for_web_20140306FM.pdf). Diagnosis of infection with this virus is difficult because infection does not kill infected poultry, but the virus may post a substantial risk for a human pandemic because of a lack of immunity in the general population (*7*). As these viruses demonstrate, reassortment of pH1N1 virus with other circulating seasonal strains can produce virulent variants that can be transmitted to and among humans and that could emerge as a future pandemic strain (*15*,*20*,*21*). Therefore, it is critical to determine whether transmitted viruses have pandemic potential in humans during the influenza season.

Multiple influenza strains are usually prevalent during an influenza season. Increasing global travel results in rapid spread of novel influenza viruses from one geographic region to another (*13*,*22*). Current approaches for screening and characterizing novel influenza viruses require many steps and multiple assays. A single test has not been available for simultaneous identification of newly emerging strains from known or unknown subtypes of influenza viruses and the characterization of unique virulence factors or putative antiviral resistance markers.

We previously described a method for detection of avian influenza A(H5N1) and swine-origin pH1N1 viruses that used a nanotechnology-based, PCR-free, whole-genome

microarray assay (nanomicroarray) (*23*,*24*). In this article, we describe a new diagnostic platform for identification and characterization of subtypes of influenza A virus that uses nanomicroarray for screening and multiplex next-generation sequencing (NGS) for laboratory confirmation. We demonstrate that this platform enables accurate and simultaneous identification of multiple subtypes in a single sample. We used this platform to evaluate clinical nasopharyngeal swab specimens from patients with influenza-like illness that had tested positive for influenza virus to determine influenza virus subtype.

Materials and Methods

Oligonucleotide Design and Nanomicroarray Assay

The sequences for multiple capture and intermediate oligonucleotides per target gene were designed and prepared as described previously (*23*,*24*). The oligonucleotide sequences and details of the nanomicroarray assays are listed and described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/3/14-1169-Techapp1.pdf).

Viruses and Clinical Samples

Information about influenza viruses used in this study is provided in the online Technical Appendix. Nasopharyngeal swab specimens from patients with symptoms of influenza-like illness were submitted to the Clinical Virology Laboratory at Yale–New Haven Hospital, New Haven, Connecticut, USA, during December 27–December 31, 2012. Samples were tested by using direct fluorescent antigen (DFA) test with SimulFluor reagents (Millipore, Billerica, MA, USA) and, in some cases, by real-time reverse transcription PCR (rRT-PCR), as requested by the patients' physicians. PCR was performed by using the Centers for Disease Control and Prevention rRT-PCR protocol for influenza as previously described (*25*). Samples for which DFA, rRT-PCR, or both gave results positive for influenza A were selected, de-identified, and sent to the Laboratory of Molecular Virology at the Food and Drug Administration in Silver Spring, Maryland, USA, for further testing (Table 1).

Viral RNA Extraction and rRT-PCR

A previously reported universal primer designed to amplify all 8 gene segments (*26*,*27*) was modified by adding 13-bp flanking sequence (5′-ACGACGGGCGACA-3′) at the 5′ end of each primer to enhance the annealing temperature and achieve high fidelity and yield in PCR amplification. Additional details of RNA extraction and rRT-PCR conditions are described in the online Technical Appendix.

NGS Assay

The concentration of PCR amplicons of all 8 gene segments of influenza A virus was measured by using the Qubit dsDNA BR Assay System (Covaris, Woburn, MA, USA); 1 ng of DNA product was processed for NGS sample preparation by using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the

Table 1. Detection of influenza A viruses in nasopharyngeal swab samples collected from naturally infected patients, Connecticut, USA, 2012–13 influenza season*

	Patient age,	Sample collection		Detection methods			
Patient ID	y/sex	Date, 2012	Location	DFA	rRT-PCR, C_t	Universal PCR	NGS
FLU001	47/F	Dec 30	Hamden, CT	+	ND	+	H3N2
FLU002	80/M	Dec 30	Milford, CT	+	17.5	+	H3N2
FLU004	35/F	Dec 30	Meriden, CT	+	ND	+	H3N2
FLU006	25/M	Dec 29	New Haven, CT	+	ND	+	H3N2
FLU007	23/F	Dec 30	New Haven, CT	+	ND	+	H3N2
FLU008	31/F	Dec 30	Trumbull, CT	+	28.1	+	H3N2
FLU009	68/M	Dec 30	Hamden, CT	+	ND	+	H3N2
FLU012	35/F	Dec 30	Rutledge, MO	+	ND	+	H3N2
FLU013	92/M	Dec 29	Woodbridge, CT	+	16.6	+	H3N2
FLU014	84/F	Dec 30	Chester, CT	+	15.5	+	H3N2
FLU017	66/F	Dec 29	Clinton, CT	+	24.3	+	H3N2
FLU018	17/M	Dec 31	New Haven, CT	+	19.9	+	H3N2
FLU021	63/F	Dec 30	New Haven, CT	+	ND	+	H3N2
FLU023	55/F	Dec 28	North Haven, CT	+	21.8	+	H3N2
FLU025	47/M	Dec 30	West Haven, CT	+	ND	+	H3N2
FLU026	32/F	Dec 28	West Haven, CT	+	ND	+	H3N2
FLU027	26/F	Dec 30	Bridgeport, CT	+	ND	+	H3N2
FLU028	89/F	Dec 29	Woodbridge, CT	I	21.0	+	H3N2
FLU033	82/F	Dec 29	Guilford, CT	+	19.8	+	H3N2
FLU034	37/F	Dec 27	New Haven, CT	+	ND	+	H3N2
FLU036	18/M	Dec 29	New Haven, CT	I	17.8	+	H3N2
FLU037	8/F	Dec 29	New Haven, CT	+	ND	+	H3N2
FLU038	21/M	Dec 27	West Haven, CT	+	ND	+	H3N2
FLU040	23/M	Dec 27	West Haven, CT	+	ND	+	H3N2

*C_t, cycle threshold value; DFA, direct fluorescent antigen test; I, inadequate cells for DFA; ID, identification; ND, not done; NGS, next-generation sequencing; rRT-PCR, real-time RT-PCR.

manufacturer's instructions. Briefly, the Nextera XT transposome fragmented PCR amplicons into a size of ≈500–700 bp and added adaptor sequences to the ends, enabling a 12-cycle PCR amplification to append additional unique dual index (i7 and i5) sequences at the end of each fragmented DNA for cluster formation. Mega-amplicons from influenza virus were internally marked with these dual-barcoded primers, which enabled multiplexing and simultaneous detection of different subtypes in the same run. After purification of PCR fragments and library normalization, sample pooling was performed by mixing equal volumes of each normalized DNA library, and the barcoded multiplexed library sequencing was performed on an Illumina MiSeq (Illumina). After automated cluster generation, sequencing was processed and genomic sequence reads obtained.

Bioinformatics Analysis

Sequencing reads of ≈300 bp were dynamically trimmed and sequence data were verified by FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before de novo assembly. The genome-contiguous assembly was constructed from MiSeq reads by using a de novo module in CLC genomics workbench software version 6.0.2 (CLC bio, Cambridge, MA, USA); minimum contiguous length was set at 800 for assembling consensus sequences (*28*). A comprehensive-read database was generated for the whole genome of the influenza virus tested. Sequences were further filtered so that the local database contained only 1 unique contig for each gene segment, and multiple contigs were generated for each sample. These representative sequences comprise the set of unique sequences from the dataset. A FASTA file with all unique contiguous sequences of each mega-amplicon was used to perform an all-by-all Identify Similae Sequences search in the Influenza Research Database (IRD, http://www.fludb.org), the Global Initiative on Sharing All Influenza Data database (http://platform.gisaid.org), and the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). The top-scoring BLAST (http://blast.ncbi.nlm.nih.gov/) match was selected to identify the specific genome. Assembled sequences were aligned in ClustalW (http://www.clustal.org), and phylogenetic analysis was performed in MEGA by using the neighbor-joining method (*29*). All amplicons were accurately categorized into a typical subtype.

Results

Verification of Capture and Intermediate Oligonucleotides

A nanomicroarray for each target gene was designed, printed in-house, and tested separately by using the PCR products as templates to verify the ability of individual capture and intermediate oligonucleotides to detect a specific target gene. Ineffective capture oligonucleotides were replaced and retested. We amplified PCR products of HA, NA, and M genes for H7N2, H7N3, and H9N2 viruses separately or simultaneously in a single reaction using the corresponding 3 sets of specific primers. To identify the correct HA and NA gene segments for multiplex influenza subtyping, we fabricated a new nanomicroarray by pooling autologous 4 to 5 capture oligonucleotides for a specific gene and then printing them on the array substrate in triplicates. Each nanomicroarray subarray contains multiple gene spots for multiplex assays. The PCR products were hybridized on the array, and the specific signal profiles were correctly observed in the areas printed with corresponding gene-specific capture oligonucleotides (online Technical Appendix Figure 1). No interference or cross-hybridization was observed when multiple targets and intermediate oligonucleotides were included in the assay. The specific signal pattern showed the assay's ability to accurately discriminate influenza subtypes.

Amplification of Whole-Genome Segments

To further confirm subtypes detected in the nanomicroarray screening assay for final laboratory diagnosis, we redesigned universal primers to amplify whole-genome segments and separately tested 22 influenza A strains covering 10 subtypes and 3 influenza B viruses. All 8 segments of influenza A viruses were simultaneously amplified in a single reaction, resulting in multiple PCR products ranging in size from 500 to 2,500 bp (mega-amplicons). We also tested 3 influenza B viruses, B/Brisbane/60/2008 (Victoria lineage), B/Pennsylvania/7/2007 (Yamagata lineage), and B/Victoria/304/2006 (Victoria lineage), and 2 influenza A viruses, A/Panama/2007/1999 (H1N1) and A/ruddy turnstone/NJ/65/1985 (H7N3), and found several faint, nonspecific small bands no larger than 1 kb (data not shown).

Evaluation of Nanomicroarray Assay by using PCR Mega-amplicons

To include M gene capture oligonucleotides for influenza B viruses, we developed a new nanomicroarray (Figure 1). As shown in Figure 2, amplified PCR products of the matrix gene from influenza A and B viruses were specifically detected in the correct spot areas without cross-hybridization. The mega-amplicons of influenza A viruses were correctly identified and found to have a unique fingerprint for each influenza A virus tested. Each spot pattern represented a typical influenza virus subtype corresponding to the gene-specific capture oligonucleotides. We conclude that the current nanomicroarray assay can simultaneously discriminate influenza A from B viruses and specifically identify influenza A subtypes H1, H2, H3, H5, H7, H9, N1, N2, and N3.

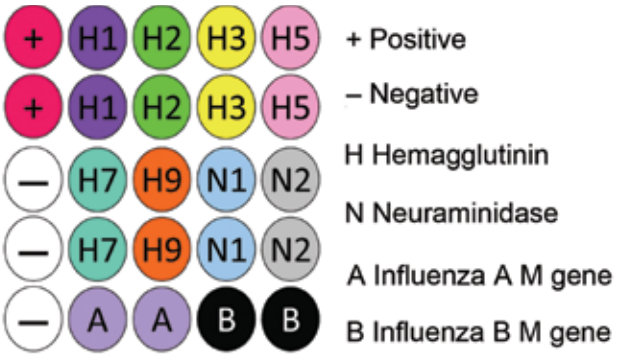

Figure 1. Nanomicroarray layout design for testing of samples for influenza A and B viruses. The microarray internal positive control capture is listed in online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/3/14-1169-Techapp1.pdf). The negative control is the printing buffer. M, matrix protein.

NGS Confirmation of Influenza A Subtypes

A total of 17 mega-amplicons representing 10 subtypes of influenza A and 1 of influenza B were tested in the NGS assay. Multiple contiguous sequences were created automatically for each mega-amplicon by using a de novo assembly program in CLC, and 4–8 contigs supported by high coverage rate of sequence reads were generated (online Technical Appendix Table 2). The mega-amplicons of the A/Vietnam/1204/2004 (H5N1) strain yielded 8 contiguous sequences supported by 90,962 reads. Further BLAST search of 8 contiguous sequences in the IRD resulted in 8 mast BLAST reports. All contiguous sequences were found to correspond to 6 proteins (PB2, PB1, PA, NP, M, and NS) of A/Puerto Rico/8/1934 (H1N1) virus and 2 proteins (HA/CIP045/CY077101 and NA/HM006761) of A/Vietnam/1203/2004 (H5N1) with 99%–100% sequence identity. These results showed that all amplicons were correctly identified as the H5N1 laboratory strain.

The mega-amplicons from A/turkey/Virginia/4529/2002 (H7N2) and A/Minnesota/10/2012 (H3N2) strains resulted in 8 contigs, all correctly identified as the correct subtype. We found strong concordance in contiguous sequences and PCR fragments for each mega-amplicon. A de novo assembly program generated at least 7 contigs from faint band mega-amplicons for influenza B virus (B/Brisbane/60/2008); 2 showed good coverage (7,077 and 10,168), but the BLAST search indicated that none matched the gene sequence from this strain. Further investigation using freshly extracted RNA may be required.

Simultaneous NGS Discrimination of Multiple Subtypes in a Single Sample

We tested 4 influenza viruses obtained from the Centers for Disease Control and Prevention, A/Puerto Rico/8/1934 (H1N1), A/Vietnam/1203/2004 (H5N1), A/Minnesota/10/2012 (H3N2), and A/Anhui/1/2013 (H7N9), to determine the presence of H1, H3, H5, H7, N1, N2, and N9 subtypes. RNA was extracted from individual or mixed viral strains. The universal rRT-PCR was performed to amplify whole-genome segments in which the PCR mega-amplicons represented a similar pattern to individual or mixed viral samples (online Technical Appendix Figure 2). After the NGS assay followed by de novo assembly, 8 contigs were generated for the H5N1 and H3N2 viruses, and 15 contigs were generated for the mixed sample, supported by a high coverage rate of reads (Table 2). These 15 contigs from mixed samples exactly matched the gene segments of the

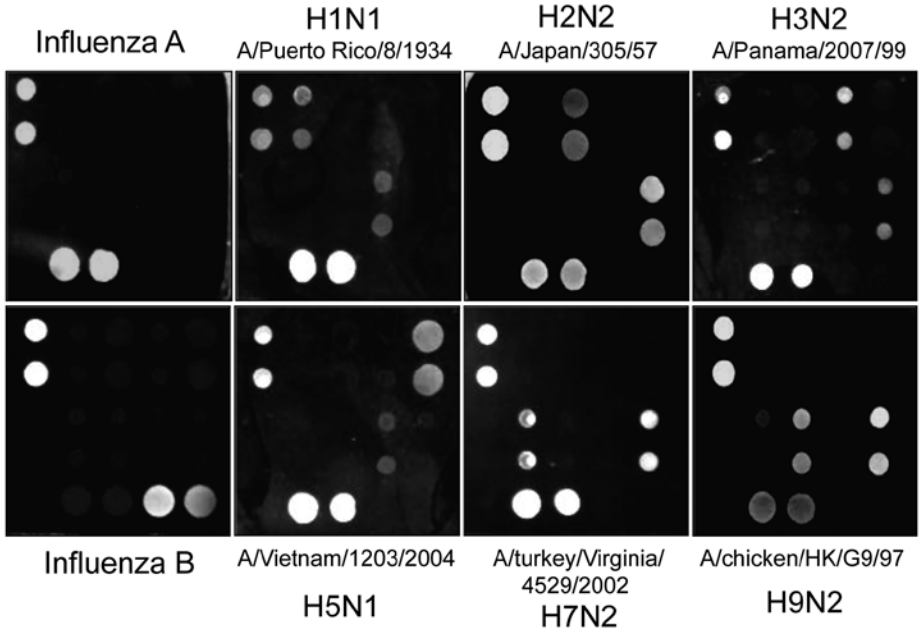

Figure 2. Portion of the microarray images for DNA oligonucleotides of influenza viruses after hybridization with PCR products. Lighter shades represent greater silver intensities for each gene. Typical nanomicroarray silver staining images represent the hits for specific types or subtypes indicated. The positive controls of influenza A and B (left panels) use PCR products amplified by pair-specific primes for matrix gene.

H5N1 and H3N2 strains, similar to the results obtained with the individual sample, as expected. HA and NA genomic sequences of the H7N9 subtype virus were identified as strain A/Anhui/1/2013 from the Global Initiative on Sharing All Influenza Data database. Because PB2 (2,341 bp), PB1 (2,341 bp), and PA (2,233 bp) genes have very similar sizes, direct separation of each gene from co-infected samples is not possible by conventional sequencing methods. These findings demonstrated that the NGS assay can simultaneously identify and confirm the presence of ≥1 influenza subtypes in a single sample.

Evaluation of Nasopharyngeal Swab Samples by using NGS Assays

We performed universal RT-PCR and NGS assays on 24 nasopharyngeal swab samples obtained from patients who had received a diagnosis of influenza. These samples were initially tested by DFA, rRT-PCR, or both and found to be positive for influenza A virus. After decoding, all samples were found to be positive by using the universal RT-PCR detection method, indicating presence of influenza A infection (Table 1). When mega-amplicons representing the 24 patient samples were tested in the NGS, a total of 32.8 million reads were obtained, and multiple contigs were generated for each sample (online Technical Appendix Table 3). A BLAST search of each contig in the IRD database identified the genome corresponding to the influenza A(H3N2) subtype. The coverage of influenza A(H3N2) genomes in the NGS assay was 96.7% (31.7/32.8 million) of raw reads and 76.6% (183/239) of total contigs. A total of 95.3% (183/192 contigs) of the influenza A(H3N2) genome was amplified and sequenced; the average depth of coverage for each contig was 3,259. Of these genomes, 71% (136/192) of segments yielded full-length sequences; HA genes were 96% (23/24); NP, 96% (23/24); NA, 88% (21/24); M, 88%, (21/24); and NS, 79% (19/24). The average breadth of coverage was 100% for HA, NA, NP, M, and NS genes and 93% for PB2, PB1, and PA genes.

Phylogenetic analysis of each of the 8 segments separately for all isolates showed that all genes clustered together in the H3N2 radiation with a high bootstrap value (data not shown). None of the M genes closely clustered with the M genes from the pH1N1 or H3N2v viruses (Figure 3), which suggests that these viruses are not H3N2v (*18*,*19*). The genotype of 24 influenza A(H3N2) viruses was determined as [A,D,B,3A,A,2A,B,1A] by using FluGenotyping (http://www.flugenome.org), which indicates that the same lineage virus is circulating in this region. The HA genes from most samples shared very high identity with A/Boston/DOA2–206/2013(H3N2) and the NA genes with A/Boston/DOA2-141/2013(H3N2) strain. After completing these analyses, 181 gene sequences were deposited into GenBank (accession nos. KJ741883–KJ742063).

Discussion

We report the development of a novel diagnostic platform for simultaneous detection, typing, and whole-genome characterization of influenza viruses that uses a combination of nanomicroarray and high-throughput NGS approaches. First, we designed capture and intermediate oligonucleotides for H1, H2, H3, H5, H7, H9, N1, N2, and N3 of influenza A virus and M genes of influenza B virus and evaluated these oligonucleotides in a nanomicroarray assay. Second, we modified previously reported universal primers (*26*,*27*) and used them to amplify the whole genome of influenza A viruses for validation of the nanomicroarray assay. Finally, we confirmed results by using the NGS assay. This protocol enables random accessing of a variety of target genes for simultaneous identification and final sequence-based confirmation of influenza virus infection.

Designing multiple capture and intermediate oligonucleotides with sequences covering the entire genome ensures specific capture of multiple target genes on the nanomicroarray and subsequent detection with a universal nanoparticle probe regardless of mutation, deletion, and

Table 2. Summary of results from NGS data analysis for influenza A(H3N2) and A(H5N1) viruses obtained from the Centers for Disease Control and Prevention*

Strain	NGS total contigs/reads	Findings	Gene segment (length, bp) PB2 (2,341)	PB1 (2,341)	PA (2,233)	HA (1,778)	NP (1,565)	NA (1,413)	M (1,027)	NS (890)
A/Vietnam/1203/ 2004(H5N1)	8/125,438	Length, bp	2,032	1,801	1,841	1,747	1,120	1,259	1,075	894
		Read count	7,604	2,019	18,762	16,925	6,490	24,192	20,127	920
A/Minnesota/10/ 2012(H3N2)	8/162,155	Length, bp	2,251	2,192	2,081	1,754	1,556	1,639	1,171	876
		Read count	30,002	16,392	19,253	15,194	21,818	8,119	25,879	18,519
A/Vietnam/1203/ 2004(H5N1) and A/Minnesota/10/ 2012(H3N2)	15/150,756	Contigs, H5	2	1	13	14	4	6	7	
		Length, bp	2,013	2,115	1,815	1,746	1,476	1,193	936	
		Read count	7,003	6,857	4,359	3,735	854	10,895	15,127	
		Contigs, H3	15	5	10	11	3	12	8	9
		Length, bp	2,530	2,393	1,959	1,465	1,565	1,633	1,103	925
		Read count	15,521	6,753	4,113	7,743	13,808	11,147	5,411	6,110

*de novo assembly module was used in CLC Genomics Workbench software version 6.0.2 (CLC bio, Cambridge, MA, USA) for result handing. Minimum contiguous length was set for 800 to assemble the consensus sequences. NGS, next-generation sequencing; PB, polybasic; PA, polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural.

Figure 3. Phylogenetic analysis of the matrix (M) gene sequences obtained from nasopharyngeal swab samples from patients who had received a diagnosis of influenza in Connecticut, USA, during the 2012–13 influenza season (see Table 1). Analysis was performed by using the neighbor-joining module in MEGA (*29*) with the Kimura 2-parameter method. The reference subtypes were fetched from the Influenza Research Database (http://www.fludb.org) and used to construct the tree. Bootstrap values >70% are shown. The M genes identified in this study are indicated by black circles; reference M genes are indicated by black squares for influenza A(H3N2)v and black triangles for pandemic influenza A(H1N1) 2009 (pH1N1) virus. Scale bar indicates 2% genetic distance.

influenza reassortment. Furthermore, this design is adaptable for other applications and enables direct detection and subtyping of an unknown sample without previous knowledge of types and subtypes. In the current format, >50 degenerate capture oligonucleotides cover 12 influenza viral target genes, enabling direct detection of any combination of ≈20 subtypes in a single sample, identification of influenza A subtypes in a single assay, and differentiation of influenza A from B viruses. An optimal nanomicroarray assay, which is a reformatted portable device modified for use in point-of-care settings, should include target genes from most influenza A and B viruses as well as for other respiratory viral pathogens. The assay should be easily performed by an untrained technician for sample testing in the field without enzymatic reactions, and results should be in a form that can easily be visualized by the naked eye. In comparison to other conventional detection methods for targeting each gene of influenza A and B viruses separately, the nanomicroarray assay is a one-test-fits-all approach for diagnosis of influenza virus infections that can provide results in <1.5 hours, making this method relatively cost- and time-effective. More important, the nanomicroarray assay can detect emerging and reassortant viruses, and those samples can be sent to centralized laboratories that perform the NGS assay for final sequence confirmation.

Gene segments in most influenza viruses isolated from humans can be adapted from animals, as shown by genetic changes in influenza A(H7N9) isolates from poultry and humans (*7*,*30*). These studies indicated that more changes were acquired during the human infection process. Determining the nature and frequency of co-infection associated with influenza A virus will be critical if an unknown sample contains a novel strain or >1 HA or NA gene subtype. NGS is a powerful tool facilitating diagnosis on a large scale, including high-throughput and simultaneous identification of ≥96 samples barcoded by using dual index primers and detection of >9,216 genes in a single sequencing run. By using a universal primer adapted to fabricate the mega-amplicons, we showed that the NGS assay is capable of accurately subtyping any influenza A virus and detecting multiple known and unknown influenza genes in a single assay. Bioinformatic skills and mathematics tools, combined with epidemiologic studies, are useful in facilitating prediction of potential subtypes according to the genetic matrix composition of influenza genomic segments for a new, emerging, and reassorted strain whereby the subtypes can be confirmed (*31*–*34*). Influenza A viruses representing 11 subtypes were accurately detected in this study, and the 2 mixed influenza viruses were discriminated by using this sequencing-based diagnostic platform.

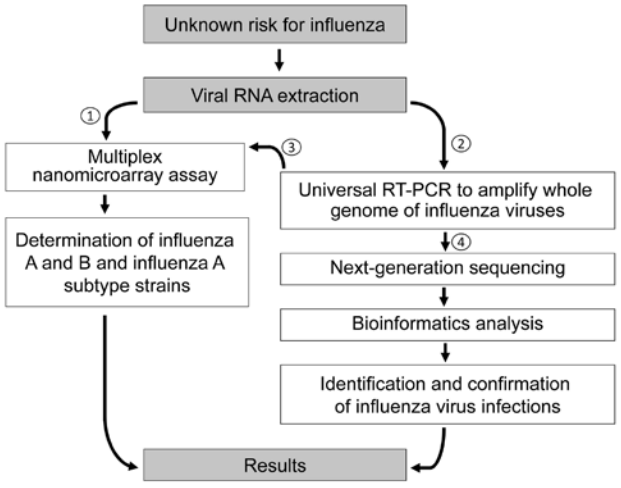

Figure 4. Diagnostic algorithm for identification of an unknown risk for influenza by using nanomicroarray and next-generation sequencing (NGS) assays. To determine the virus type for a suspected influenza virus infection, viral RNA is extracted from a patient sample and initially analyzed in nanomicroarray assay for screening and determining the influenza A and B viruses (*1*). Once a novel, emerging, or co-infected influenza A and B virus is found, universal reverse transcription PCR (RT-PCR) is performed to generate whole-genome mega-amplicons (*2*), which can then be retested on the nanomicroarray assay to confirm the initial finding (*3*) or sent to the central laboratory performing the NGS assay and data analysis for final sequence confirmation (*4*).

Sequence analysis of 24 clinical samples revealed that 23 (92%) segments contained an amino acid substitution at position E627K in PB2 gene. Mutation of glutamic acid (E) at PB2 residue 627 to lysine (K) favors adaptation to the mammalian host; such mutations have been found in human isolates of highly pathogenic avian influenza viruses of the H7N7 and H7N9 subtypes (*7*,*35*,*36*). These mutations might confer high virulence to the virus by enhancing replication efficiency, increasing polymerase activity and disease severity of avian influenza viruses in mammals (*37*).

Of the 24 M genes of the samples we tested, 21 (88%) had a single S31N mutation in the transmembrane region of the M2 protein, which has been found to confer resistance to amantadine (*7*,*38*). The emergence of E627K(PB2) and S31N(M2) mutations in tested samples suggests that human host infection in the Connecticut region in the 2012–2013 seasons might be poultry-to-human transmission associated with disease severity (*39*,*40*). This observation highlights an increased risk to public health and the need to continually monitor isolates obtained from mammal reservoirs for genetic variation. This information may help guide clinical treatment and assessment of epidemiology during the epidemic season.

The assay we evaluated is a minimally manipulated procedure that greatly reduces the number of amplifications and omits fragment separation and purification. It is therefore suitable for identification of any strains of influenza virus. An ongoing study using this assay has simultaneously detected and confirmed influenza A(H3N2), pH1N1, and influenza B viruses in >100 nasopharyngeal swab samples (J. Zhao et al., unpub. data).

This detection platform provides a new, accurate, and rapid method to refine the differential diagnosis of influenza by selecting a single test or a small set of tests to determine the strain or strains present in a single clinical sample. We propose a new diagnostic algorithm based on this combined platform for identification and characterization of infection risks of unknown influenza strains (Figure 4). For testing a suspected influenza virus infection, this detection platform takes 2–3 days to perform NGS assay and data analysis. However, it provides whole-genome characterization and a final report in matrix type by which a potential pandemic prevalence strain can be predicted, with data including the genetic variant, amino acid signatures for virulence factors, and drug-resistance– and host-adaptation–associated mutations.

Future studies need to be conducted to reformat the current microarray to a point-of-care setting and to expand testing of clinical samples to other geographic regions and additional influenza virus types/subtypes. The NGS assay involves sample preparation and generates massive sequence data for the final report for test interpretation, which requires a higher level of performance for clinical assay

validation. Development of an automated assembly and analysis pipeline can make the bioinformatics analysis of transferring raw reads to the specific genomic identification more efficient. This molecular diagnostic platform has the potential for monitoring newly emerging or re-emerging viral reassortants derived from different precursors and could be included as a part of pandemic influenza surveillance strategies for efficient prevention and timely implementation of treatment to protect and improve public health.

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Multidrug-Resistant Tuberculosis in Europe, 2010–2011

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Drug-resistant *Mycobacterium tuberculosis* is challenging elimination of tuberculosis (TB). We evaluated risk factors for TB and levels of second-line drug resistance in *M. tuberculosis* in patients in Europe with multidrug-resistant (MDR) TB. A total of 380 patients with MDR TB and 376 patients with non–MDR TB were enrolled at 23 centers in 16 countries in Europe during 2010–2011. A total of 52.4% of MDR TB patients had never been treated for TB, which suggests primary transmission of MDR *M. tuberculosis*. At initiation of treatment for MDR TB, 59.7% of *M. tuberculosis* strains tested were resistant to pyrazinamide, 51.1% were resistant to ≥1 second-line drug, 26.6% were resistant to second-line injectable drugs, 17.6% were resistant to fluoroquinolones, and 6.8% were extensively drug resistant. Previous treatment for TB was the strongest risk factor for MDR TB. High levels of primary transmission and advanced resistance to second-line drugs characterize MDR TB cases in Europe.

Emergence of drug-resistant tuberculosis (TB) threatens the goal of TB elimination (*1*). Multidrug-resistant (MDR) TB is defined by in vitro resistance of *Mycobacterium tuberculosis* to at least both of the 2 most effective drugs for treatment (rifampin and isoniazid). Extensively drug-resistant TB (XDR TB) is defined as MDR TB plus in vitro resistance to at least 1 second-line injectable drug (amikacin, capreomycin, or kanamycin) plus resistance to any of the fluoroquinolones (e.g., ofloxacin, levofloxacin, or moxifloxacin) (*2*). In the World Health Organization (WHO) European Region, the estimated incidence of patients with MDR TB differs markedly: 1.6 cases/100,000 persons in the 29 European Union/European Economic Area countries and 16.8 cases/100,000 persons in the 24 other countries of the region in 2012 (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/3/14-1343-Techapp1.pdf) (*3*). The actual number of patients with

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MDR TB living in this region may be much higher because a substantial proportion of patients are never screened for drug-resistant TB before starting treatment, partly because of a lack of diagnostic capacity (*3*).

MDR TB is associated with poor treatment outcomes (*1,2,4*). The proportion of treatment success in patients with MDR TB was only 54% in an individual patient data metaanalysis of >9,000 patients from 32 observational studies (*5*). Results from this cohort showed that additional resistance to fluoroquinolones in patients with MDR TB reduced treatment success to 48%; patients with XDR TB were treated successfully in 40% of cases (*6*), which approached treatment outcomes similar to those of the pre–antimicrobial drug era (*4*). A recent surveillance report from the EU reported 32.2% treatment success for MDR TB and 19.1% treatment success for XDR TB (*7*).

Detailed information about characteristics, management, and outcomes of patients with MDR TB in Europe is scarce but essential to inform health policy makers and optimize disease management (*8*). We compared baseline characteristics and risk factors for patients with MDR TB, as well as availability and results of drug susceptibility testing (DST) for second-line drugs for treatment of TB, in a cohort of patients from 16 countries in Europe with low, intermediate, and high incidence of TB, who had started first-line or second-line TB treatment.

Methods

Participating Sites

TBNET is a European consortium for clinical research in the field of TB (*9*). This study was conducted at 23 TBNET sites in 16 countries in Europe: 2 with a high (>100 cases/100,000 persons) incidence of TB, 4 with an intermediate (20–100 cases/100,000 persons) incidence, and 10 with a low (<20 cases/100,000 persons) incidence (Figure). Stratification was based on WHO/European Centre for Disease Prevention and Control estimates of TB incidence during the study period (2010–2011) (*10*). The number of study participants per country is shown in online Technical Appendix Table 1.

Study Population

After informed consent was obtained, patients starting treatment for a new episode of culture-confirmed TB with resistance to at least rifampin and isoniazid (MDR TB cohort) were eligible for enrollment. Patients were included prospectively by using consecutive inclusion during January 2010–December 2011 at each site. In Belarus, Latvia, Moldova, and Romania, additional enrollment was stopped when a preagreed number of patients were enrolled to avoid overrepresentation of patients from these countries in the cohort.

For each MDR TB patient, 1 patient with non–MDR TB (pan drug–susceptible, monoresistant, or polydrug-resistant TB [*11*]) was enrolled at each center at the time of enrollment of the MDR TB patient: these additional patients were denoted as controls. Controls were selected on the basis of DST results that identified non–MDR TB, and that were obtained at the closest date before enrollment of an MDR TB patient at the same site.

Because of this selection process, a limited number of patients (41, 5.4%) started treatment before the study began in January 2010, but none started treatment before

Figure. TBNET study sites in the Pan European network for study and clinical management of drug-resistant tuberculosis (TBPAN-NET) project. Stratification is based on the incidence of tuberculosis (TB) reported during 2010–2011, which matched the inclusion period of the study. Data for 2011 were obtained from the European Centre for Disease Control and Prevention (*10*). Low TB incidence, <20 cases/100,000 persons; intermediate TB incidence, 20–100 cases/100,000 persons; high TB incidence, >100 cases/100,000 persons.

January 2007. However, we maintained consecutive inclusion for MDR TB patients. This feature ensured an acceptable sample size for countries with a low incidence of TB during the inclusion period.

Data Collection

Data collection used an electronic case record form designed in Open Clinica (http://www.openclinica.com). A paper version of this form was used in Moldova, Romania, Estonia, and Belarus, where internet access was not always available. All investigators were initially trained onsite, and continuous training was ensured through annual investigator meetings, regular site visits, and newsletters.

Laboratory Testing

Routine data were obtained from local laboratory reports for sputum smear microscopy, sputum culture, and DST for first-line and second-line drugs and, when available, *M. tuberculosis*–specific nucleic acid amplification tests. All laboratories at study sites were subjected to quality control through the WHO Supranational Reference Laboratory Network.

Study Outcome

We analyzed characteristics of the cohort at the time of enrollment. We also assessed factors associated with MDR TB in a cross-sectional approach.

Data Management

Data management included regular data checks on key variables for missing data and inconsistencies. The study coordinator, a study monitor, and a trained study nurse performed routinely manual plausibility checks and clarified inconsistencies with the investigators.

Statistical Analysis

Descriptive statistics are reported as frequencies or medians, where appropriate. Risk factor analysis was performed by using univariable and multivariable logistic regression. We used robust SEs to adjust for clustering by country. All variables with <20% missing data were assessed for inclusion in the models. Missing data for included variables were coded as separate categories to prevent listwise deletion of observations. Age was dichotomized at 45 years to align with values in a previous study (*12*). The variables age and sex were purposefully included in the multivariable analysis in which other variables were included on the basis of the Wald statistic (<0.1) and the change in the pseudo R^2 (>10%) because a robust SE precludes formal use of the log-likelihood ratio test. In a sensitivity analysis, we repeated multivariable logistic regression with the inclusion of a sampling weight for the MDR TB patients (inverse of the sampling fraction with expected number of MDR TB patients in the country as denominator) (online Technical Appendix Table 5). Non–MDR TB patients were given a weight of 1. The weighted analyses assessed the potential effect of unbalanced contribution of countries in the cohort. Goodness-of-fit was assessed by using the F-adjusted mean residual test.

Drug resistance was expressed as the proportion of isolates tested and the proportion of isolates that were resistant. Corresponding frequencies when applying sampling weights and analysis by a complex survey approach (*13,14*) are shown in online Technical Appendix Table 4.

Ethics

Patient information and consent forms were translated into local languages when needed. The study was approved by the Ethics Committee of the University of Lübeck (Lübeck, Germany). The study protocol was approved by the local ethics committee at all participating centers. Written informed consent was obtained from all patients according to site-specific regulations. Data were collected pseudonymously and stored on a secured server.

Results

Cohort Characteristics

The cohort consisted of 380 MDR TB patients and 376 non–MDR TB controls. Descriptive characteristics of the MDR TB cohort are shown in Table 1 (http://wwwnc.cdc.gov/EID/article/21/3/14-1343-T1.htm) and those for the non–MDR TB cohort in online Technical Appendix Table 2. Both groups had predominantly male patients. The median age was 36 years (interquartile range 27–47 years) for the MDR TB patients and 41 years (interquartile range 31–54 years) for the controls. The proportion of foreign-born patients with MDR TB in countries of low, intermediate, and high TB incidence was 85.4%, 5.8% and 0.5%, respectively. Similar proportions were observed in controls (56.3%, 5.7% and 2.1%, respectively). Of 94 foreign-born patients, 60 (64%) were from countries of the European region of WHO, 17 (18%) from Russia, 18 (19%) from Southeast Asia, 11 (12%) from sub-Saharan Africa, 1 (1%) from North Africa, and 4 (4%) from South America. Smoking was common in both groups (50.5% for MDR TB patients and 41.5% for controls).

HIV infection and diabetes mellitus were infrequently observed: 6.6% in MDR TB patients and 4.3% in controls for HIV, and 4.2% in MDR TB patients and 5.3% in controls for diabetes mellitus. The percentage of patients with MDR TB whose episode of active TB was their first was 52.4% (59.2%, 74.4%, and 38.7% in countries with low, intermediate, and high TB incidence, respectively).

Drug Resistance Profiles

Among 380 patients with MDR TB, second-line *M. tuberculosis* DST profiles were available for 356 patients.

Reasons for unavailable baseline DST results were 1) an initial diagnosis of MDR TB at a peripheral hospital and subsequent patient transfer to a central hospital where *M. tuberculosis* could not be grown in culture (n = 6); 2) contamination of cultures (n = 12); 3) insufficient growth in cultures (n = 4); 4) patient death between the first and second cultures (n = 1), and 5) unknown reason (n = 1). Among patients with MDR TB, 6.8% of cases fulfilled the definition of XDR TB. Drug resistance profiles for first-line and second-line drugs other than rifampin and isoniazid are shown in Table 2 for the MDR TB cohort, in online Appendix Table 3 for the MDR TB cohort compared with the non–MDR TB cohort, and in online Technical Appendix Table 4 for the MDR TB cohort by weighted analysis.

DST for pyrazinamide and ethambutol was performed for 45.0% (177/380) and 97.6% (371/380) of strains from MDR TB patients and controls, respectively. Testing was performed for 94.7% (360/380) of strains for resistance to any second-line drug, 93.7% (356/380) for any second-line injectable drug, 92.6% (352/380) for any fluoroquinolone, and 93.2% (356/380) for ethionamide/prothionamide. Strains from MDR TB patients showed additional resistance to pyrazinamide (59.7%, 105/177), ethambutol (59.3%, 220/371), ≥1 second-line injectable drug (26.1%, 93/356), ≥1 fluoroquinolone (17.6%, 62/352), and ethionamide/prothionamide (31.3%, 119/354) (Table 2). The weighted analysis showed higher proportions of resistance to all drugs, except capreomycin, moxifloxacin, and ethionamide/prothionamide (online Technical Appendix Table 4).

Risk Factors for MDR TB

Risk factors for TB were compared between patients with MDR TB and controls. Previous treatment for TB (odds ratio 10.7, 95% CI 7.3–15.6) and age <45 years (OR 1.90, 95% CI 1.23–2.93) were identified as independent risk factors for MDR TB by multivariable analysis (Table 3). There was also a moderate association for sex and current homelessness with MDR TB by weighted analysis (online Technical Appendix Table 5). No association was found between MDR TB and abnormal body mass index (<18 or >25), employment status, birth in a foreign country, history of imprisonment, injectable drug use, co-infection with HIV, or diabetes. The role of TB contact was not evaluated because data were not sufficiently robust because of a high percentage of unknown/unreliable results for self-reporting. Weighted analyses showed similar results with only minor differences in effect size.

Table 2. Drug resistance profiles for first-line and second-line drugs used for treatment of multidrug-resistant tuberculosis in TBNET study in Europe, 2010–2011*

	Incidence of TB in region						All MDR TB patients, n = 380	
	Low, n = 103†		Intermediate n = 86‡		High n = 191§			
Drug†	Tested	Resistant	Tested	Resistant	Tested	Resistant	Tested	Resistant
First-line								
Pyrazinamide	97 (94.2)	52 (53.6)	70 (81.4)	49 (71.0)	10 (5.2)	4 (40.0)	177 (45.0)	105 (59.7)
Ethambutol	99 (96.1)	50 (50.5)	85 (98.9)	55 (64.7)	187 (97.9)	115 (61.5)	371 (97.6)	220 (59.3)
Streptomycin	93 (90.3)	78 (83.9)	85 (98.9)	82 (96.5)	187 (97.9)	171 (91.4)	365 (96.1)	331 (90.7)
≥1 non–first line	101 (97.1)	66 (65.4)	86 (100)	64 (74.4)	173 (86.4)	64 (37.0)	360 (94.7)	194 (51.1)
Class II								
Amikacin	95 (92.2)	17 (17.9)	85 (98.8)	25 (29.4)	1 (0.5)	0	181 (47.6)	42 (23.2)
Kanamycin	39 (37.9)	8 (20.5)	79 (91.9)	37 (46.8)	170 (89.0)	23 (13.5)	288 (75.8)	68 (23.6)
Capreomycin	88 (85.4)	15 (17.0)	84 (97.7)	26 (31.0)	94 (49.2)	4 (4.3)	266 (71.1)	45 (16.9)
≥1 second-line inj.	100 (97.1)	24 (24.0)	86 (100)	42 (48.8)	170 (89.0)	27 (15.9)	356 (93.7)	93 (26.1)
Class III								
Ofloxacin	69 (67.0)	16 (23.2)	86 (100)	26 (30.2)	169 (88.5)	14 (8.3)	324 (85.3)	56 (17.3)
Levofloxacin	16 (15.5)	1 (6.2)	7 (8.1)	1 (14.3)	10 (5.2)	2 (20.0)	32 (8.4)	4 (12.5)
Moxiflocacin	61 (59.2)	14 (23.0)	12 (14.0)	3 (25.0)	0		73 (19.2)	17 (23.3)
≥1 fluoroquinolone	96 (96.2)	21 (21.9)	86 (100)	26 (30.2)	170 (89.0)	15 (8.8)	352 (92.6)	62 (17.6)
Class IV								
ETO/PTO	98 (95.1)	47 (48.0)	86 (100)	36 (41.9)	170 (89.0)	36 (21.2)	354 (93.2)	119 (31.3)
PAS	54 (52.4)	10 (18.5)	68 (79.1)	10 (14.7)	175 (91.6)	2 (1.1)	295 (77.6)	22 (7.5)
DCS/TRD	53 (51.5)	6 (11.3)	69 (80.2)	13 (18.8)	100 (52.4)	5 (5.0)	220 (57.9)	23 (10.6)
Class V								
Linezolid	62 (60.2)	2 (3.2)	6 (7.0)	0	1 (0.5)	0	69 (18.2)	2 (2.9)
Imipenem	0	0	0	0	0	0	0	0
Meropenem	1 (1.0)	1 (100)	0	0	0	0	1 (0.3)	1 (100)
AMX/CLV	0	0	0	0	0	0	0	0
Clarithromycin	17 (16.5)	3 (17.7)	0	0	0	0	17 (4.5)	3 (17.6)

*Values are no. (%) samples. Unweighted analysis was used. TB, tuberculosis; MDR TB, multidrug-resistant tuberculosis; inj, injectable; ETO/PTO, ethionamide/prothionamide; PAS, para-aminosalicylic acid; DCS/TRD, cycloserine/terizidone; AMX/CLV, amoxicillin/clavulanic acid.
†Austria, Belgium, Czech Republic, Denmark, Germany, Great Britain, Ireland, Netherlands, Italy, and Spain.
‡Belarus, Estonia, Latvia, and Portugal.
§Moldova and Romania.

Table 3. Risk factors for multidrug-resistant tuberculosis in patients in TBNET study in Europe, 2010–2011*

Factor	Non–MDR TB, n = 376	MDR TB, n = 380	Univariable analyisis		Multivariable analysis	
			OR (95% CI)	p value	OR (95% CI)	p value
Sex						
F	111	141	1	NA	1	NA
M	265	239	0.71 (0.52–0.97)	0.031	0.78 (0.53–1.14)	0.195
Age, y						
<45	212	258	1.73 (1.16–2.58)	0.007	1.90 (1.23–2.93)	0.004
≥45	155	109	1	NA	1	NA
Unknown	4	10	NA	NA	NA	NA
Body mass index						
<18	31	48	1.64 (0.94–2.85)	0.082	NA	NA
18–<25	276	261	1	NA	NA	NA
≥25	38	49	1.36 (0.65–2.87)	0.414	NA	NA
Currently employed						
Yes	144	144	1	NA	NA	NA
No	211	222	1.03 (0.71–1.49)	0.886	NA	NA
Unknown	16	14	NA	NA	NA	NA
Foreign born						
Yes	63	94	1.63 (1.12–2.38)	0.011	1.52 (0.89–2.61)	0.120
No	313	286	1	NA	1	NA
Imprisonment before current diagnosis						
Yes	15	30	2.05 (0.75–5.66)	0.164	1.27 (0.82–1.97)	0.280
No	345	336	1	NA	1	NA
Unknown	16	14	NA	NA	NA	NA
Current homelessness						
Yes	21	16	0.73 (0.43–1.24)	0.248	NA	NA
No	346	359	1	NA	NA	NA
Unknown	9	5	NA	NA	NA	NA
Injectable drug user						
Yes	13	24	1.87 (0.92–3.83)	0.084	1.32 (0.54–3.21)	0.541
No	332	327	1	NA	1	NA
Unknown	31	29	NA	NA	NA	NA
HIV infected						
Yes	16	25	1.57 (0.86–2.87)	0.146	1.78 (0.81–3.89)	0.151
No	320	345	1	NA	1	NA
Not tested	36	9	NA	NA	NA	NA
Unknown	4	1	NA	NA	NA	NA
Diabetes						
Yes	20	16	0.80 (0.32–1.98)	0.622	NA	NA
No	354	356	1	NA	NA	NA
Unknown	2	8	NA	NA	NA	NA
Previous TB treatment						
Yes	33	133	9.49 (7.05–12.76)	<0.001	10.71 (7.33–15.63)	<0.001
No	339	244	1	NA	1	NA
Unknown	4	3	NA	NA	NA	NA

*MDR TB, multidrug-resistant tuberculosis; OR, odds ratio; NA, not applicable.

Discussion

We studied a multicenter cohort of patients with MDR TB at 23 referral centers across Europe and found high rates of drug resistance to second-line drugs for treatment of TB in circulating *M. tuberculosis* strains, and limited availability of second-line drug resistance testing in several countries with a high incidence of TB. Furthermore, we found evidence of ongoing transmission of MDR strains of *M. tuberculosis* in eastern Europe: 52.4% of patients with MDR TB were experiencing their first episode of TB. In countries in western Europe with a low incidence of TB, MDR TB is predominantly a disease of immigrants (*15*), which reflects the epidemiology of MDR TB in the country of origin. Only a few (8.9%) MDR TB patients were born outside the European region of WHO. Thus, interventions for the control of MDR TB should be specific for countries with high incidence of MDR TB, especially in eastern Europe (*16*).

Mathematical and epidemiologic models indicate that early diagnosis, effective treatment, and improved access to laboratory infrastructure could have a strong effect on the incidence of MDR TB in high-prevalence regions (*17*). However, few of such programmatic requirements are met at many sites in Europe at the present time (*18*).

Possible active transmission of strains causing MDR TB, as reflected by the large proportion of patients never having received TB treatment before in this European cohort, is consistent with recently reported data and deserves attention. A drug resistance survey conducted in Belarus in 2011 showed that 32.3% of new TB infections and 75.6% of previously treated TB infections had an MDR strain of

M. tuberculosis (*19*). In Moldova, for which adequate surveillance data are available, 23.7% of new TB cases involve an MDR strain (*3*). A recent report of surveillance data in countries with >700 estimated MDR TB cases per year indicated that more than half of the reported pulmonary MDR TB cases were new cases (*20*).

More than 90% of strains from MDR TB patients had undergone DST for ≥1 second-line injectable drug and fluoroquinolone. The role of ethambutol and pyrazinamide for treatment of MDR TB is unclear. In our cohort, 97.6% and 45.0% of MDR TB strains were tested for resistance to ethambutol and pyrazinamide, respectively. In countries with a high incidence of TB, only 5.2% of MDR TB cases were tested for pyrazinamide resistance because of limited availability of liquid culture methods and special pH media requirements for pyrazinamide DST. Less than half of the strains tested were susceptible to these drugs. Currently, the mechanism of action of pyrazinamide in combination therapy and the relevance of in vitro DST for pyrazinamide are uncertain. Findings from this study raise questions about a universal recommendation to treat MDR TB with pyrazinamide throughout the entire course of treatment (*21*).

In our study cohort, 1 of 3 *M. tuberculosis* strains with resistance to at least rifampin and isoniazid were also resistant to protionamide/ethionamide, 1 of 4 were resistant to any second-line injectable drug, and 1 of 5 were resistant to a fluoroquinolone. Of all MDR TB cases, 6.8% fulfilled the definition of XDR TB. Surveillance data from the European Centre for Disease Prevention and Control indicated that 9.1% of cases of XDR TB in patients with MDR TB underwent second-line DST. Given the high proportion of strains that received a second-line DST, it is unlikely that these percentages are overstated because of preferred testing of patients at high risk for acquiring TB.

Our results are consistent with those from the Preserving Effective TB Treatment Study (PETTS) (*22*), which investigated second-line drug resistance in strains from 1,278 patients in 8 countries, including Latvia and Estonia, which were countries with study sites in this cohort. The main difference between PETTS study and ours was a high frequency of *M. tuberculosis* resistance to prothionamide/ethionamide in our cohort, which reflected the relatively higher frequency of treatment with this drug combination in eastern Europe than in other parts of the world (*23*). Recently published data from the PETTS study showed an increased risk of acquiring resistance to second-line drugs during treatment and increased baseline resistance (*24*). Increased resistance to second-line drugs is associated with higher proportions of treatment failures (*6*). It can be assumed, if one considers the findings from the PETTS study, that many of the patients in our cohort are at high risk for treatment failure.

Of particular concern is resistance to fluoroquinolones because these drugs are the core of new treatment regimens (*25*,*26*), including regimens for patients with drug-susceptible strains of *M. tuberculosis* (*26*). In our study, the capacity to perform DST for later-generation fluoroquinolones (levofloxacin and moxifloxacin) was only present for 19.2% of strains for levofloxacin and 8.4% of strains for moxifloxacin. Later generations of fluoroquinolones may still be effective for treatment of MDR TB in some patients when drug resistance to ofloxacin is documented (*27*). The capacity to perform DST for later generations of fluoroquinolones needs to be improved in the region.

Multivariable analysis showed that previous TB treatment and patient age <45 years showed an association with MDR TB (male sex and current homelessness showed an association in a weighted model). However, none of the other traditional risk factors for drug-resistant TB, such as HIV infection or birth in a foreign country (*12*), showed this association. Although previous treatment for TB and contact with persons infected with drug-resistant strains have been reported as strong risk factors for MDR TB, the role of HIV infection, young age, sex, and previous imprisonment are less clear (*12*,*28*). The high proportion of new cases and the lack of association of other traditional risk factors with drug-resistant TB suggest an increased role of ongoing transmission in the community outside established risk groups for becoming infected with drug-resistant strains of *M. tuberculosis* (*19,20,29*).

Our study has several major limitations. First, baseline data were obtained from an observational cohort study and were not derived from routine surveillance. Only 14 of 28 countries from the European Union and 2 countries outside the European Union were represented in the study. Site selection was based on voluntary participation in the study and being a center for the management of MDR TB. Because a high number of patients in Europe are being treated outside such centers, the generalizability of data might be limited. However, the included centers adhered to national policies regarding diagnosis and treatment of MDR TB patients and therefore reflect current practice. To provide a better estimation of representativeness of data for the situation in Europe, we additionally performed weighted analyses based on the sampling fraction and the expected number of reported MDR TB patients in the countries from which patients were recruited (online Technical Appendix). Results suggest that frequencies of drug resistance to second-line drugs might be underestimated by our analysis.

Second, some data collected were self-reported by patients and are prone to information bias. This limitation particularly applies to information on previous TB treatment in foreign-born patients, who might fear stigmatization in the country where treatment was provided.

Third, DST was performed at laboratories that used external quality control practices. However, quality control for testing of second-line drugs varies among sites and

respective laboratories (*30*). Incompleteness of DST data for second-line drugs demonstrates the situation with which clinicians are confronted in making their management decisions and shows the need for scale up in laboratory testing, even in MDR TB reference centers in Europe.

Despite these limitations, our study identified 3 major concerns regarding TB in Europe. First, transmission of MDR strains of *M. tuberculosis* is ongoing. Second, diagnostic capacity is poor, especially for DST. Third, levels of resistance to second-line TB drugs are high. These factors must be addressed in any TB surveillance and control programs that are implemented.

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Risk Factors for Death from Invasive Pneumococcal Disease, Europe, 2010

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We studied the possible association between patient age and sex, clinical presentation, *Streptococcus pneumoniae* serotype, antimicrobial resistance, and death in invasive pneumococcal disease cases reported by 17 European countries during 2010. The study sample comprised 2,921 patients, of whom 56.8% were men and 38.2% were ≥65 years of age. Meningitis occurred in 18.5% of cases. Death was reported in 264 (9.0%) cases. Older age, meningitis, and nonsusceptibility to penicillin were significantly associated with death. Non–pneumococcal conjugate vaccine (PCV) serotypes among children <5 years of age and 7-valent PCV serotypes among persons 5–64 years of age were associated with increased risk for death; among adults ≥65 years of age, risk did not differ by serotype. These findings highlight differences in case-fatality rates between serotypes and age; thus, continued epidemiologic surveillance across all ages is crucial to monitor the long-term effects of PCVs.

Streptococcus pneumoniae causes severe invasive disease that results in considerable illness and death. The incidence of invasive pneumococcal disease (IPD) is higher during the early years of life and among elderly persons (*1*). Geographic and ethnic differences also exist (*1*,*2*). Environmental factors (i.e., ambient temperature, humidity, and air pollution) affect IPD incidence (*3*,*4*). IPD has also been related to recent respiratory viral infection (*4*).

The ability of the different *S. pneumoniae* serotypes to cause disease has been related to serotype-specific characteristics and the molecular size of the capsular polysaccharide and chemical composition, among other factors (*5*). Therefore, it seems plausible that different serotypes exhibit different virulence and propensity to cause certain clinical presentation (*5*).

Brueggemann et al. studied the invasive disease potential of different *S. pneumoniae* serotypes (*6*). They concluded that so-called "highly invasive" serotypes (including 4, 1, 14, 18C, and 7F), convey a higher risk for invasive disease than do the "low invasive" serotypes (including 3, 15B/C, and 6B), which are more frequently isolated as colonizers (*7*). Furthermore, serotype distribution varies with patient age, both in disease and in nasopharyngeal colonization (*2*,*8*–*10*). However, evidence exists that pneumococcal invasiveness does not necessarily mean lethality (*7*). Low invasive serotypes usually account for higher case-fatality rates (CFRs).

The availability of 7-valent, 10-valent, and 13-valent pneumococcal conjugate vaccines (PCV7, PCV10, and PCV13, respectively) and their introduction as part of national immunization schedules have contributed to reducing illnesses and death from IPD (*10*–*12*). Nevertheless, the subsequent replacement of vaccine serotypes by nonvaccine serotypes is an accepted and global phenomenon (*13*,*14*).

The incidence of drug- and multidrug-resistant *S. pneumoniae* strains is increasing worldwide (*15*). Antimicrobial use and abuse is a main driver for the emergence of antimicrobial resistance in respiratory pathogens. Persons who carry (nasopharyngeal colonization), and hence share the potential to transmit resistant pneumococci, also are more susceptible to IPD caused by resistant strains (*16*).

Monitoring antimicrobial resistance trends and serotype distribution is paramount because this information is essential in helping to determine risk factors and optimizing the appropriate clinical management of cases and public health interventions. We studied the possible association between age, sex, serotype, clinical presentation, antimicrobial resistance, and death among persons reported to have IPD in European countries during 2010.

Materials and Methods

Data

IPD data derived from passive national surveillance case notification systems were collected during 2010 by 26 European Union (EU)/European Economic Area countries

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(Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Malta, Netherlands, Norway, Poland, Romania, Slovakia, Slovenia, Spain, Sweden, and United Kingdom); data were submitted to The European Surveillance System. The platform of The European Surveillance System is a metadata-driven system for the collection, validation, cleaning, and analysis of data hosted by the European Centre for Disease Prevention and Control. Surveillance systems differ across Europe, and data were reported with varying levels of completeness. Countries reported only laboratory-confirmed cases based on the EU 2008 case definition.

Study Sample

The study sample was the subsample of cases for which information was available about both serotype and outcome (Figure 1). The sample represents data from 17 European countries (Table 1).

Study Variables

An episode of IPD was defined as the isolation of a strain or detection of nucleic acid or antigen of *S. pneumoniae* from a normally sterile site. Countries reported IPD outcome according to their national surveillance and guidelines. The following age groups were defined for the study: <5 years, 5–64 years, and ≥65 years. For purpose of this analysis, clinical presentation was recoded as "meningitis" and "nonmeningitis." Clinical presentation was grouped on the basis of a literature review (*5*), which suggested that meningitis and nonmeningitis had different degrees of severity and conveyed different rates of death.

Serotypes were grouped into 3 categories: PCV7 serotypes (serotypes in PCV7: 4, 6B, 9V, 14, 18C, 19F, and 23F), PCV13-specific serotypes (serotypes in PCV13 but not in PCV7: 1, 3, 5, 6A, 7F, and 19A), and non-PCV serotypes (serotypes not in any PCV). Results of antimicrobial susceptibility testing to penicillin and erythromycin were reported as "susceptible," "intermediate," or "resistant" by the countries according to their national standards and protocols. Therefore, information was not available about the breakpoints and guidelines used for antimicrobial susceptibility testing in each country. For example, in the European Antimicrobial Resistance Surveillance Network report for 2010 (*17*), 66% of reporting laboratories in Europe used Clinical and Laboratory Standards Institute standards, whereas 29% applied the European Committee on Antimicrobial Susceptibility Testing guidelines.

For this study, we redefined the variable to include just 2 categories: "susceptible" (cases reported as susceptible

Table 1. Characteristics of patients with invasive pneumococcal disease, Europe, 2010*

Characteristic	No. cases (% of total), N = 17,549	Sample size, no. (%), n = 2,921†
Sex		
F	7,915 (45.3)	1,257 (43.2)
M	9,565 (54.7)	1,651 (56.8)
Age group, y		
<5	1,980 (11.3)	570 (19.7)
5–64	7,819 (44.7)	1,222 (42.1)
≥65	7,684 (44.0)	1,108 (38.2)
Outcome		
Nonfatal	4,146 (89.4)	2,657 (91.0)
Fatal	491 (10.6)	264 (9.0)
Clinical presentation		
Nonmeningitis	6,047 (79.4)	1,722 (81.5)
Meningitis	1,572 (20.6)	391 (18.5)
Serotype		
PCV13-specific‡	4,185 (42.1)	1,235 (42.7)
PCV7	1,772 (17.8)	517 (17.9)
Non-PCV	3,989 (40.1)	1,137 (39.4)
Antimicrobial susceptibility		
Penicillin		
Susceptible	8,420 (91.1)	1,949 (94.1)
Nonsusceptible§	827 (8.9)	122 (5.9)
Erythromycin		
Susceptible	6,911 (82.5)	1,573 (76.4)
Nonsusceptible	1,471 (17.5)	486 (23.6)

*Numbers do not add to the total in each category because of missing data. See Figure 1. PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV.
†Defined as patients for whom information was available about serotype and outcome.
‡Serotypes contained in PCV13 but not in PCV7.
§Either resistant or intermediate resistance.

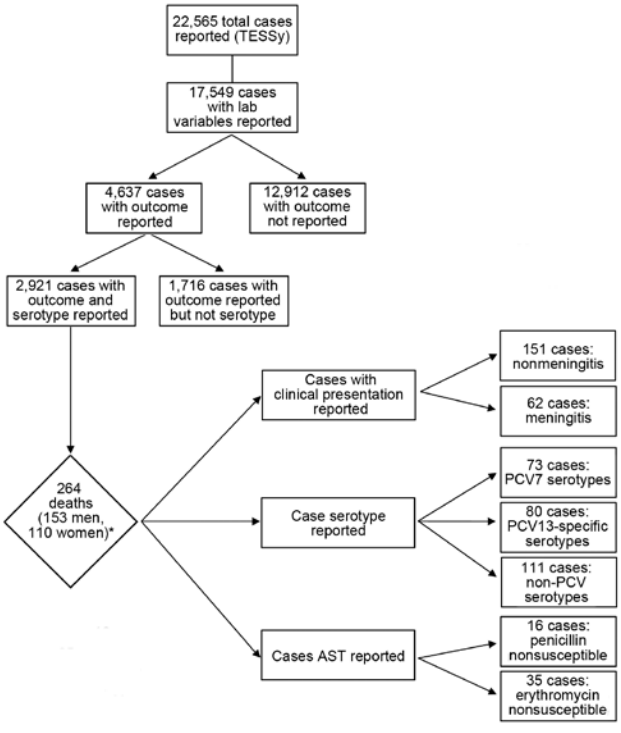

Figure 1. Flow of invasive pneumococcal disease cases through the study, Europe, 2010. *Sex was unknown for 1 patient. AST, antimicrobial susceptibility testing; PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV; TESSy, The European Surveillance System.

by the countries) and "nonsusceptible" (intermediate and resistant), both for penicillin and erythromycin. Methods for the characterization of isolates and for antimicrobial susceptibility testing are provided in detail in the 2010 IPD enhanced surveillance report by the European Centre for Disease Prevention and Control (*18*).

Statistical Analysis

Categorical variables are presented as number of cases and percentages. We used the Pearson χ^2 test to compare the proportion of deaths by PCV7, PCV13-specific, and non-PCV serotypes; the proportion of deaths by the defined age groups and by sex; the proportion of deaths by clinical presentation; and the proportion of deaths in antimicrobial-susceptible and -nonsusceptible cases, according to antimicrobial drug type. We used the Fisher exact test to analyze the association between penicillin-susceptible/penicillin-nonsusceptible IDP and outcome for patients <5 years of age and non-PCV serotypes and to assess differences between penicillin-susceptible/penicillin-nonsusceptible cases and outcome for serotype 35B. In addition, we assessed the associations between each serotype and death using a generalized linear model with log-link function. This analysis was performed for the 28 serotypes that accounted for up to 80% of cases with fatal outcomes; each individual serotype was also compared with all the others.

Univariable analysis was performed for the 264 fatal cases to identify factors associated with a fatal outcome. To test the association between age, serotype, clinical presentation, and death, a generalized linear model with robust SEs accounting for the country effect was fitted because data came from different national surveillance systems and vaccination policies and practices differ widely across Europe. We studied the role of variables as potential confounders/modifiers, but only age was statistically significant. Age was an effect modifier of the association between serotype and risk for death, and thus the analysis was stratified by age group.

We also conducted regression analysis. The regression model comprised factors that were significant by univariable analysis and that had previously been hypothesized to affect IPD CFRs.

All p values were 2 tailed, and statistical significance was defined as $p<0.05$. We conducted statistical analyses by using STATA 12.0 (StataCorp, College Station, TX, USA).

Results

Case Characteristics

In 2010, the European countries reported 22,565 IPD cases. Of these, information was available about laboratory variables for 17,549 cases (Figure 1); outcome was known for 4,637 of these. The study sample comprised 2,921 cases for which information was available about serotype and outcome.

A total of 56.8% of cases (Table 1) occurred in men, and 38.2% of cases were among adults ≥65 years of age. Children <5 years of age accounted for 19.7% of cases. A total of 264 (9.0%) persons died. Meningitis occurred in 18.5% of cases. PCV13-specific serotypes (1, 3, 5, 6A, 7F, 19A) accounted for 42.7% of cases. Nonsusceptibility (intermediate + resistant) to penicillin was reported in 122 (5.9%) of 2,071 cases; nonsusceptibility to erythromycin was reported in 486 (23.6%) of 2,059 cases (Table 1).

PCV13-specific serotypes caused 57.7% ($p<0.001$) of cases among children <5 years of age (Figure 2). Non-PCV serotypes accounted for 48.0% of cases among adults ≥65 years of age. Meningitis cases were predominantly caused by non-PCV serotypes (41.4%, $p<0.001$) (Figure 2). Nonsusceptibility to penicillin was highest among PCV7 serotypes (64.8%, $p<0.001$) (Figure 2).

The Pearson χ^2 analysis (Table 2) demonstrated a lack of statistical association between sex and death ($p = 0.631$). The CFR was highest for adults ≥65 years of age (13.7%, $p<0.001$); 2.3% of children <5 years of age died.

Clinical presentation was associated with death. The CFR for persons with meningitis was 15.9% compared with 8.8% for those without meningitis ($p<0.001$).

Death was also associated with nonsusceptibility to penicillin. Death occurred in 13.1% of cases in which *S. pneumoniae* was not susceptible to penicillin ($p = 0.010$) (Table 2). Nonsusceptibility to erythromycin was not significantly associated with death ($p = 0.837$).

We determined the association between individual serotype and death (Table 3). Serotype 35B (relative risk [RR] 4.98, 95% CI 2.49–9.95), serotype 4 (RR 2.03, 95% CI 1.04–3.95), and serotype 11A (RR 1.97, 95% CI 1.33–2.94) were most associated with death. Serotype 3 (RR 1.39, 95% CI 0.88–2.21) accounted for the highest number and the highest percentage (13.3%) of serotype-specific deaths, but the association with death was not statistically significant ($p = 0.161$). In contrast, for serotype 1 (RR 0.25, 95% CI 0.13–0.48) and serotype 5 (RR 0.15, 95% CI 0.09–0.26), the association with death was significant. Subanalysis of the association between susceptibility to penicillin and outcome for serotype 35B found no significant differences in risk for death between susceptible and nonsusceptible cases.

Risk Factors for IPD-Associated Death

Univariable analysis showed differences between nonfatal and fatal cases (Table 4). Persons 5–64 years of age (RR 3.55, 95% CI 1.66–7.61) and ≥65 years of age (RR 4.79, 95% CI 3.08–11.76) had a higher risk for death than did children <5 years of age. In the univariable

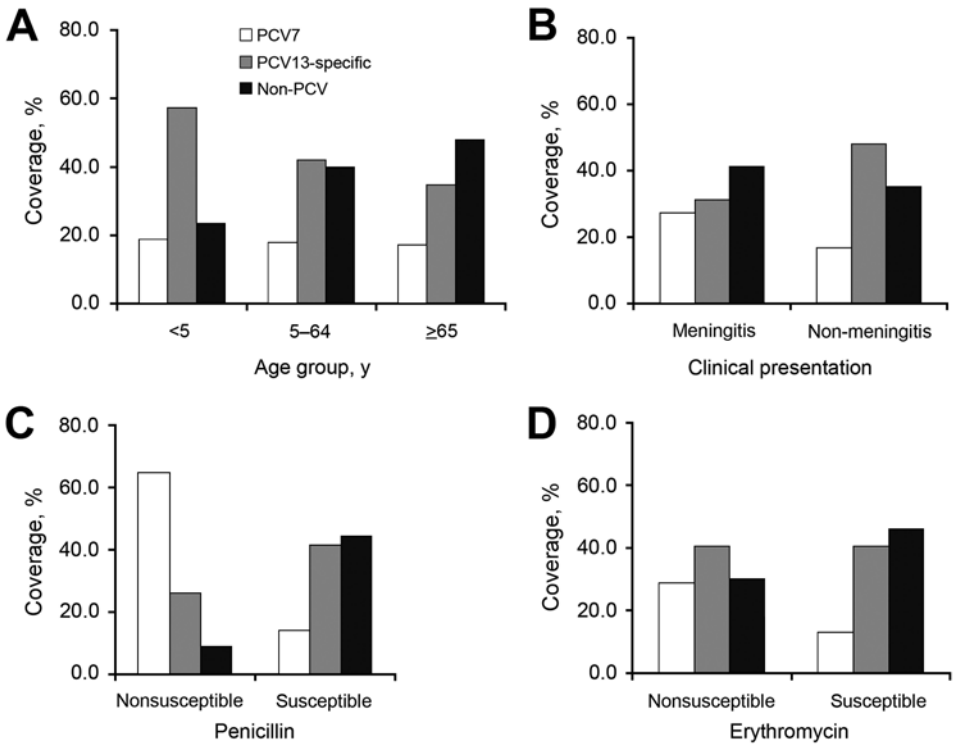

Figure 2. Invasive pneumococcal disease study variables and PCV coverage of *Streptococcus pneumoniae* serotypes, Europe, 2010. A) Age group. B) Clinical presentation. C) Penicillin susceptible. D) Erythromycin susceptible. For all 4 variables, p<0.001. White bars, PCV7 serotypes; gray bars, PCV13 serotypes; black bars, non-PCV serotypes. PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV.

analysis, meningitis (RR 1.81, 95% CI 1.25–2.61, p = 0.002) was significantly associated with death. PCV7 serotypes were also significantly associated with death (RR 2.18, 95% CI 1.06–4.48, p = 0.034). Conversely, non-PCV serotypes were not associated with death (RR 1.47, 95% CI 0.94–2.28).

Table 2. Associations between invasive pneumococcal disease study variables and death, Europe, 2010*

Variable	Outcome: Nonfatal, no. (%)	Outcome: Fatal, no. (%)	p value†
Sex			
F	1,147 (91.3)	110 (8.8)	0.631
M	1,498 (90.7)	153 (9.3)	
Age group, y			
<5	557 (97.7)	13 (2.3)	
5–64	1,123 (91.9)	99 (8.1)	<0.001
≥65	956 (86.3)	152 (13.7)	
Clinical presentation			
Nonmeningitis	1,571 (91.2)	151 (8.8)	<0.001
Meningitis	329 (84.1)	62 (15.9)	
Serotype			
PCV13-specific‡	1,155 (93.5)	80 (6.5)	<0.001
PCV7	444 (85.9)	73 (14.1)	
Non-PCV	1,028 (90.5)	111 (9.5)	
Antimicrobial susceptibility			
Penicillin			
Susceptible	1,815 (93.1)	134 (6.9)	
Nonsusceptible§	106 (86.9)	16 (13.1)	
Erythromycin			0.010
Susceptible	1,464 (93.1)	109 (6.9)	
Nonsusceptible	451 (92.8)	35 (7.2)	

*PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV;
†Pearson χ^2 test.
‡Serotypes contained in PCV13 but not in PCV7.
§Either resistant or intermediate resistance.

Nonsusceptibility to penicillin was associated with increased risk for death (RR 1.91, 95% CI 1.16–3.13). Nonsusceptibility to erythromycin was not significantly associated with death (RR 1.04, 95% CI 0.84–1.29).

Our comparison of susceptibility to penicillin and outcome for clinical presentation showed that the association with the outcome remained statistically significant only for meningitis cases (RR 1.82, 95% CI 1.27–2.62, p = 0.001). These factors were not associated with nonmeningitis cases (RR 1.31, 95% CI 0.28–6.01).

Age was an effect modifier. In the stratified analysis, we found that among children <5 years of age, risk for death from non-PCV serotypes increased (RR 3.68, 95% CI 1.27–10.69) (Table 5), whereas among persons 5–64 years of age, PCV7 serotypes conveyed the highest risk for death (RR 2.68, 95% CI 1.37–5.23). Among adults ≥65 years of age, risk for death among the serotypes did not differ significantly.

We analyzed the association between susceptibility to penicillin and outcome for non-PCV serotypes. Children <5 years of age showed no differences between susceptible and nonsusceptible cases.

Discussion

Our analysis of IPD surveillance data from Europe in 2010 unveiled a significant association between death and older age, meningitis, serotypes contained in PCV7, and non-

Table 3. *Streptococcus pneumoniae* serotype in invasive pneumococcal disease and association with death, Europe, 2010*

Serotype	PCV†	Fatal, %	Nonfatal, %	RR (95% CI)	p value‡
3	PCV13-specific§	13.3	9.6	1.39 (0.88–2.21)	0.161
4	**PCV7**	**6.1**	**2.8**	**2.03 (1.04–3.95)**	**0.038**
19A	PCV13-specific	6.1	7.6	0.80 (0.41–1.57)	0.515
14	PCV7	5.7	4.6	1.23 (0.78–1.85)	0.369
7F	PCV13-specific	4.9	8.3	0.59 (0.35–1.01)	0.053
6B	PCV7	3.8	1.7	2.01 (0.79–5.16)	0.144
19F	PCV7	3.8	1.9	1.85 (0.93–3.65)	0.078
22F	Non-PCV	3.8	2.8	1.35 (0.89–2.03)	0.157
9V	PCV7	3.4	2.2	1.50 (0.95–2.38)	0.081
23F	PCV7	3.4	2.3	1.42 (0.60–3.32)	0.423
1	**PCV13-specific**	**3.4**	**13.1**	**0.25 (0.13–0.48)**	**<0.001**
11A	**Non-PCV**	**2.3**	**1.1**	**1.97 (1.33–2.94)**	**0.001**
10A	Non-PCV	2.3	1.4	1.52 (0.86–2.68)	0.147
6A	PCV13-specific	2.3	2.3	1.01 (0.39–2.57)	0.990
6C	Non-PCV	1.9	0.7	2.33 (0.93–5.86)	0.072
9N	Non-PCV	1.9	1.5	1.21 (0.52–2.82)	0.664
12F	Non-PCV	1.9	1.8	1.07 (0.51–2.23)	0.867
35B	**Non-PCV**	**1.5**	**0.2**	**4.98 (2.49–9.95)**	**<0.001**
33F	Non-PCV	1.5	0.9	1.53 (0.55–4.28)	0.414
18C	PCV7	1.5	1.2	1.23 (0.40–3.76)	0.713
8	Non-PCV	1.5	3.1	0.59 (0.25–1.06)	0.073
23A	Non-PCV	1.1	0.7	1.51 (0.66–3.45)	0.323
15A	Non-PCV	0.8	0.7	1.05 (0.46–2.43)	0.909
15B	Non-PCV	0.8	1.0	0.79 (0.26–2.41)	0.677
24F	Non-PCV	0.4	0.6	0.69 (0.12–4.09)	0.683
5	**PCV13-specific**	**0.4**	**2.6**	**0.15 (0.09–0.26)**	**<0.001**

*PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV; RR, relative risk. Boldface indicates statistical significance.
†Classification of serotypes according to study group.
‡Generalized linear model with log-link function.
§Serotypes contained in PCV13 but not in PCV7.

susceptibility to penicillin. As have many other studies, we found an association between increased age and death (*19*–*22*). The risk was higher for adults ≥65 years of age (RR 4.79, 95% CI 3.08–11.76) than for persons 5–64 years of age (RR 3.55, 95% CI 1.66–7.61). However, the lack of information about patients' clinical characteristics impedes accurate assessments of these differences.

Elderly persons have been postulated to have an increased susceptibility to—in addition to co-ocurring conditions—pneumococcal disease because of reduced splenic function (*23*), age-related changes in respiratory tract, immunosenescence, and cellular senescence related to age-associated inflammation (*23*). The higher incidence and death rates for IPD in this age group is remarkable and highlights the need to direct vaccination toward the elderly. These findings may present an opportune moment to revisit adult vaccination recommendations and programs in European countries (*24*).

We did not find sex to be significantly associated with death. However, other studies have shown association either with men (*25*) or women (*23*,*26*).

In our study, presence of meningitis was significantly associated with death. Harboe et al. obtained similar results in a large population-based cohort study (*25*). In Denmark, another study concluded that patients with pneumococcal meningitis had increased death rates, but these rates derived from severe underlying conditions (*27*). CFRs for pneumococcal meningitis are usually higher than for nonmeningitis (*28*). More recently, Ladhani et al. found that the CFR was higher for children with meningitis in England and Wales (*29*). This study showed that infecting serotype was not associated with death (*29*), whereas meningitis and co-occurring conditions were significantly associated with death. In our analysis, meningitis was predominantly caused by non-PCV serotypes; this finding could be an effect of PCV introduction, as observed in other studies (*30*). Another analysis of susceptibility to penicillin by clinical presentation showed a higher risk for death among persons with nonsusceptible IPD than for those with susceptible IPD who had meningitis. Therefore, in the absence of information about clinical management of cases and existing co-occurring conditions, the association between meningitis and nonsusceptibility to penicillin might be an explanation.

Capsular differences between serotypes affect clinical presentation and outcome (*10*,*31*,*32*). These differences are in accordance with our study, which found PCV7 serotypes were associated with death in the univariable analysis. Among children <5 years of age, PCV13-specific serotypes were most frequently identified, compared with PCV7 and non-PCV serotypes, as defined in our study. In 2010, PCV13 was already licensed, and many European countries began moving from PCV7 to the higher-valent vaccine, although with different schemes, policies, and dates

Table 4. Association between invasive pneumococcal disease study variables and death, Europe, 2010*

Variable	Relative risk† (95% CI)
Sex	
F	Reference
M	1.06 (0.88–1.28)
Age group, y	
<5	Reference
5–64	3.55 (1.66–7.61)
≥65	4.79 (3.08–11.76)
Clinical presentation	
Nonmeningitis	Reference
Meningitis	1.81 (1.25–2.61)
Serotype	
PCV13-specific‡	Reference
PCV7	2.18 (1.06–4.48)
Non-PCV	1.47 (0.94–2.28)
Antimicrobial susceptibility	
Penicillin	
Susceptible	Reference
Nonsusceptible§	1.91 (1.16–3.13)
Erythromycin	
Susceptible	Reference
Nonsusceptible	1.04 (0.84–1.29)

*PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV.
†Generalized linear model with log-link function.
‡Serotypes contained in PCV13 but not in PCV7.
§Either resistant or intermediate resistance.

of introduction. Nevertheless, these changes are unlikely to have affected our study findings because we analyzed data from 2010.

After stratification, the highest risk for death among children <5 years of age corresponded to non-PCV serotypes. This finding could be attributed to serotype replacement after pneumococcal vaccination (*29*,*30*). Our analysis found no differences between penicillin-susceptible and -nonsusceptible cases among children <5 years of age and non-PCV serotypes subgroup with respect to death. However, the overall percentage of meningitis cases was high (18.5% of the study sample), and meningitis was predominantly caused by non-PCV serotypes (p<0.001) (Figure 2). Hence, vaccines with enhanced serotype coverage (higher valency) might be needed to prevent IPD in this age group in the near future.

Among persons 5–64 years of age, the risk for death was highest for PCV7 serotypes, which were predominantly nonsusceptible to penicillin (p<0.001) (Figure 2). Reductions in IPD caused by PCV7 serotypes in non–vaccine-eligible age groups in countries with universal use of PCV7 might indicate the indirect effect of PCV7 (*33*). However, because vaccine policies differed among European countries at the time of the study, this indirect effect might not be reflected in the pooled data (Table 6).

Serotypes 1, 5, and 7F have been described as having high potential for invasiveness (these serotypes are carried for a short time) but are associated with milder disease and lower CFRs (*7*,*9*,*19*,*34*). As in those studies, we found that serotypes 1 and 5 caused IPD but were not associated with death.

Serotype 35B has been reported as nonsusceptible to penicillin (*35*). The subanalysis on susceptibility to penicillin for serotype 35B showed that penicillin nonsusceptibility did not affect the risk for death for serotype 35B. Nevertheless, the increased risk for death of non-PCV serotypes 11A and 35B merits further monitoring.

We found penicillin nonsusceptibility to be significantly associated with death, as described by others (*20*,*36*). Nevertheless, in other large studies, this association was not found (*21*,*26*,*34*,*37*), and the effect of multidrug-resistant strains remains to be determined. Conversely, we found that erythromycin nonsusceptibility did not significantly affect death, as described by Song et al. (*37*) and Martens et al. (*20*). A plausible explanation might be the additional benefits of macrolides (i.e., their immunomodulatory/antiinflamatory properties), which might be important when these drugs are used in combination with other therapeutic agents (*38*).

Antimicrobial resistance to *S. pneumoniae* is increasing in many countries in Europe (*17*), and the prudent use of antibacterial drugs, apart from immunization, is pivotal in preventing and controlling IPD. Furthermore, these findings underpin the importance of antimicrobial susceptibility testing to assist with the clinical management of cases and to provide data on prevalence of antimicrobial resistance.

Table 5. Stratified analysis of *Streptococcus pneumoniae* serotype distribution in a study of invasive pneumococcal disease, Europe, 2010*

Age group, y	Survived, no. (%)	Died (%)	RR (95% CI)	p value
<5				
PCV13-specific	325 (98.8)	4 (1.2)	1	
PCV7	104 (97.2)	3 (2.8)	2.31 (0.35–15.02)	0.382
Non-PCV	128 (95.5)	6 (4.5)	3.68 (1.27–10.69)	0.017
5–64				
PCV13-specific	486 (94.4)	29 (5.6)	1	
PCV7	186 (84.9)	33 (15.1)	2.68 (1.37–5.23)	0.004
Non-PCV	451 (92.4)	37 (7.6)	1.35 (0.64–2.82)	0.429
≥65				
PCV13-specific	338 (87.8)	47 (12.2)	1	
PCV7	154 (80.6)	37 (19.4)	1.59 (0.90–2.79)	0.108
Non-PCV	464 (87.2)	68 (12.8)	1.05 (0.64–1.72)	0.856

*PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV; RR, relative risk.

Table 6. Characteristics of national pneumococcal vaccination programs in European Union/European Economic Area countries, 2010*

Country	Date of PCV7 introduction	Scope of PCV vaccination program	Immunization schedule	Dose				Vaccine coverage†	Year of measurement
				First, mo	Second, mo	Third, mo	Fourth, mo		
Austria	2004 Jul	Universal	3+1 dose	3	5	7	12–24	NA	NA
Belgium	2005 Jan	Universal	2+1 dose	2	4	12		97	2010
Bulgaria	2010 Apr	Universal	3+1 dose/2+1 dose	2	3	4	12	NA	NA
Cyprus	2008 Aug	Universal	3+1 dose	2	4	6	12–15	NA	NA
Czech Republic	2010 Jan	Risk-based	3+1 dose	2	4	6	18	86.3	2010
Denmark	2007 Oct	Universal	2+1 dose	3	5	12		85	2010
Estonia	NA	NA	not decided	NA	NA	NA	NA	NA	NA
Finland	2009 Jan	Risk-based	2+1 dose	3	5	12		NA	NA
France	2006 Jun	Universal	2+1 dose	2	4	12		81	2008
Germany	2006 Jul	Universal	3+1 dose	2	3	4	11–14	52.9	2010
Greece	2006 Jan	Universal	3+1 dose	2	4	6	12–15	NA	NA
Hungary	2008 Oct	Universal	2+1 dose	2	4	15		81.1	2009
Iceland	2006 Dec	Risk-based	2+1 dose	3	5	12		NA	NA
Ireland	2002 Oct	Universal	2+1 dose	2	6	12		89	2009
Italy	2005 May	Universal/risk-based	2+1 dose	3	5	11		55	2008
Latvia	2010 Jan	Universal	3+1 dose	2	4	6	12–15	51	2010
Lithuania	NA	NA	3+1 dose	2	4	6	24	NA	NA
Luxembourg	2003 Feb	Universal	3+1 dose	2	3	4	12–15	86	2010
Malta	2007 Jan	Risk-based	3+1 dose	2	4	13	None	NA	NA
Netherlands	2006 Jun	Universal	3+1 dose	2	3	4	11	94	2009
Norway	2006 Jul	Universal	2+1 dose	3	5	12		90	2009
Poland	2008 May	Risk-based	3+1 dose/2+1 dose	NA	NA	NA	NA	1.70	2008
Portugal	2010 Jun	Risk-based	2+1 dose	2	4	12–15		52	2009
Romania‡			3+1 dose	2	4	6	15–18		
Slovakia§	2006 Jan	Risk-based	2+1 dose	2	4	10		99.2	2009
Slovenia	2005 Sep	Risk-based	3+1 dose	2-3	4	6	24	NA	NA
Spain¶	2001 Jun	Risk-based	3+1 dose	2	4	6	15	NA	NA
Sweden	2009 Jan	Universal	2+1 dose	3	5	12		NA	NA
United Kingdom	2006 Sep	Universal	2+1 dose	2	4	13		90	2010

*NA, not available; PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV. Blank cells indicate not applicable.
†Sources: Vaccine European New Integrated Collaboration Effort II project and World Health Organization estimates of PCV7 coverage.
‡PCV7 was registered in September 2007 for voluntary use on a private basis.
§Universal as of April 2008.
¶Universal introduction in the autonomous region of Madrid in November 2006.

The major strength of our study is its large sample size; data were from national surveillance systems across Europe (i.e., we analyzed IPD individual-level data from populations in a large geographic area). In 2010, European IPD surveillance collected data corresponding to ≈82% of the total population of EU/European Economic Area countries. This enhanced surveillance for IPD data pooled together at supranational level enables comparisons with other parts of the world.

Despite its strengths, our study has some limitations. Surveillance of IPD varies markedly in Europe, including differences in laboratory methods to confirm cases, reporting, and medical practices. Therefore, a certain degree of underdiagnosis and underreporting are likely to exist in this dataset. Moreover, surveillance systems for IPD differ in sensitivity, representativeness, and specificity across Europe; these variations might have influenced the results because some countries were major contributors (Table 7) and ascertainment bias might have also occurred. Information about co-occurring conditions or clinical management of cases that might have affected outcome was also missing. European countries introduced pneumococcal vaccination at different times and with different policies, which might have affected the serotype distribution throughout Europe. Furthermore, the incomplete information about the vaccination status of cases makes difficult the interpretation of results. These limitations emphasize the need for continued and improved surveillance of IPD throughout Europe.

In conclusion, we found that older age, meningitis, non-PCV serotypes among children <5 years of age and PCV7 serotypes among persons 5–64 years of age, and penicillin nonsusceptibility were risk factors for death from IPD in Europe. The stratified analysis highlighted differences in risk for death according to *S. pneumoniae* serotype and age group. This knowledge may assist in decision making when implementing vaccination strategies as new immunization strategies are needed to tackle the considerable IPD and associated death in adults (*39*) and in designing new extended valency vaccines or protein-based pneumococcal vaccines that may confer serotype-independent immunity (*40*).

Table 7. Geographic distribution of cases and deaths of invasive pneumoccal disease for which *Streptococcus pneumoniae* serotype and disease outcome were known, Europe, 2010

Reporting country	No. (%) cases	No. (%) deaths
Austria	190 (6.5)	15 (7.9)
Belgium	1,255 (43.0)	67 (5.3)
Cyprus	3 (0.1)	0
Czech Republic	242 (8.3)	43 (17.8)
Denmark	35 (1.2)	0
Greece	20 (0.7)	1 (5.0)
Hungary	26 (0.9)	7 (26.9)
Ireland	78 (2.7)	4 (5.1)
Italy	209 (7.2)	31 (14.8)
Lithuania	3 (0.1)	0
Malta	7 (0.2)	0
Netherlands	45 (1.5)	4 (8.9)
Norway	357 (12.2)	41 (11.5)
Poland	205 (7.0)	43 (21.0)
Romania	21 (0.7)	2 (9.5)
Slovenia	224 (7.7)	6 (2.7)
Slovakia	1 (0)	0
Total	2,921 (100.0)	264 (9.04)

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Mycoplasma pneumoniae and *Chlamydia* spp. Infection in Community-Acquired Pneumonia, Germany, 2011–2012

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Mycoplasma pneumoniae and *Chlamydia* spp., which are associated with community-acquired pneumonia (CAP), are difficult to propagate, and can cause clinically indistinguishable disease patterns. During 2011–2012, we used molecular methods to test adult patients in Germany with confirmed CAP for infection with these 2 pathogens. Overall, 12.3% (96/783) of samples were positive for *M. pneumoniae* and 3.9% (31/794) were positive for *Chlamydia* spp.; *C. psittaci* (2.1%) was detected more frequently than *C. pneumoniae* (1.4%). *M. pneumoniae* P1 type 1 predominated, and levels of macrolide resistance were low (3.1%). Quarterly rates of *M. pneumoniae*–positive samples ranged from 1.5% to 27.3%, showing a strong epidemic peak for these infections, but of *Chlamydia* spp. detection was consistent throughout the year. *M. pneumoniae*–positive patients were younger and more frequently female, had fewer co-occurring conditions, and experienced milder disease than did patients who tested negative. Clinicians should be aware of the epidemiology of these pathogens in CAP.

Community-acquired pneumonia (CAP) is associated with high rates of illness and hospitalization; annual CAP incidence among adults in Europe has ranged from 1.5 to 1.7 per 1,000 population (*1*). Studies have shown that that a broad range of pathogens can cause CAP (*2*). Among these is *Mycoplasma pneumoniae*, a common agent of respiratory tract infections that is transmitted from person to person through aerosolization. The infection occurs in all age groups, but older children and young adults are affected at a higher frequency than other age groups. Clinical manifestations range from mild cases of tracheobronchitis to severe atypical pneumonia and can be followed by a broad spectrum of extrapulmonary complications.

The epidemiology of *M. pneumoniae* infection is characterized by incidence peaks every 4–7 years; during these periods, *M. pneumoniae* is responsible for up to 25% of all cases of CAP (*3*). Between epidemic periods, proportions of 1%–8% are more typical (*4*). Reports from Europe and Asia have shown a notable increase in the frequency of infections caused by *M. pneumoniae* during 2011–2012 (*5*–*12*).

For clarification of the epidemiology of *M. pneumoniae* infection and identification of the relevant periods of incidence peaks, molecular typing of the prevalent strains can be an efficient tool. *M. pneumoniae* strains can be divided into subtypes and variants according to sequence differences in the gene coding for the immunodominant main adhesin P1. It has been hypothesized that the specific antibody level in the host population can influence further infections and lead to a change of the dominating P1 type (*13*). The recently developed multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) enables differentiation of strains with higher discriminatory power (*14*). Further studies are necessary to determine associations between P1 and MLVA typing. However, knowledge of the strain's genotype identity currently has no therapeutic consequences. Because mycoplasmas, which do not have cell walls, are not susceptible to β-lactam antimicrobial drugs, macrolides are generally accepted as first-choice agents for treatment, especially in children. However, mutations in the 23S rRNA locus of *M. pneumoniae* have been shown to result in complete macrolide resistance (*15*). Resistance rates range from >90% in China (*16*) to <10% in Europe (*15*), requiring periodic monitoring of strains to identify possible new resistance or resistant strains.

Chlamydia pneumoniae is another agent associated with CAP that can also be involved in pharyngitis,

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bronchitis, and sinusitis. Reports have attributed 6%–20% of CAP cases to this bacterium (*17*), and its role in chronic respiratory illness (*18*) and exacerbation of asthma (*19*) has also been studied. *C. pneumoniae* infection is regarded as widely distributed, if not ubiquitous, with antibody prevalence rates >50% (*19*). The clinical course of infection varies from subclinical to mild and, more rarely, to severe manifestations of pneumonia. The outcome of infection is often dependent on the patient's immune competence, but co-infection by other bacteria has been suggested to be relevant in 30% of adult cases of CAP (*20*). However, a discrepancy exists between the elevated serologic prevalence and the low figures obtained through DNA-based detection methods (*21*); a recent publication from Germany reported a prevalence <1% (*22*).

Other *Chlamydia* spp. have not usually been included in epidemiologic studies of pneumonia. In particular, *C. psittaci*, the causative agent of human psittacosis (or ornithosis), has not been investigated except in severe clinical pneumonia cases. As is the case for infections caused by by other chlamydiae, an asymptomatic or mild clinical course of *C. psittaci* is far more frequent than a fulminant outbreak of disease. Nevertheless, infections that do not result in overt illness may have long-term implications for the patient's health, as was shown in cattle that were carriers of *Chlamydia* spp. but did not show signs of disease (*23*).

In this study, we used molecular diagnostic approaches to investigate the occurrence of *M. pneumoniae* and *Chlamydia* spp. in adult patients in Germany who had confirmed CAP. The use of molecular typing methods for *M. pneumoniae* in combination with the determination of macrolide resistance was intended to obtain a nationwide overview of circulating strains in a period of high incidence of infections. Parallel testing for *Chlamydia* spp. was included to explore the frequency of co-infections with 2 microorganisms that are difficult to propagate and that can cause disease patterns that may be clinically indistinguishable.

Methods

Patient Population, Samples, and Data Collection

The CAPNETZ study is a multicenter, prospective, epidemiologic cohort study initiated by the German Competence Network for Community-Acquired Pneumonia (http://www.capnetz.de [*24*]). The network comprises clinical centers throughout Germany representing hospitals and outpatient departments at all levels of health care provision that are involved in the management of CAP. The decision on timing and type of treatment for each patient is left to the discretion of the attending physician. No attempt is made to implement standardized criteria or rules for the assessment of pneumonia severity or for the decision to hospitalize.

For this study, we prospectively recorded all consecutive and nonselected patients who sought treatment for signs and symptoms of CAP during March 2011–December 2012. Eligible participants were adult patients (≥18 years of age) who had CAP confirmed by a new pulmonary infiltrate on chest radiograph and ≥1 sign or symptom of lower respiratory tract infection (i.e., fever, cough, purulent sputum, focal chest signs). Exclusion criteria were the following: hospital admission within 28 days before sampling, presence of immunosuppression (defined as chemotherapy and/or neutropenia <1,000 10^6/L during the previous 28 days), therapy with corticosteroids >20 mg for >14 days, known HIV infection, immunosuppressive therapy after organ or bone marrow transplant, or active tuberculosis. All patients gave written informed consent and received a pseudonym from an independent third party to ensure data security. The study is registered at the German Clinical Trial Register (DRKS-ID: DRKS00005274).

All patients provided pharyngeal swab specimens for the determination of the presence of *M. pneumoniae* and *Chlamydia* spp. Follow-up consultations by phone call to patient or next of kin or family physician were conducted 28 days and 180 days after enrollment. All demographic, clinical, and diagnostic data for patients were recorded using standardized Web-based data sheets created by 2mt (Ulm, Germany). The study was approved by the Institutional Review Board of the Otto-von-Guericke University (Magdeburg, Germany) under ID 104/01 in 2001 and subsequently by all local institutional review boards.

Sample Processing and Microbiological Investigations

DNA Extraction

Swab specimens were shipped overnight in transport medium to the Friedrich-Loeffler-Institut (Jena, Germany) for testing. DNA extraction was performed by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Testing for *Chlamydia* spp.

To test for *Chlamydia* spp., we first conducted a real-time PCR specific for the family *Chlamydiaceae* (*25*). Positive samples were further examined by using a *C. psittaci*–specific real-time PCR (*26*) and a DNA microarray assay in ArrayStrip format that covered all *Chlamydia* spp., *Waddlia chondrophila*, and *Simkania negevensis* (*27*,*28*).

Testing for *M. pneumoniae*

Aliquots of the DNA extracts were examined by using a previously described real-time PCR assay targeting copies of the repetitive element RepMP1 (*29*). Positive samples were further tested for macrolide resistance by methods

previously reported (*15*). P1 and MLVA type were determined by nested PCR approaches and sequencing (*30*,*31*).

Statistical Analysis

Categorical data are presented as frequencies and were compared by χ^2 or Fisher exact test, as appropriate. The Yates correction procedure was applied to all comparisons.

Continuous variables are presented as median and range. Differences were analyzed by using the Mann-Whitney U test; p values <0.05 were considered significant. All analyses were carried out in SPSS version 20 software (IBM/SPSS, Chicago, IL, USA).

Results

Overall, 783 CAP patients were enrolled during March 2011–December 2012 and were tested for *M. pneumoniae*; 96 (12.3%) were positive. Patients who had *M. pneumoniae* infection were significantly younger and more frequently female, had fewer co-occurring conditions, and experienced significantly milder disease that did those who did not have *M. pneumoniae* infection (Table 1). *M. pneumoniae*–positive patients were more frequently pretreated with antimicrobial drugs; however, we found no significant differences in the classes of antimicrobial drugs administered, particularly not in the use of macrolides.

Using real-time PCR targeting the RepMP1 copies in the *M. pneumoniae* genome, we calculated a median of 7.8 × 10^3 copies (range 4.1 × 10^1 to 1.5 × 10^6) in the *M. pneumoniae*–positive samples. *M. pneumoniae* positivity showed a clear season-dependent trend; low positivity of ≈4% was found at the beginning and the end of the investigation period, but high positivity was found during October 2011–December 2011. Quarterly incidence ranged from 1.5% (quarter 3, 2012) to 27.3% (quarter 4, 2011) (Figure 1).

The percentage of *M. pneumoniae*–positive patients from each age group ranged from 28.1% for the 18- to 29-year age group to 13.5% for the ≥60-year age group (Figure 2). The prevalence of *M. pneumoniae* decreased by age group: 18–29 years, 38%; 30–39 years, 31%; 40–49 years, 17%; 50–59 years, 13%; ≥60 years, 3%. More than half (55%) of *M. pneumoniae*–positive patients were female; only the 18- to 29-year age group had more *M. pneumoniae*–positive men than women.

Regarding the P1 genotype, all strains in the 96 *M. pneumoniae*–positive samples could be typed culture independently. Subtype 1 strains dominated (60.4%; Figure 1, panel B), followed by variant 2a (19.8%), variant 2b (9.4%), variant 2c (8.3), and subtype 2 strains (2.1%). A high proportion of subtype 1 strains were found during the entire 22-month investigation period, and all P1 types detected during the period were found at nearly the same proportion during the high-incidence period of October–December 2011.

Highly discriminatory MLVA was carried out on all positive samples using nested PCR. For 87 of the 96 samples, the complete recommended panel of 5 tandem-repeat regions could be amplified and sequenced successfully. Overall, 23 MLVA types were identified. The most common MLVA types were 4/4/5/7/2 (n = 12, 13.8%) and 5/4/5/7/2 (n = 11, 12.6%). During the high-incidence period (quarter

Table 1. Demographic and clinical characteristics of patients with community-acquired pneumonia whose respiratory tract samples were tested for *Mycoplasma pneumoniae*, Germany, 2011–2012*

Characteristic	All, n = 783	*M. pneumoniae*–positive, n = 96	*M. pneumoniae*–negative, n = 687	p value†
Median age, y (range)	61 (18–102)	39.5 (18–84)	64 (18–102)	<0.001
Male sex	56.8	43.8	58.7	0.008
BMI (range)	25.6 (13.9–56.5)	24.2 (18.8–44.5)	25.8 (13.9–56.5)	
Co-occurring conditions				
Chronic pulmonary disease	19.2	6.2	23.1	<0.001
Chronic renal disease	19.2	0	20.6	0.004
Chronic heart failure	26.1	11.3	27.2	
Diabetes mellitus	24.9	11.1	25.9	
Cerebro-vascular disease	9.5	3.7	9.3	
CURB scores‡				<0.001
0	57.3	77.0	54.7	
1	31.0	21.8	32.2	
2	10.5	1.1	11.8	
3	1.2	0	1.4	
4	0	0	0	
Antimicrobial drug pretreatment	29.3	51.0	26.2	<0.001
Macrolides	8.0	7.3	8.3	§
Mortality rate				
28 d	1.8	0	2.0	
180 d	4.6	0	5.2	

*Values are percentages except as indicated. BMI, body mass index.
†Only significant values are shown. Comparison between *M. pneumoniae*-positive and -negative patients by χ^2 or Mann-Whitney U test as appropriate.
‡Clinical evaluation of the following risk factors (each scores 1 point): Confusion; blood Urea >7 mmol/L; Respiratory rate ≥30 bpm; Blood pressure <90 (systolic) or ≤60 mm Hg (diastolic).
§Also no significant differences for any other antimicrobial drug classes.

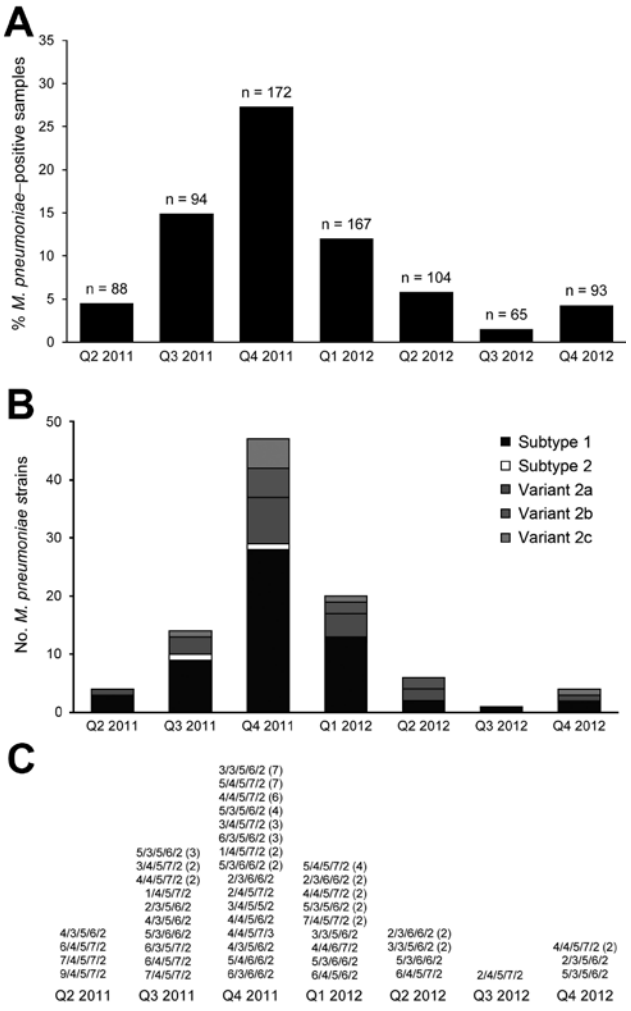

Figure 1. Results of molecular detection of *Mycoplasma pneumoniae* from 783 respiratory tract specimens from adult patients with pneumonia, Germany, March 2011–December 2012. A) Quarterly incidence of *M. pneumoniae* infection. n values indicate number of samples investigated by real-time PCR. B) *M. pneumoniae* P1 genotypes. C) *M. pneumoniae* multilocus variable-number tandem-repeat analysis types. Numbers of strains belonging to a given type are indicated in parentheses.

4 of 2011), the 42 *M. pneumoniae* strains that showed valid MLVA results belonged to 16 MLVA types. No clear correlation was found between P1 and MLVA typing. The 51 classifiable subtype 1 strains can be assigned to 16 MLVA types (Table 2). Five of these MLVA types can also be found in subtype 2 and the related variant 2 strains.

On the basis of sequencing data, macrolide resistance can be assumed in 3 of the 96 *M. pneumoniae*–positive specimens (3.1%). All strains showed an A→G mutation at position 2063 of the 23S rRNA. The macrolide-resistant strains belonged to the predominant subtype 1 in P1 typing but differed in MLVA type (2/3/6/6/2, 2/4/5/7/2, and 5/4/5/7/2). The specimens containing resistant strains were sampled at different points of the study: December 2011, April 2012, and August 2012. All affected patients were female (ages 31, 42, and 42 years). For 2 of these patients, treatment with macrolides during the month before sampling was reported.

We further tested 794 patients for *Chlamydia* spp. (Table 3); 31 (3.9%) patients tested positive, 6 with dual infections (Table 4). In contrast to the *M. pneumoniae* findings, we found no significant differences in clinical characteristics between *Chlamydia*-positive and -negative patients (Table 3). Notably, no *Chlamydia*-positive patients received macrolides, whereas 8.3% of *Chlamydia*-negative patients did.

Test results identified *C. psittaci* (2.1%) as the most prevalent chlamydial species, followed by *C. pneumoniae* (1.4%). In addition, *C. trachomatis*, *Simkania negevensis*, and the animal pathogens *C. suis*, *C. abortus* and *Waddlia chondrophila* were identified in individual samples. The use of a DNA microarray assay combined with real-time PCR assays enabled us to detect multiple chlamydial infections; all 6 dual chlamydial infections (representing 19.3% of *Chlamydia*-positive patients) involved *C. psittaci*, 3 in conjunction with *C. pneumoniae* and 1 each with *C. abortus*, *S. negevensis*, and *W. chondrophila*. Co-infections with *M. pneumoniae* and *Chlamydia* spp. were detected in 3 samples (3.1% of *M. pneumonia*–positive and 8.1% of *Chlamydia*-positive specimens): 1 *M. pneumoniae* + *C. psittaci*, 1 *M. pneumoniae* + *C. pneumoniae*, and 1 *M. pneumoniae* + *C. psittaci* + *C. pneumoniae.*

Discussion

Many studies have described the strictly time-dependent epidemiology of infections caused by *M. pneumoniae* (*3*). Although we did not include serologic testing to provide further information for the differentiation of colonization of patient from infection, the results of our study confirm a strong epidemic peak of *M. pneumoniae*–positive

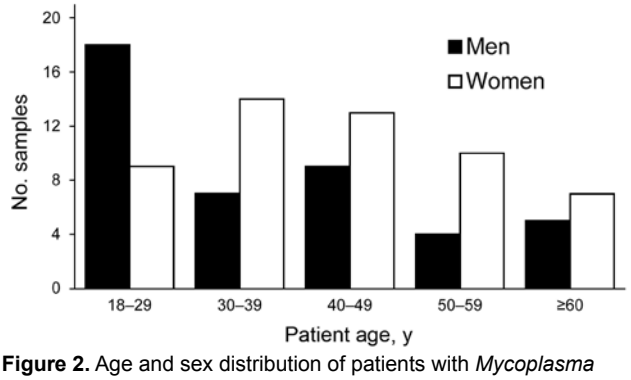

Figure 2. Age and sex distribution of patients with *Mycoplasma pneumoniae*–positive respiratory tract samples (n = 96), Germany, March 2011–December 2012. Percentage of positive samples for each age group: 18–29 y, 28.1%; 30–39 y, 21.9%; 40–49 y, 22.9%; 50–59 y, 14.6%; ≥60 y, 13.5%. (Total >100% due to rounding.)

Table 2. Comparison of the results of P1 and MLVA typing of 87 *Mycoplasma pneumoniae* strains from patients with community-acquired pneumonia, Germany, 2011–2012*

P1 type	MLVA types (no. strains)
Subtype 1, n = 51	1/4/5/7/2 (3), 2/3/6/6/2, 2/4/5/7/2 (2), 3/3/5/6/2 (2), 3/4/5/5/2 (2), 3/4/5/7/2 (5), 4/4/5/6/2 (2), 4/4/5/7/2 (11), 4/4/6/7/2, 5/3/5/6/2, 5/3/6/6/2, 5/4/5/7/2 (11), 6/3/5/7/2, 6/4/5/7/2 (3), 7/4/5/7/2 (3), 9/4/5/7/2
Subtype 2, n = 2	5/3/6/6/2 (2)
Variant 2a, n = 19	2/3/5/6/2 (2), 3/3/5/6/2 (6), 4/3/5/6/2 (2), 5/3/5/6/2 (5), 6/3/5/6/2 (2), 6/4/5/6/2, 7/4/5/7/2
Variant 2b, n = 8	2/3/6/6/2 (4), 5/3/6/6/2 (2), 5/4/6/6/2, 6/3/5/6/2
Variant 2c, n = 7	3/3/5/6/2 (2), 4/3/5/6/2, 5/3/5/6/2 (3), 6/3/5/6/2

*Underlining indicates MLVA types that occur in both subtype 1 and subtype 2/variant 2 strains. MLVA, multilocus variable-number tandem-repeat analysis.

respiratory samples among adult patients with suspected CAP in Germany during 2011–2012. The incidence of infections temporarily rose to ≈27% during the fourth quarter of 2011, a level that is in accordance with other reports (*5*–*12*). Despite limited comparability (e.g., target used), the number of RepMP1 copies measured with real-time PCR (median 7.8×10^3) is in the range of results of other studies (*32*,*33*). Because of the short duration of the epidemic peak and known deficiencies in testing routines for symptomatic *M. pneumoniae* patients, an increase in incidence could easily escape the notice of public health authorities. Moreover, because patients with *M. pneumoniae* infection were significantly younger than those without infection, it is possible that the true incidence might even be higher, given the fact that younger persons visit physicians less frequently and are admitted to hospitals less often than older patients. In addition, β-lactams are often used as the first-line antimicrobial drugs for CAP but are known to be inefficient in treatment of *Mycoplasma* infections; this conflict might represent another reason for the spread of this pathogen. In light of these results, revision of recent guidelines for management of CAP with antimicrobial drugs should be considered.

Typing of strains can help clarify the dynamics of epidemic peaks. There is no evidence that the incidence peak we registered was related to the genotype of circulating strains. *M. pneumoniae* is a genetically conserved organism, which implies limits to potential typing targets; most frequently used is P1, the main adhesin and most immunogenic protein, where sequence variation occurs mainly in the 2 copies of repetitive elements RepMP2/3 and RepMP4 of the P1-encoding locus *mpn141*. The epidemiologic importance of P1 genotypes is based on their ability to generate a specific host immune response (*13*). Therefore, P1 genotyping of circulating mycoplasma strains is helpful for understanding host-pathogen interactions and the infections ensuing. The current dominance of subtype 1 strains in combination with a rare occurrence of subtype 2 was also described in Europe and Asia (*11*,*34*). Subtype 2 strains have been replaced with the phylogenetically related variant 2 strains, which occurred in our study in different types. The variant 2c of this group was described in 2011 in several isolates from China (*34*); our detection of variant 2c strains in Germany confirms the parallel circulation of most variant 2 strains described this far.

Table 3. Demographic and clinical characteristics of patients with community-acquired pneumonia whose respiratory tract samples were tested for *Chlamydia* spp., Germany, 2011–2012*

Characteristic	All, n = 794	*Chlamydia*-positive, n = 31	*Chlamydia*-negative, n = 763	p value†
Median age, y (range)	61 (18–102)	64 (18–89)	61 (18–102)	
Male sex	56.8	54.8	56.9	
BMI (range)	25.6 (13.9–56.5)	24.3 (17.7–45.3)	25.7 (13.9–56.5)	
Co-occurring				
Chronic pulmonary disease	21.2	32.3	20.7	
Chronic renal disease	19.2	0	19.9	p = 0.05
Chronic heart failure	25.7	12.5	26.2	
Diabetes mellitus	24.8	25.0	24.8	
Cerebro-vascular disease	9.3	12.5	9.2	
CURB scores‡				
0	56.8	54.8	56.9	
1	31.5	41.9	31.0	
2	10.5	3.2	10.8	
3	1.2	0	1.2	
4	0	0	0	
Antimicrobial drug pretreatment	29.1	25.8	29.3	
Macrolides	7.9	0	8.3	§
Mortality rate				
28 d	1.8	6.5	1.6	
180 d	4.7	6.5	4.6	

*Values are percentages except as indicated. BMI, body mass index.
†Only significant values are shown. Comparison between *Chlamydia*-positive and -negative patients by χ^2 or Mann-Whitney U test as appropriate.
‡Clinical evaluation of the following risk factors (each scores 1 point): Confusion; blood Urea >7 mmol/L; Respiratory rate ≥30 bpm; Blood pressure <90 (systolic) or ≤60 mm Hg (diastolic).
§Also no significant differences for other antibiotic classes.

In this study, only particular regions of the P1 gene were selected for analysis, and the occurrence of additional P1 types showing further sequence variations cannot be ruled out. The recently characterized variant 2d (*35*) shares an identical 3′ part of the RepMP2/3 with variant 2a but differs in the 5′ region of the repetitive element; on the basis of those results, we retested all variant 2a strains, but we could not confirm the occurrence of variant 2d.

For epidemiologic reasons, it is important that the period with a high proportion of *M. pneumoniae*–positive samples (October–December 2011) was not associated with a change of the dominating genotype nor with the presence of a particular P1 type. Previous reports have hypothesized that the circulation of genotype-specific antibodies in the human population can influence the number of infections and the dominating P1 type (*13*), but our data do not support this hypothesis.

In recent years, MLVA was introduced for typing of *M. pneumoniae* isolates (*14*) and extended to culture-independent typing from clinical samples (*31*). The determination of the number of 5 VNTR markers (Mpn1, 13–16) enables characterization of strains with a higher discriminatory power in comparison with P1 typing. Whereas the genomic regions used for MLVA are located mainly between genes and within genes of unknown function, the P1 protein plays a critical role in host–pathogen interaction. The most common MLVA types (4/4/5/7/2 and 5/4/5/7/2) we detected were also found in high abundance in strains recently identified in France, China, and the United States (*11*,*16*,*36*), thus indicating a worldwide dissemination of particular MLVA types.

Regarding the strains included in our study, the assignment of P1 types to MLVA types confirmed that no clear relationship between the typing methods exists (Table 2). In agreement with the results of other reports (*11*,*14*,*16*), a low number of P1 subtype 1 strains belong to MLVA types that are typical for subtype 2 or variant strains (e.g., 5/3/5/6/2) and vice versa. In our opinion, both typing approaches are of practical importance and complement each other; the circulating P1 types reflect a more host-dependent pattern, whereas MLVA can differentiate strains with higher discriminatory power, enabling a better understanding of epidemiologic relationships.

Instability of VNTR marker 1 has been reported (*36*), and the removal of Mpn1 from the current MLVA scheme has been suggested (*16*). For our data, the removal of Mpn1 would reduce the number of MLVA genotypes to 9, with >80% of strains belonging to 2 types, 3/5/6/2 and 4/5/7/2. This change would result in a substantial decrease of the discriminatory power of the MLVA typing method and would require efforts to include further VNTR markers showing a stable number of repeats within a given strain.

Table 4. Results of testing for *Chlamydia* spp. in 780 respiratory samples from patients with community-acquired pneumonia, Germany, 2011–2012

Species	No. (%) positive
C. psittaci	17 (2.2)
C. pneumoniae	11 (1.4)
C. trachomatis	3 (0.4)
C. suis	2 (0.3)
C. abortus	1 (0.1)
Other *Chlamydia* sp.	1 (0.1)
Simkania negevensis	1 (0.1)
Waddlia chondrophila	1 (0.1)
Total	37* (4.7)

*A total of 31 patients tested positive; 6 were infected with >1 *Chlamydia* species.

The data from our study confirm that a nationwide peak of infections caused by *M. pneumoniae* is polyclonal, which is in agreement with results of other reports (*11*). In contrast, the clonal spread of *M. pneumoniae* can only be expected for small-scale endemic outbreaks with person-to-person transmission in close communities, such as families (*37*).

Since 2000, an increase of the worldwide occurrence of macrolide resistance in *M. pneumoniae* strains to 90% and higher has reported. Compared with data from Asia (*16*), the proportion of 3.1% of macrolide-resistant *M. pneumoniae* strains we detected is low. Results of studies from Germany in recent years have showed results in the same range (*15*), thus indicating a nearly constant prevalence of resistance. Nevertheless, resistant strains are circulating in the population we investigated, which requires further monitoring of strains to provide an updated overview of drug resistance. Several reports have confirmed that resistant strains were selected during antimicrobial drug treatment (*38*). However, in our study, increased prescription of macrolide antimicrobial drugs, which can be expected during a period of high CAP incidence, did not result in a measurable increase of resistant strains. In addition, because most current resistance data originate from pediatric patients, the results of this study are of general interest because we report on adult patients.

Although further sequence differences of the 23S rRNA locus of *M. pneumoniae* have been described, the A→G mutation at position 2063 is most common (*15*,*16*). Comparison of sequencing results with antimicrobial susceptibility testing confirmed that the mutation at positions 2063/2064 led to a high level of macrolide resistance (*15*). Thus, with the mutation detected, resistance of these strains can be assumed. We did not find macrolide resistance to be associated with a certain MLVA type, which confirms the findings of other reports (*16*), but the low number of resistant strains in our study is insufficient for us to draw a final conclusion.

The results of *Chlamydia* testing are remarkable for the comprehensive methodologic approach. In the past, the choice of diagnostic tests was usually limited: that is, either

only *C. pneumoniae* was analyzed or all *Chlamydia* spp. identified were thought to be *C. pneumoniae*. Although the positivity of 1.4% for *C. pneumoniae* we found is in line with previous data from Germany (*22*), the proportions of positive samples of 2.1% for *C. psittaci* and 4.7% for all *Chlamydiae* spp. considered here deserve specific attention (Table 4). Chlamydial co-infection involving ≥2 *Chlamydia* spp. has been shown to be frequent in trachoma patients (*39*), but data from pneumonia patients remain scarce. Our finding that 19.3% of *Chlamydia*-positive patients harbored ≥2 *Chlamydia* spp. is in the same range as the 24% found in a recent trachoma study (*40*).

One unexpected finding in our study was that *C. psittaci* positivity could not be correlated with the patients having regular contact with birds at home or at work. Moreover, no seasonal patterns were identified. Nevertheless, our findings indicate that chlamydial species other than *C. pneumoniae* should be included in testing of CAP patients; in particular, *C. psittaci* should be included but also *C. trachomatis*. The relatively high prevalence of *C. psittaci* we found raises questions about its epidemiologic and etiologic importance, which should be addressed in future studies.

In summary, we confirmed a strong epidemic peak of *M. pneumoniae*–positive respiratory samples among adult patients with confirmed CAP in Germany during 2011–2012. During the fourth quarter of 2011, the incidence of infections temporarily rose to ≈27% of all patients investigated. *Chlamydia* spp. were found in 3.9% of samples, without epidemic peaks. Throughout the study period, the dominant *M. pneumoniae* P1 type was subtype 1; only 3.1% of *M. pneumoniae* strains were macrolide resistant. Both bacteria represent relevant pathogens in CAP, and awareness of their epidemiology, particularly among clinicians, is clearly warranted.

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Epidemiology of Human *Mycobacterium bovis* Disease, California, USA, 2003–2011

Mark Gallivan, Neha Shah, Jennifer Flood

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Learning Objectives

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

- Describe the epidemiology of *Mycobacterium bovis* infection in California, based on a retrospective review
- Identify risk factors for *Mycobacterium bovis* infection in California
- Distinguish the public health implications of the findings of this retrospective review regarding control of *Mycobacterium bovis* infection.

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We conducted a retrospective review of California tuberculosis (TB) registry and genotyping data to evaluate trends, analyze epidemiologic differences between adult and child case-patients with *Mycobacterium bovis* disease, and identify risk factors for *M. bovis* disease. The percentage of TB cases attributable to *M. bovis* increased from 3.4% (80/2,384) in 2003 to 5.4% (98/1,808) in 2011 (p = 0.002). All (6/6) child case-patients with *M. bovis* disease during 2010–2011 had ≥1 parent/guardian who was born in Mexico, compared with 38% (22/58) of child case-patients with *M. tuberculosis* disease (p = 0.005). Multivariate analysis of TB case-patients showed Hispanic ethnicity, extrapulmonary disease, diabetes, and immunosuppressive conditions, excluding HIV co-infection, were independently associated with *M. bovis* disease. Prevention efforts should focus on Hispanic binational families and adults with immunosuppressive conditions. Collection of additional risk factors in the national TB surveillance system and expansion of whole-genome sequencing should be considered.

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Mycobacterium bovis, part of the *Mycobacterium tuberculosis* complex, is a zoonotic pathogen that can cause tuberculosis (TB) disease in a broad range of mammalian hosts (*1*). TB disease caused by *M. bovis* is clinically, radiographically, and pathologically indistinguishable from TB caused by *M. tuberculosis* (*2*). *M. bovis* transmission to humans most frequently occurs through consumption of unpasteurized, contaminated dairy products, but person-to-person transmission has been reported (*3*,*4*). The consumption of contaminated unpasteurized dairy products has been suggested as a major contributor to human *M. bovis* disease for several reasons: 1) the near absence of *M. bovis* disease among infants <12 months of age; 2) a high percentage of extrapulmonary disease, particularly abdominal disease, among patients with *M. bovis* disease; and 3) an association between positive interferon-γ release assay results and consumption of unpasteurized dairy products (*5*–*11*).

In the United States, 1%–2% of all human TB cases are attributable to *M. bovis* infection (*7*), but in certain geographic regions and communities, human *M. bovis* infection accounts for a much higher percentage of the cases. During 2001–2005, *M. bovis* accounted for nearly 10% of culture-positive TB isolates in San Diego, California, USA, including 54% of those from children (<15 years of age) and 8% of those from adults (≥15 years of age) (*8*). Nearly all (97%) case-patients with *M. bovis* disease were among the Hispanic population, and 60% of those case-patients were born in Mexico (*8*). During 2001–2004, an investigation in New York, New York, USA, showed a high prevalence of TB caused by *M. bovis* among the Hispanic community. New York investigators reported that 57% of *M. bovis* case-patients were born in Mexico, and 83% of the interviewed case-patients consumed unpasteurized cheeses produced in Mexico while living in the United States (*12*).

The internationally recognized genotypic method for identifying *M. bovis* is spacer oligonucleotide typing (*13*). In 2004, the CDC (Centers for Disease Control and Prevention) Tuberculosis Genotyping Program (now called the National Tuberculosis Genotyping Service, http://www.cdc.gov/tb/publications/factsheets/statistics/genotyping.htm) began spoligotyping *M. tuberculosis* complex isolates from US patients with culture-positive TB (*14*). In California, the percentage of culture-positive isolates spoligotyped each year has gradually increased from 35% in 2004 to 92% in 2011. The incompleteness and variability (by geographic location) of spoligotype testing over this period exclude trend analysis and population-based *M. bovis* studies using this genotypic method. However, pyrazinamide monoresistance can serve as a proxy measure for *M. bovis* because *M. bovis* is intrinsically resistant to pyrazinamide but pyrazinamide monoresistance is rare among *M. tuberculosis* isolates (*7*,*15*,*16*). A recent national study on pyrazinamide resistance showed that 0.7% (196/27,428) *M. tuberculosis* isolates were pyrazinamide monoresistant (*15*). Since 2003, ≈97% of all culture-positive TB isolates in California have had initial (i.e., pretreatment) drug susceptibility testing for pyrazinamide, isoniazid, and rifampin.

M. bovis disease is of particular concern because of the high percentage of cases among children and because of its association with zoonotic and foodborne transmission, HIV co-infection, and poor treatment outcomes compared with *M. tuberculosis* disease (*6*,*17*–*19*). Further investigation into *M. bovis* disease is needed to understand the epidemiology of cases among children and adults, its association with immunosuppressive conditions, and the association of those conditions with treatment outcomes. We conducted a retrospective review of California TB surveillance data to evaluate trends for TB cases attributable to *M. bovis*, evaluate epidemiologic differences between *M. bovis* TB cases in adults and children, and identify risk factors associated with *M. bovis* disease compared with *M. tuberculosis* disease. We also conducted an evaluation of the accuracy of pyrazinamide monoresistance as a proxy measure for *M. bovis* disease by using surveillance and genotyping data.

Methods

The study population included all patients with culture-confirmed TB reported to the California TB registry during 2003–2011 and who had initial drug susceptibility testing results for isoniazid, rifampin, and pyrazinamide. Patients were classified as having *M. bovis* disease if the initial drug susceptibility results indicated resistance to pyrazinamide and susceptibility to isoniazid and rifampin. Sociodemographic, clinical, and treatment outcome information for all case-patients was abstracted from the TB registry. TB case registry data were matched to the California HIV/AIDS registry to identify HIV co-infection status. This analysis was conducted as part of the California Department of Public Health's mandate to routinely collect and analyze surveillance data for public health purposes. The CDC (Atlanta, GA, USA) determined that the project was not human subject research and did not require approval by an institutional review board.

To compare the differences between child and adult populations, we stratified *M. bovis* case-patients by their age at the time TB was reported. The child population consisted of patients <15 years of age, and the adult population consisted of patients ≥15 years of age. To evaluate characteristics associated with TB disease caused by *M. bovis* compared with TB disease caused by *M. tuberculosis*, we conducted bivariate analysis with variables that were added to the national TB surveillance system

in 2010. These variables include birth country of parents/guardians (for child case-patients), primary reason evaluated for TB disease, diabetes mellitus, and other immunosuppressive conditions. Immunosuppressive conditions, excluding HIV co-infection and diabetes mellitus, comprised end-stage renal disease, anti–tumor necrosis factor-α therapy–associated immunosuppression, solid organ transplant–associated immunosuppression, or other immunosuppressive condition as indicated by medical records or a health care provider. In addition, patient variables previously shown to be associated with TB disease caused by *M. bovis* (i.e., country of birth, race/ethnicity, age, HIV co-infection, site of disease, and death before treatment completion) were included in the bivariate analysis (*7*,*19*). Sociodemographic and clinical variables shown to be significantly associated with *M. bovis* disease at the bivariate level were put into a logistic regression model. The final logistic regression model was constructed by using the backward stepwise elimination procedure, removing predictors with $p>0.05$.

For TB cases during 2004–2011, the sensitivity and positive predictive value of the pyrazinamide monoresistance case definition were calculated by using spoligotyping data as the reference standard. The signature spoligotypes differentiating *M. bovis* and *M. tuberculosis* have been described previously in detail (*20*). Spoligotyping was conducted at the California Microbial Diseases Laboratory (Richmond, CA, USA), and the resulting data were entered into the TB Genotyping Information Management System (http://www.cdc.gov/tb/programs/genotyping/tbgims/default.htm). The TB registry and TB Genotyping Information Management System databases were merged by patients' unique case numbers after spoligotyping data were deduplicated. Spoligotyping results indicating a genotype other than *M. bovis* or *M. tuberculosis* (including *M. bovis* bacillus Calmette-Guérin (BCG) strains) and isolates missing spoligotyping data or initial drug susceptibility testing results for pyrazinamide, isoniazid, or rifampin were excluded from analysis.

Analyses were conducted by using SAS version 9.3 (SAS Institute, Cary, NC, USA). Separate Poisson regression models were used to identify temporal trends in the incidence of TB caused by *M. tuberculosis* and *M. bovis*. Year of TB case report was used as the explanatory variable, TB case number as the dependent variable, and state population size as the offset variable. Population denominators were obtained from the US Census Bureau's current population survey (*21*). Trends of the annual percentage of TB cases attributable to *M.* bovis were examined by using the Cochran-Armitage trend test. Epidemiologic differences between *M. bovis* and *M. tuberculosis* were compared by using the χ^2 test or Fisher exact test. Differences in median time to completion of therapy from start of therapy were analyzed by using the Wilcoxon rank-sum test. Trends and differences in disease characteristics were considered statistically significant if $p<0.05$ (2-sided).

Results

Burden and Trends

During 2003–2011, a total of 24,424 verified TB cases were reported in California. Of these, 5,061 (21.0%) culture-negative cases were excluded (Figure 1). Approximately 3.0% (611/19,363) of culture-positive cases were excluded because results of initial drug susceptibility testing for isoniazid, rifampin, or pyrazinamide were absent. *M. bovis* was identified by pyrazinamide monoresistance in 4.0% (742/18,752) of all eligible TB cases. *M. bovis* accounted for 22.0% (82/379) of culture-positive TB cases in children, 3.9% (519/13,397) of cases in adults 15–64 years of age, and 2.8% (141/4,976) of cases in adults ≥65 years of age. A total of 163 children <12 months of age had TB disease, and among those with culture-positive cases (81 children), none had *M. bovis* disease. The highest cumulative incidence of human *M. bovis* disease occurred in lower southern California (Figure 2).

During 2003–2011, TB incidence attributable to *M. tuberculosis* declined significantly ($p<0.0001$), but TB incidence attributable to *M. bovis* did not change ($p = 0.92$). The annual percentage of TB cases attributable to *M. bovis* among all age groups increased from 3.4% (80/2,384) in 2003 to 5.4% (98/1,808) in 2011 ($p = 0.002$; Figure 3). The annual percentage of TB cases attributable to *M. bovis* among the child population did not change significantly ($p = 0.15$), but the percentage among adults increased from 3.0% to 5.5% ($p<0.001$).

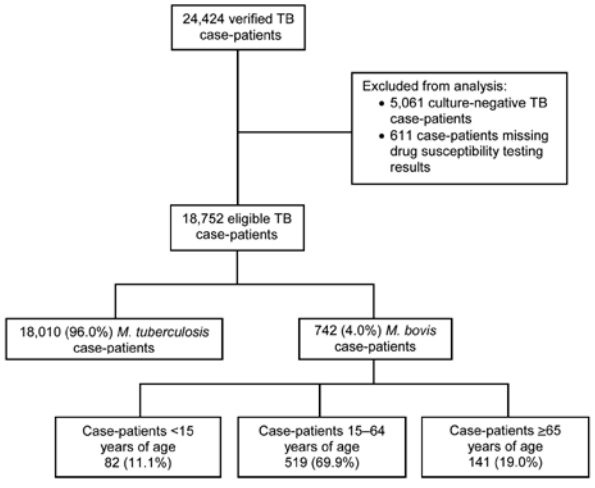

Figure 1. Number of verified tuberculosis case-patients eligible for inclusion in an epidemiologic study of human *Mycobacterium bovis* disease, California, USA, 2003–2011.

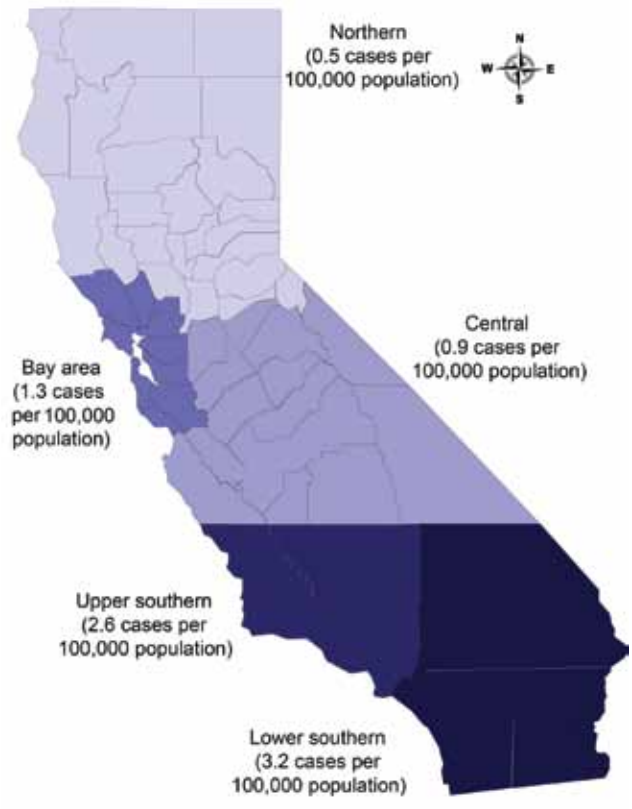

Figure 2. Cumulative incidence of human *Mycobacterium bovis* disease, by region, California, USA, 2003–2011.

Epidemiology of *M. bovis* Disease in Children versus Adults

Several differences were observed between child and adult case-patients with *M. bovis* disease (Table 1). Child case-patients were more likely to be Hispanic and US-born and to have extrapulmonary disease. All *M. bovis* case-patients with concurrent HIV co-infection were adults. During 2003–2011, a total of 11.4% (80/699) of all case-patients with *M. bovis* disease died before treatment was completed. Among children, 1.2% (1/82) died before treatment was completed, compared with 12.8% (79/617) of adults (p = 0.002). Among case-patients who completed treatment, the median time to completion did not differ significantly between children and adults.

M. bovis Epidemiology versus *M. tuberculosis* Epidemiology

Bivariate analysis showed that the following patient characteristics were associated with TB caused by *M. bovis* but not by TB caused by *M. tuberculosis*: Hispanic ethnicity, birth in Mexico, incidental laboratory result as primary reason for TB evaluation, HIV co-infection, presence of extrapulmonary TB disease, and diabetes and other immunosuppressive conditions. Results were similar when we repeated the bivariate analysis using the spoligotyping method to identify *M. bovis* disease. A higher percentage of *M. bovis*–than *M. tuberculosis*–infected patients had diabetes; however, this association was no longer significant (p = 0.08) because the study population was smaller. The association between *M. bovis* disease and other immunosuppressive conditions remained significant (p = 0.002).

During 2010–2011, case-patients with *M. bovis* disease were more likely than those with *M. tuberculosis* disease to die before treatment completion (15.8% vs. 8.6%, p = 0.006) (Table 2). Among case-patients who died before treatment completion, those with *M. bovis* disease were more likely than those with *M. tuberculosis* disease to have had ≥1 concurrent immunosuppressive condition (73% vs. 53%, p = 0.05). Of the 26 *M. bovis* case-patients who died before treatment completion, 19 (73%) had ≥1 concurrent immunosuppressive condition: diabetes and end-stage renal disease (n = 6), diabetes (n = 5), HIV co-infection (n = 1), end-stage renal disease (n = 1), post–organ transplantation–associated immunosuppression (n = 1), end-stage renal disease and post-organ transplantation–associated immunosuppression (n = 1), or any other immunosuppressive condition (n = 4).

Subanalysis of child case-patients during 2010–2011 showed that 100% (6/6) of children with *M. bovis* disease had ≥1 parent/guardian who had been born in Mexico, compared with 38% (22/58) of children with *M. tuberculosis* disease (p = 0.005). In addition, 66% (4/6) of the children with *M. bovis* disease were born in the United States and 33% (2/6) were born in Mexico, compared with 93.1% (54/58) and 1.7% (1/58), respectively, of the children with *M. tuberculosis* disease (p = 0.02). Moreover, 66% (4/6) of the children with *M. bovis* disease had lived in Mexico for >2 months, compared with 3% (2/58) of the children with *M. tuberculosis* disease (p<0.001).

Multivariate analysis showed that Hispanic ethnicity (compared with non-Hispanic white), extrapulmonary disease, and diabetes and other immunosuppressive

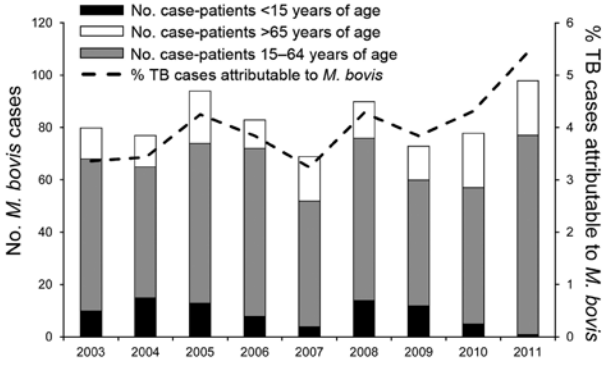

Figure 3. Annual number of case-patients with *Mycobacterium bovis* disease and percentage of tuberculosis cases attributable to *M. bovis*, California, USA, 2003–2011.

Table 1. Sociodemographic and clinical characteristics of and treatment outcomes for case-patients with tuberculosis attributable to *Mycobacterium bovis*, California, USA, 2003–2011*

Characteristic or outcome	No. (%) patients, by age group		p value	No. patients/no. total (%), N = 742†
	<15 y, n = 82	≥15 y, n = 660		
Sex			0.08	
F	47 (57.3)	311 (47.1)		
M	35 (42.7)	349 (52.9)		384 (52)
Race/ethnicity			0.0007	
Hispanic	81 (98.8)	518/659 (78.6)		599/741 (80.7)
Asian, non-Hispanic	1 (1.2)	99/659 (15.0)		100/741 (13.5)
Black, non-Hispanic	0	8/659 (1.2)		8/741 (1.1)
White, non-Hispanic	0	29/659 (4.4)		29/741 (3.9)
Other	0	5/659 (0.8)		5/741 (0.7)
Country of birth			<0.0001	
United States	70 (85.4)	112 (17.0)		182 (24.5)
Mexico	11 (13.4)	426 (64.5)		437 (58.9)
Other†	1 (1.2)	122 (18.5)		123 (16.6)
Disease site			<0.0001	
Extrapulmonary only	71 (86.6)	248 (37.6)		319 (43.0)
Pulmonary only	3 (1.1)	266 (40.3)		269 (36.3)
Extrapulmonary and pulmonary	8 (5.2)	146 (22.1)		154 (20.7)
Positive acid-fast bacilli sputum smear‡	2/7 (28.6)	223/390 (57.2)	0.25	225/397 (56.7)
Presence of cavitation on chest radiographs‡	1/8 (12.5)	89/383 (23.2)	0.69	90/391 (23.0)
Reported HIV co-infection	0 (0.0)	83 (12.6)	<0.0001	83 (11.2)
Health care provider type			0.002	
Health department	16 (19.5)	252/644 (39.2)		268/726 (37.0)
Private/other	43 (52.4)	271/644 (42.0)		314/726 (43.2)
Health department and private/other	23 (28.1)	121/644 (18.8)		144/726 (19.8)
Dead at diagnosis	0 (0.0)	20 (3.5)	0.15	20 (3.1)
Treatment outcome			0.002	
Patient died before completion	1/82 (1.2)	79/617 (12.8)		80/699 (11.4)
Completed treatment	79/82 (96.4)	511/617 (82.8)		590/699 (84.4)
Patient lost during treatment	1/82 (1.2)	12/617 (2.0)		13/699 (1.9)
Refused treatment	1/82 (1.2)	2/617 (0.3)		3/699 (0.4)
Other	0/82 (0.0)	13/617 (2.1)		13/699 (1.9)
Median no. months to treatment completion (IQR)§	10.1 (9.1, 12.4)	9.4 (8.9, 12.0)	0.16	9.5 (8.9, 12.0)

*Data are no. (%) case-patients, except as noted. n values are indicated for categories with missing or incomplete data. AFB, acid-fast bacilli; IQR, interquartile range.
†Other countries (no. cases): Vietnam (33), Philippines (18), El Salvador (13), Cambodia (11), China (9), India (7), Indonesia (4), Guatemala (3), Peru (3), North Korea (3), Fiji (2), South Korea (2), Thailand (2), Cameroon (1), Chile (1), Egypt (1), Ethiopia (1), Honduras (1), Iran (1), Japan (1), Laos (1), Malaysia (1), Nicaragua (1), Taiwan (1), Tanzania (1), and Ukraine (1).
‡Among tested patients with any pulmonary disease involvement.
§Among patients who completed therapy.

conditions were independently associated with *M. bovis* disease compared with *M. tuberculosis* disease (Table 3). Birth in a country other than the United States, excluding Mexico, was negatively associated with *M. bovis* disease compared with *M. tuberculosis* disease. Birth in Mexico was not independently associated with *M. bovis* disease (adjusted odds ratio 1.1, 95% CI 0.7–1.8).

Evaluation of the Pyrazinamide Monoresistance *M. bovis* Definition

Among all isolates with spoligotype and drug resistance data available during 2004–2011, the pyrazinamide monoresistance case definition had a sensitivity of 92% (95% CI 90%–95%) and positive predictive value of 82% (95% CI 79%–86%) for *M. bovis*. Among the Hispanic population, the sensitivity and positive predictive value of the pyrazinamide monoresistance definition was 94% (95% CI 92%–97%) and 96% (95% CI 94%–98%), respectively. When the analysis was restricted to the non-Hispanic Asian population, the sensitivity and positive predictive value dropped to 45% (95% CI 16%–75%) and 8% (95% CI 1%–14%), respectively. In the non-Hispanic Asian population, 92% (61/66) of pyrazinamide-monoresistant isolates were genotyped as *M. tuberculosis*.

Discussion

In this large population-based epidemiologic study of *M. bovis* disease in California, we observed an increase in the annual percentage of TB cases attributable to *M. bovis* from 2003 through 2011. *M. bovis* disease accounted for nearly 25% of culture-positive TB cases in children. Patients with *M. bovis* disease were more likely than those with *M. tuberculosis* disease to die during treatment, and most deaths were among adults with concurrent immunosuppressive conditions. Hispanic ethnicity (compared with non-Hispanic white), extrapulmonary disease, and diabetes and other concurrent immunosuppressive conditions (excluding HIV co-infection) were independently

Table 2. Sociodemographic and clinical characteristics of and treatment outcomes for case-patients with tuberculosis attributable to *Mycobacterium bovis* and *M. tuberculosis*, California, USA, 2003–2011

Patient group, characteristic or outcome	No. (%) cases*		p value
	M. bovis, n = 176*	*M. tuberculosis*, n = 3,445	
Child case-patients†			
Country of birth			0.02
United States	4/6 (66.7)	54/58 (93.1)	
Mexico	2/6 (33.3)	1/58 (1.7)	
Other‡	0/6	3/58 (5.2)	
Any parent/guardian born in Mexico	6/6 (100.0)	22/58 (38.0)	0.005
Lived in Mexico for >2 mo	4/6 (66.7)	2/58 (3.4)	<0.001
All patients			
Age, years			0.24
0 to <15	6 (3.4)	58 (1.7)	
15–24	16 (9.1)	304 (8.8)	
25–44	46 (26.1)	990 (28.7)	
45–64	66 (37.5)	1,112 (32.3)	
≥65	42 (23.9)	981 (28.5)	
Race/ethnicity			<0.001
White, non-Hispanic	3 (1.7)	277 (8.0)	
Black, non-Hispanic	3 (1.7)	204 (5.9)	
Asian, non-Hispanic	42 (23.9)	1,743 (50.6)	
Hispanic	127 (72.2)	1,187 (34.5)	
Other	1 (0.6)	34 (1.0)	
Country of birth			<0.001
United States	33/175 (18.9)	643/3,438 (18.7)	
Mexico	94/175 (53.7)	740/3,438 (21.5)	
Other country‡	48/175 (27.4)	2,057/3,438 (59.8)	
Main site of disease			<0.001
Pulmonary	110 (62.5)	2,938 (85.2)	
Extrapulmonary only	66 (37.5)	507 (14.8)	
Presence of cavitation on chest radiographs§	23/99 (23.2)	666/2,781 (24.0	0.87
Positive acid-fast bacilli sputum smear§	57/101 (56.4)	1,678/2,785 (60.3)	0.44
Reported HIV co-infection	13 (7.4)	140 (4.1)	0.03
Presence of diabetes mellitus	59 (33.5)	826 (24.0)	0.004
Presence of other immunosuppressive condition(s)¶	35 (19.9)	352 (10.2)	<0.001
Primary reason evaluated for tuberculosis			<0.001
Presence of tuberculosis symptoms	116 (65.9)	2,243/3,435 (65.3)	
Abnormal results on chest radiograph	26 (14.8)	608/3,435 (17.7)	
Contact investigation	0	86/3,435 (2.5)	
Targeted testing	2 (1.1)	60/3,435 (1.7)	
Immigration medical examination	3 (1.7)	113/3,435 (3.3)	
Incidental laboratory result	29 (16.5)	285/3,435 (8.3)	
Other	0	40/3,435 (1.2)	
Treatment outcome			0.006
Patient died before completion	26/165 (15.8)	285/3,299 (8.6)	
Completed treatment	126/165 (76.3)	2,786/3,299 (84.4)	
Other	13/165 (7.9)	228/3,299 (6.9)	

*n values are indicated for categories with missing or incomplete data.
†Case-patients <15 y of age.
‡Other countries (no. cases): Ethiopia (1), Malaysia (1), Vietnam (1).
§Of case-patients with any pulmonary involvement.
¶Includes end-stage renal disease, immunosuppression associated with receiving anti–tumor necrosis factor-α therapy, immunosuppression associated with receiving solid-organ transplant, or other immunosuppressive conditions, but excludes diabetes and HIV co-infection.

associated with *M. bovis* disease compared with *M. tuberculosis* disease.

During 2010–2011, all (6/6) child case-patients with *M. bovis* disease had ≥1 parent/guardian who had been born in Mexico, and 4 of the 6 children had lived in Mexico for >2 months. These results, in conjunction with the a high percentage of extrapulmonary disease among the case-patients and the lack of *M. bovis* disease among children <12 months of age, are consistent with findings in previous epidemiologic studies that suggested that human *M. bovis* disease in the United States often results from consumption of unpasteurized dairy products originating from foreign countries, including Mexico (*6*,*12*,*22*,*23*). National pasteurization requirements, strict regulations on the importation of dairy cattle from Mexico into the United States, and an effective US bovine TB eradication program have substantially reduced *M. bovis* infection in US-born cattle (*22*,*24*). In the past century, the prevalence of *M. bovis* disease in US dairy herds has decreased from ≈5.0% to <0.001% (*25*). Furthermore, *M. bovis* isolates from humans in San

Table 3. Multivariate model of sociodemographic and clinical characteristics associated with *Mycobacterium bovis* disease versus *M. tuberculosis* disease, California, USA, 2010–2011

Risk factors	Adjusted odds ratio (95% CI)
Diabetes status	
Absent	Reference
Present	1.5 (1.1–2.1)
Other immunosuppressive condition(s)*	
Absent	Reference
Present	1.8 (1.2–2.8)
Race/ethnicity	
White, non-Hispanic	Reference
Black, non-Hispanic	1.2 (0.2–6.2)
Asian, non-Hispanic	3.6 (1.0–12.8)
Hispanic	7.3 (2.2–24.3)
Other	2.3 (0.2–23.3)
Country of birth	
United States	Reference
Mexico	1.1 (0.7–1.8)
Other	0.4 (0.2–0.7)
Main site of disease	
Pulmonary†	Reference
Extrapulmonary only	3.9 (2.8–5.5)

*Excludes diabetes mellitus and HIV co-infection but includes end-stage renal disease, immunosuppression associated with receiving anti–tumor necrosis factor-α therapy, immunosuppression associated with receiving solid-organ transplant, or other immunosuppressive condition.
†Includes case-patients with pulmonary and extrapulmonary disease.

Diego have been found to be genetically related to *M. bovis* strains from cattle in Mexico (*22*). Expansion of *M. bovis* disease surveillance and genotyping to include whole-genome sequencing may add discriminatory power beyond traditional genotyping and help to identify the source and route of transmission of *M. bovis* (*26*). Sharing of whole-genome sequencing data between countries and different health agencies may enhance future national and international prevention interventions.

Despite the relatively high percentage of extrapulmonary disease, 57% of *M. bovis* case-patients in our study had pulmonary involvement; this percentage is consistent with findings in other studies in the United States (*7*,*8*). Person-to-person transmission of *M. bovis* is considered infrequent, but the magnitude of such transmission has not been precisely quantified (*27*). Our findings are consistent with those from past research, which has shown that case-patients with *M. bovis* and *M. tuberculosis* pulmonary disease do not differ significantly in 2 of the key indicators of infectivity: presence of lung cavitation on chest radiographs and presence of acid-fast bacilli in sputum smears (*6*,*7*,*28*). In addition, previous research from pulmonary TB contact investigations showed that TB infection conversion rates among contacts did not differ significantly by mycobacterial species of the source case, suggesting that *M. bovis* is equally as transmissible as *M. tuberculosis* (*28*). However, current TB contact investigation guidelines do not include risk factors for *M. bovis* transmission. Although it is recommended to prioritize immunocompromised contacts of *M. tuberculosis* patients for evaluation, our data suggest that it may be even more important to prioritize immunocompromised contacts of *M. bovis* patients during contact investigations.

In our evaluation of the definition of pyrazinamide monoresistance, we found that isolates genotyped as *M. tuberculosis* may have been misclassified as *M. bovis* because of the pyrazinamide monoresistance definition. Misclassifications occurred most notably among the non-Hispanic Asian patients and might be explained by host, environmental (e.g., regional programmatic differences in TB treatment), and bacterial characteristics. From a recent national multivariate analysis of pyrazinamide resistance, Kurbatova et al. (*15*) suggested that bacterial lineage, not host characteristics, was the primary association between pyrazinamide monoresistance and *M. tuberculosis* disease.

Despite the possible definition-associated overestimation of *M. bovis* disease, the results from our study may still underestimate the true burden of *M. bovis* disease in California. Cases in children may be underestimated in the study population because sputum or gastric aspirate specimens are not consistently obtained from young children (*29*). In addition, *M. bovis* disease may be present in case-patients in the culture-negative subpopulation. We found case-patients with culture-negative TB to be similar to case-patients with culture-confirmed *M. bovis* disease with respect to age, extrapulmonary disease, and Hispanic ethnicity (data not shown).

Given the limitations of traditional genotyping and surveillance, we could not assess whether the increase in the number and percentage of adult *M. bovis* case-patients was to the result of recently acquired TB or reactivation of a previous infection. In addition, analysis of the new variables that were added to the national TB surveillance system in 2010 was hindered by the small number of child case-patients with *M. bovis* disease during 2010–2011. Also, because we did not conduct a medical chart review, we could not determine the cause of death among TB case-patients who died before the completion of TB treatment. Because of limitations in the national TB surveillance report form, we were unable to assess several possible relevant risk factors, including consumption of unpasteurized dairy products and the potential protective effect of the BCG vaccine against *M. bovis* disease. Although there is little information in the literature on the efficacy of the BCG vaccine in protecting against human *M. bovis* disease, the vaccine is notably protective against extrapulmonary disease and childhood TB disease, both of which are characteristic of *M. bovis* disease (*30*–*33*).

In summary, human *M. bovis* disease incidence has not declined in California, and the percentage of TB cases attributable to *M. bovis* has increased, exceeding the overall average for the United States. In California, there are ongoing interventions designed to limit the demand for and distribution of unpasteurized and contaminated dairy

products, which are associated with *M. bovis* disease and other foodborne diseases (*23*). Elimination of human *M. bovis* disease in California likely requires further implementation of programs to reduce *M. bovis* contamination of dairy products in countries that have bovine TB, including pasteurization and test and cull interventions. In the interim, actions in the United States can help facilitate this effort. For example, *M. bovis* genotyping surveillance could be expanded and key risk elements (e.g., consumption of unpasteurized dairy products) could be captured in the national TB surveillance system to help determine *M. bovis* transmission routes. In addition, current TB contact investigation guidelines should be changed to include risk factors for *M. bovis* transmission. Last, future education efforts to prevent acquisition of *M. bovis* should focus on Hispanic binational families and adults with concurrent immunosuppressive conditions, including diabetes.

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etymologia

Mycobacterium bovis [*mī″ko-bak-tēr-eəm bō′-vis*]

From the Latin bos ("ox" or "cow") *Mycobacterium bovis* is a virulent bacterial species originally isolated from tubercules in cattle. Robert Koch, who discovered the tubercle bacillus in 1882, believed that *M. bovis* was not a danger to humans. Theobald Smith and others established beyond doubt that, contrary to Koch's belief, *M. bovis* could infect humans but was not the usual source of human infection. In 1908, French scientists Albert Calmette and Camille Guérin chose an *M. bovis* strain for their work on a tuberculosis vaccine. They repeatedly subcultured the isolate on a mixture of glycerol, potato, and bile for 13 years until it was sufficiently attenuated to be used as a vaccine. The bacillus Calmette-Guérin (BCG) vaccine was adopted by the League of Nations as the standard tuberculosis vaccine in 1928 and continues to be used in most developing countries.

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Regional Spread of Ebola Virus, West Africa, 2014

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To explain the spread of the 2014 Ebola epidemic in West Africa, and thus help with response planning, we analyzed publicly available data. We found that the risk for infection in an area can be predicted by case counts, population data, and distances between affected and nonaffected areas.

The first cases of the 2014 Ebola epidemic in West Africa (49 cases in Guinea) were reported on March 21 (*1*). By November 4, the World Health Organization had reported 13,241 cases in the 3 primarily stricken countries of Guinea, Sierra Leone, and Liberia and single cases in Senegal and Mali (*2*). Although virus transmission to other countries (Nigeria, United States, and Spain) has occurred via air travel, most infections have spread regionally via ground movement of sick persons. To aid with response planning, we sought to explain this regional spread by assessing publicly available information.

The Study

The data analyzed were case counts, population data, and distances between affected and nonaffected districts (these distances are influential predictors in the spread of infectious diseases) (*3*–*5*). We first classified as affected those districts within Guinea (prefectures), Sierra Leone (districts), and Liberia (counties) that had reported to the World Health Organization ≥1 suspected, probable, or confirmed case of Ebola virus infection from the weeks ending March 29, 2014 (epidemiological week 13), through August 16, 2014 (epidemiological week 33) (*2*). For each district, we considered the week of its first reported case as the week it became affected (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/3/14-1845-Techapp1.pdf). We also identified the population-weighted geographic centroid (center of an area, adjusted for its population density) in each district and computed the distance from these centers to similar centers in each affected district.

We then created 4 regression models to calculate the weekly risk of a district being affected as a function of combinations of its population, the sum of inverse distances (SID) from all affected districts, and SID weighted by the number of new cases in affected districts over the preceding 3 weeks (online Technical Appendix Table 2). We chose the best model by examining how well it fit the data available through week 33 (August 16). We then evaluated how well the chosen model predicted that districts would become affected as the outbreak continued by comparing calculated probabilities that a district would become affected (at weeks 33, 36, and 39) to actual reports of newly affected districts over the subsequent 3-week periods (weeks 34–36 [period 1], weeks 37–39 [period 2], and weeks 40–42 [period 3], respectively). By using data available through week 42, we calculated probabilities that districts in countries bordering the 3 primarily affected countries (departments in Côte D'Ivoire, circles in Mali, departments in Senegal, sectors in Guinea-Bissau, and divisions in Gambia) would become affected.

We assumed that country and district borders were porous and that infected persons could not be prevented from moving into nonaffected areas (*6*–*8*). Reports from the field support this assumption, even after country borders were officially closed (*9*). We also assumed no heterogeneities in the capabilities of the different areas to identify and report cases and that aggregating case count reports into a weekly unit of analysis would blunt the effects of reporting delays. Our last assumption, for identifying an affected district, was that suspected and probable cases were as sensitive and specific as confirmed cases.

Among the 3 primarily affected countries, 39 districts were affected in 12 weeks (during weeks 13–33). The model that best explained this pattern was one in which the risk of a district becoming affected depended on its population and the SID from all affected districts to a nonaffected district and in which each inverse distance is multiplied by the sum of new cases within the past 3 weeks (weighted SID) (online Technical Appendix Table 2 and Figure 2). The overall average weighted SID was greater for districts during the weeks in which they became affected than for districts that had not yet reported cases by the same week (online Technical Appendix Figure 3, panel A).

Figure 1 shows the probabilities for specific districts becoming affected at weeks 33, 36, and 39. The ranking of districts by their probabilities on week 33 (Figure 1, panel A) illustrates the good fit of the model because 27 (87%) of the 31 districts ranked in the top half (most likely to become affected) were actually affected.

During weeks 34–36 (period 1), 4 districts became affected; during weeks 37–39 (period 2), 4 districts became affected; and during weeks 40–42 (period 3), 5 districts became affected. The model predicted well which districts would become affected during periods 1 and 3 (Figure 1,

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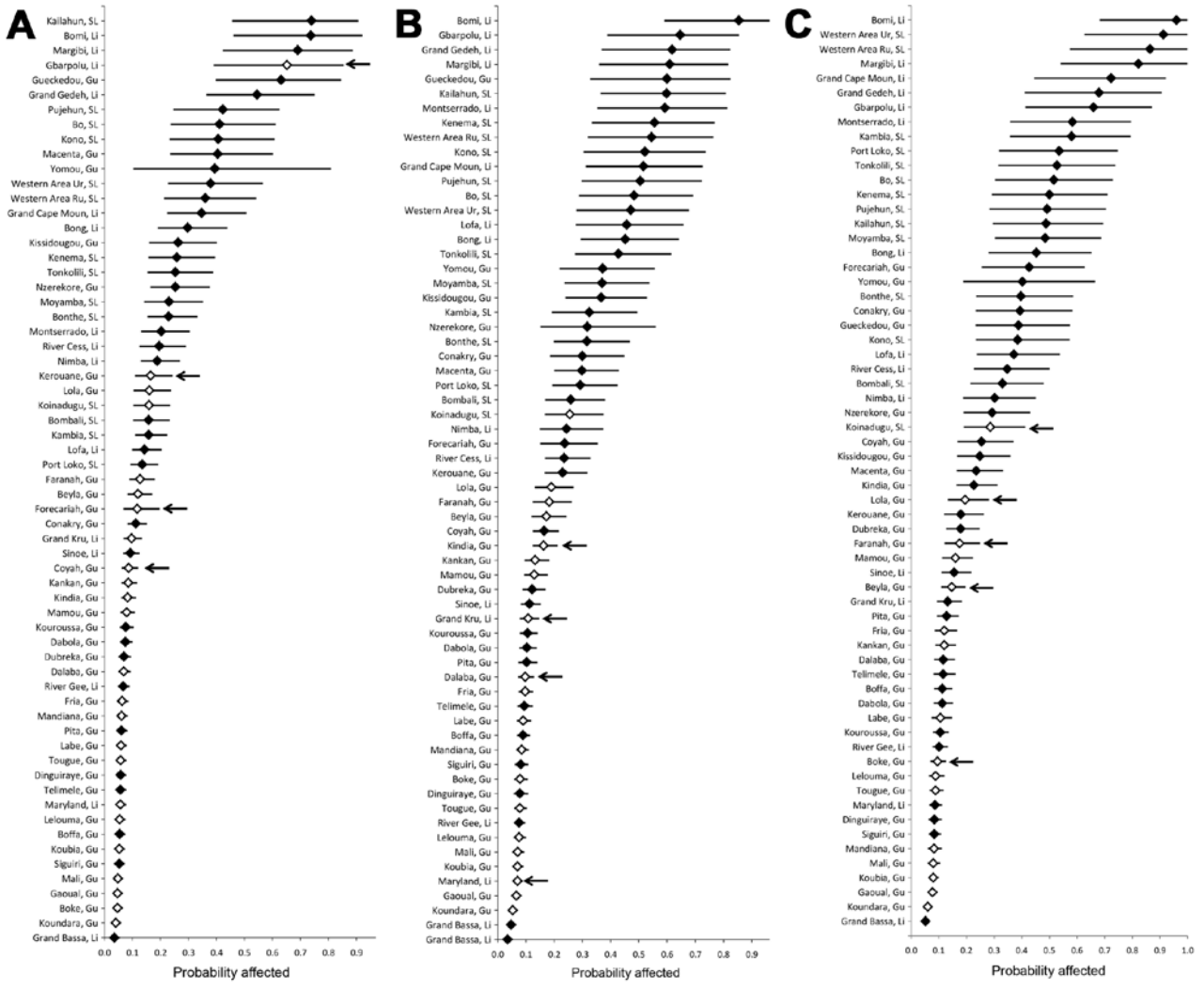

Figure 1. Probability predictions (with 95% CIs) for districts in countries primarily affected by Ebola virus infection in 2014, by week of analysis. A) Data available through week 33 (August 16). B) Data available through week 36 (September 6). C) Data available through week 39 (September 27). Diamonds indicate the probability that the districts should be affected at the time of the analysis. Filled diamonds indicate districts that were affected (i.e., had reported at least 1 case) at the date of the analysis. Black arrows identify those districts that became affected within 3 weeks of the date of analysis. SL, Sierra Leone; Gu, Guinea; Li, Liberia.

panels A, C); districts that became affected were predominantly among those with the highest calculated probabilities of becoming affected. The model did not predict as well which districts would become affected during period 2 (Figure 1, panel B).

Of 167 districts in the countries bordering the primarily affected countries, the predicted probability of becoming affected was >20% for 9 districts (calculated at week 42). The 3 top-ranked districts had the largest populations in their respective countries: Abidjan (Côte D'Ivoire), Bamako (Mali), and Pikine (Senegal); Bamako and Pikine reported cases in weeks 43 and 35, respectively. Also, among the top 10 districts, 5 were on or near the Côte D'Ivoire–Liberia border (Figure 2).

Conclusions

We identified spatial influences on the regional spread of Ebola virus infections. The risk of becoming affected by Ebola was significantly higher for nonaffected districts that had a larger population and that were closer to affected districts with higher case counts (online Technical Appendix Table 2 and Figure 2). Thus, it seems that data on population size and straight-line distances can serve as pragmatic alternatives to data on travel patterns between Guinea, Liberia, and Sierra Leone during the first 8 months of the outbreak. The correlation between the risk of becoming affected and distances and population size was sufficiently accurate for predicting which districts would next become affected. Furthermore, a high calculated probability of becoming affected

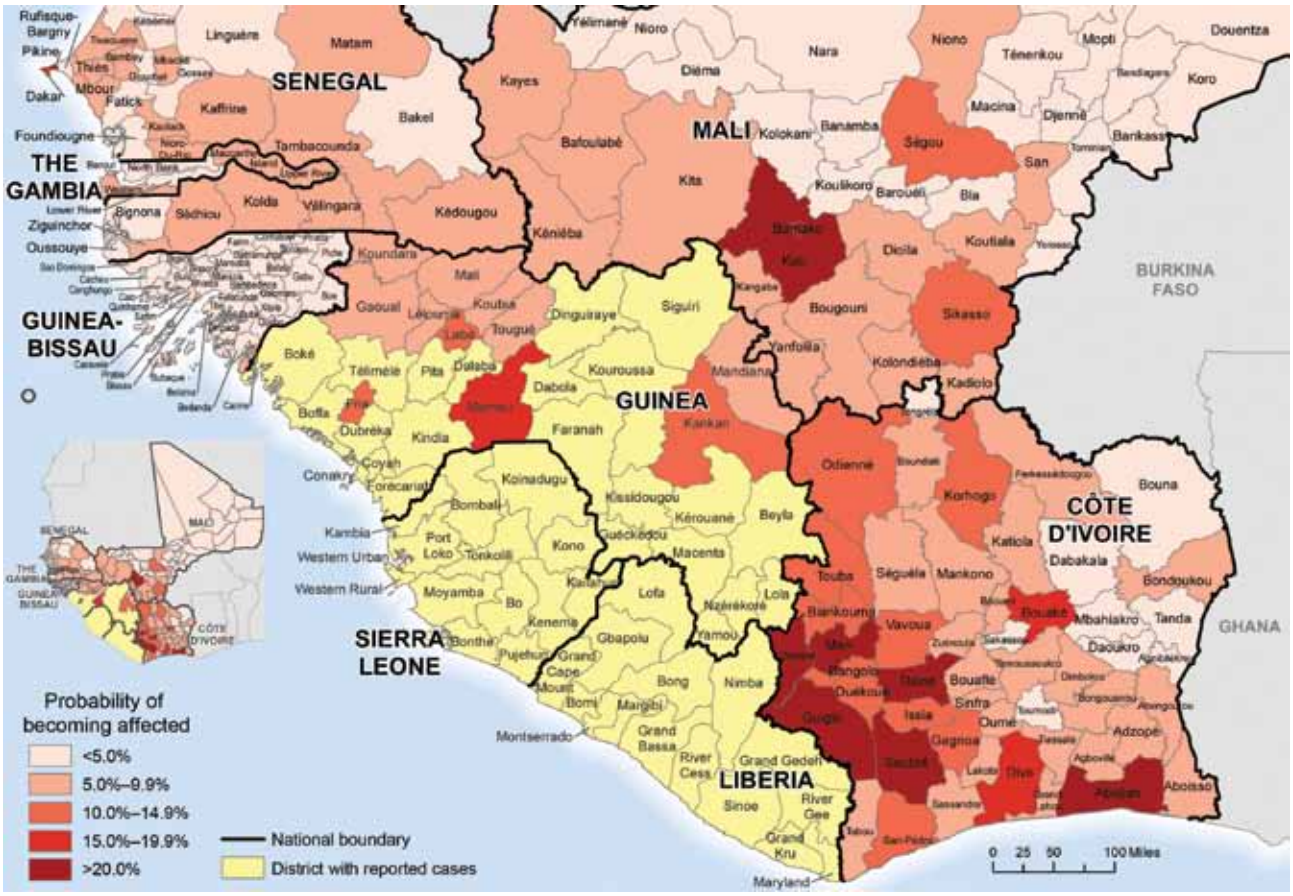

Figure 2. Predicted risk of districts becoming affected by Ebola virus infection (neighboring countries included) in 2014, based on data available through epidemiological week 42 (October 18, 2014).

for a district considered not affected might indicate the presence of undetected cases.

This analysis relied heavily on the accuracy of case reports and their timely documentation, but there are indications that extreme conditions in the affected countries resulted in incomplete records and reporting delays (*10*). These factors potentially contributed to errors in the identification of which week a district became affected. Consequently, we examined the potential effects of reporting delays (online Technical Appendix Table 2). Also, our results might have been influenced by our choice of administrative unit level to use for defining districts. (In our analysis, countries with smaller district units have less risk of being affected than countries with larger district units, if population densities are generally comparable.)

The good fit of our model, absent predictors for the influence of interventions, suggests that interventions (including border closings) were minimally effective at stemming regional spread of Ebola virus infection during the period analyzed. As the spread of the epidemic changes because of interventions and changes in human behavior, there is need to update and reevaluate the model fit and the parameters used.

We chose to not pursue data on travel patterns, despite their potential utility for explaining the spread of Ebola virus infection. Travel patterns may evolve as the outbreak progresses, and obtaining accurate data during an ongoing outbreak is challenging. We, therefore, focused on producing the simplest model.

Overall, our simple model shows that available case reports, population data, and distance data can be used to identify areas at risk of being affected in an outbreak of Ebola virus infection. Additionally, if the current pattern of spread in this outbreak continues, or if the outbreak takes hold in new countries, this model can be used to advocate for allocation of surveillance and control resources to nonaffected areas.

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Spillover of *Mycobacterium bovis* from Wildlife to Livestock, South Africa

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During August 2012–February 2013, bovine tuberculosis was detected in communal livestock bordering the Greater Kruger National Park Complex (GKNPC) in South Africa. Using spacer oligonucleotide and variable number tandem repeat typing, we identified the *Mycobacterium bovis* strain endemic in GKNPC wildlife. Our findings indicate bovine tuberculosis spillover from GKNPC wildlife to neighboring livestock.

Bovine tuberculosis is an infectious disease caused by *Mycobacterium bovis*. The wide host range of the pathogen comprises humans and domestic and wild animals. Great strides in controlling bovine tuberculosis have drastically reduced its prevalence in livestock and humans, particularly in industrialized countries. However, in developing countries in southern Africa and elsewhere, bovine tuberculosis remains a challenge to animal health because of a total or partial lack of bovine tuberculosis control, limited by a lack of funds (*1*,*2*). The control and/or elimination of bovine tuberculosis in both developing and industrialized countries can be complicated by wildlife reservoirs of the disease, which pose a threat of re-infection in livestock (*3*). In sub-Saharan Africa, particularly South Africa and Uganda, African buffalos (*Syncerus caffer*) serve as wildlife reservoirs of bovine tuberculosis; in Zambia, lechwe antelopes (*Kobus leche Kafuensis*) have been identified as wildlife reservoirs (*4*,*5*). New reports have suggested greater kudu (*Tragelaphus strepsiceros*) and common warthog (*Phacochoerus africanus*) as potential wildlife reservoirs of bovine tuberculosis (*4*).

M. bovis is endemic in buffaloes and has spilled into other wildlife species, particularly in the Kruger National Park (KNP) and adjacent game reserves that form part of the Greater Kruger National Park Complex (GKNPC) in South Africa (*6*,*7*). Except for data from sporadic regulatory bovine tuberculosis surveillance activities in cattle adjacent to the GKNPC, no data exist on the transmission of bovine tuberculosis from the GKNPC, where it is endemic, into livestock in neighboring communities (*3*). Because of the potentially negative implications of livestock–wildlife interactions on livestock and human health, the presence and role of zoonotic diseases in these communities needs to be investigated (*5*). We report on an investigation into the status and genotype of bovine tuberculosis in livestock in rural communities bordering the bovine tuberculosis–endemic GKNPC.

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The Study

The study was conducted in a rural community under the Mnisi Tribal Authority. The community is situated in Mpumalanga Province, South Africa, and borders the GKNPC in the west and 1 private game reserve (Figure 1). We constructed maps for this study using ArcGIS version 10.2 (http://www.arcgis.com). The KNP and private game reserves are fenced and have buffer zones established by double fencing (*8*).

Cattle farming, an essential part of the livelihood of the Mnisi community, is practiced primarily on a communal basis. Approximately 12,000 cattle live within the Mnisi area with 15 dip tanks, where the national government provides veterinary extension services and which represent epidemiologic units for sampling (Figure 1). Farmers in the study area are assigned and registered to a particular dip tank by the agricultural authorities by a stock card system. On a stock card, number of cattle, births/deaths, and animal movement are recorded. For this study, a dip tank is considered a whole herd because animals in a particular dip tank interacted extensive during grazing and dip tank inspections, and usually 1 herdsman was in charge of multiple stock cards.

During August 2012–February 2013, a total of 1,166 cattle at the 15 dip tanks in the study area were tested for bovine tuberculosis by using the comparative intradermal tuberculin test (CIDT). Animals selected for testing were chosen randomly from a list of stock cards at each dip tank. From each stock card chosen, we selected 10% of registered animals; however, a minimum of 2–3 animals per stock card were tested. All stock card owners willing to participate were included to reach a target of 10% of cattle assigned to each dip tank. We calculated the bovine tuberculosis status and 95% CI per dip tank assuming a binomial distribution of the data (Table 1).

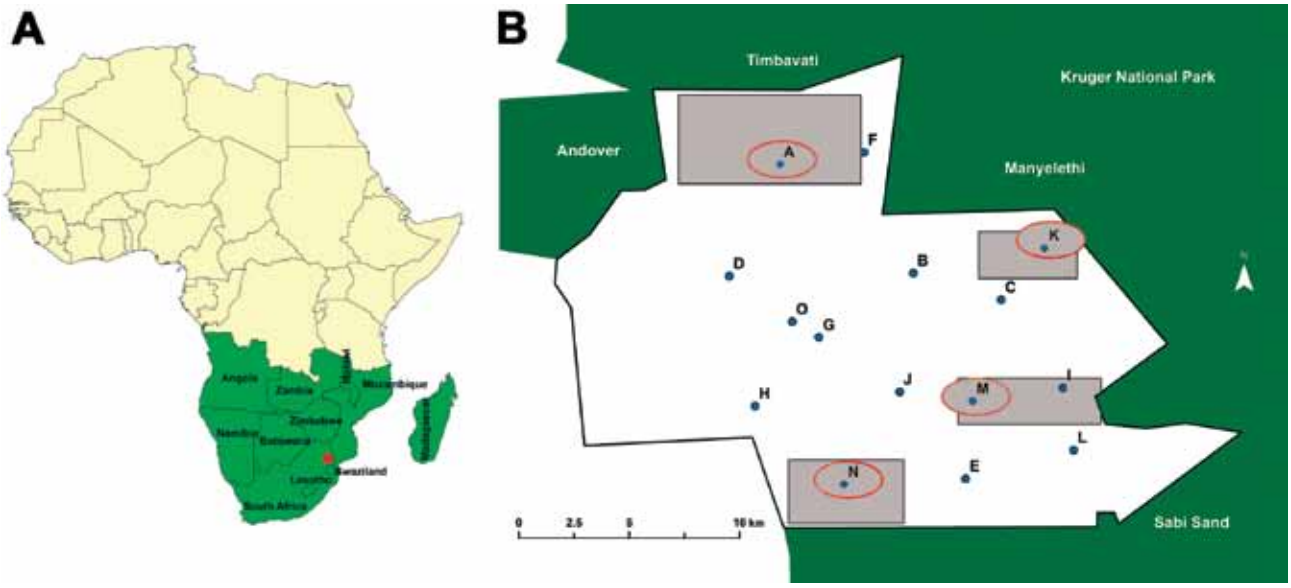

Figure 1. Location of study area (A, red dot) and location of dip tanks (B) in study of bovine tuberculosis transmission, Greater Kruger National Park Complex, South Africa, August 2012–February 2013. Parentheses used below indicate the shortest distance between individual dip tanks and the game fence, as follows: dip tank A (3.1 km), B (3 km), C (4.2 km), D (7.3 km), E (2.3 km), F (1 km), G (6.1 km), H (5.8 km), I (0.5 km), J (6 km), K (1.2 km), L (1 km), M (4.3 km), N (2 km), O (6.4 km). Blue dots indicate dip tanks sampled; red circles indicate dip tanks at which bovine tuberculosis–positive cattle were detected. Gray boxes indicate observed cattle grazing range for dip tanks at which bovine tuberculosis was detected.

A whole-blood interferon-γ (IFN-γ) assay was performed as an ancillary test to the CIDT on all 4 CIDT-positive cattle and all 5 cattle with inconclusive reactions (defined as a difference between the bovine and avian increase in skin-fold thickness of ≥3 mm) (Table 2). Among the 9 cattle, 4 animals were classified as bovine tuberculosis reactors on the basis of the IFN-γ assay response (*9*), 1 each in 4 of the 15 dip tanks (Figure 1).

Animals classified as bovine tuberculosis reactors were purchased and slaughtered. These animals included a 1-month-old calf born to a CIDT- and IFN-γ assay–positive cow (animal no. K1). Standard sets of tissue samples were collected and cultured as previously described (*10*). Pathologic examination and culture results are shown in Table 2.

M. bovis was isolated from the 5 animals, and the isolates were characterized by using spacer oligonucleotide typing (spoligotyping) (*11*). Spoligotypes were named according to the *M. bovis* spoligotype database (http://www.mbovis.org). Variable number tandem repeat (VNTR) typing of the isolates was performed as previously described (*12*). Spoligotyping showed a single *M. bovis* spoligotype,

Table 1. Status of bovine tuberculosis detected by using comparative intradermal tuberculin testing at 15 dip tanks, Greater Kruger National Park Complex, South Africa, August 2012–February 2013

			Test results	
Dip tank	No. cattle	No. cattle tested (%)	Inconclusive, no. animals (%; 95% CI)	Positive, no. animals (%; 95% CI)
A	1,648	178 (10.8)	6 (3.4; 1.5–7.3)	1 (0.6; 0.1–3.9)
B	556	55 (9.9)	3 (5.5; 1.8–15.6)	0 (0; 0–5.3)
C	963	104 (10.8)	2 (1.9; 0.5–7.4)	0 (0;0–2.8)
D	706	72 (10.2)	3 (4.2;1.4–12.1)	0 (0;0–4.1)
E	585	82 (14.0)	1 (1.2; 0.2–8.1)	0 (0; 0–3.6)
F	786	75 (9.5)	0 (0; 0–3.9)	0 (0; 0–3.9)
G	1,092	86 (7.9)	3 (3.5; 1.1–10.3)	0 (0;0–3.4)
H	709	70 (9.9)	3 (4.3; 1.4–12.5)	0 (0; 0.0–4.2)
I	850	75 (8.8)	1 (1.3; 0.2–8.9)	0 (0; 0–3.9)
J	545	48 (8.8)	1 (2.1; 0.3–13.4)	1 (2.1; 0.3–13.4)
K	436	49 (11.2)	3 (6.1; 2–17.3)	1 (2; 0.3–13.1)
L	812	79 (9.7)	2 (2.5; 0.6–9.6)	0 (0; 0.0–3.7)
M	903	50 (5.5)	1 (2; 0.3–12.9)	0 (0; 0.0–5.8)
N	1,298	83 (6.4)	2 (2.4; 0.6–9.1)	1 (1.2; 0.2–8.1)
O	943	60 (6.4)	1 (1.7; 0.2–10.9)	0 (0; 0–4.9)
Total	12,832	1,166 (9.1)	32	4

Table 2. CIDT results, whole-blood IFN-γ results, pathologic examination, and culture results of cattle tested for *Mycobacterium bovis* infection, Greater Kruger National Park Complex, South Africa, August 2012–February 2013*

Animal ID	Bovine bias†	CIDT	IFN-γ assay	Macropathology	Culture
N1	8.2	Positive	ND	NVL	*M. bovis*
A1	5.5	Positive	Positive	Multiple lesions in mediastinal and bronchial lymph nodes; single lung lesion	*M. bovis*
J1	5.4	Positive	Negative	NA	NA
K1	4.8	Positive	Positive	Multiple lesions in bronchial, lumbar and renal lymph nodes	*M. bovis*
K1 calf	ND	ND	ND	Single lung lesion	*M. bovis*
MI	3.8	Inconclusive	Positive	NVL	*M. bovis*
OI	3.8	Inconclusive	Negative	NA	NA
HI	3.5	Inconclusive	Negative	NA	NA
AI	3.5	Inconclusive	Negative	NA	NA
GI	3.1	Inconclusive	Negative	NA	NA

*CIDT, comparative intradermal tuberculin test results; ID, identification; IFN-γ, interferon-γ; NA, not applicable (animals were not slaughtered); ND, not done because of poor sample quality; NVL, nonvisible lesions.
†Difference in skin thickness increase elicited by bovine and avian purified protein derivatives.

SB 0121, in all isolates. VNTR analysis using a 13-loci panel identified all isolates as the KNP VNTR 1, which constitutes the *M. bovis* outbreak strain responsible for the bovine tuberculosis epidemic in the KNP and the larger GKNPC (*12*) (Figure 2).

Conclusions

We detected bovine tuberculosis in livestock directly bordering the GKNPC ecosystem. All 5 animals examined were infected with the same spoligotype and VNTR genotype of *M. bovis* as wildlife species in the adjacent GKNPC (*12*). This

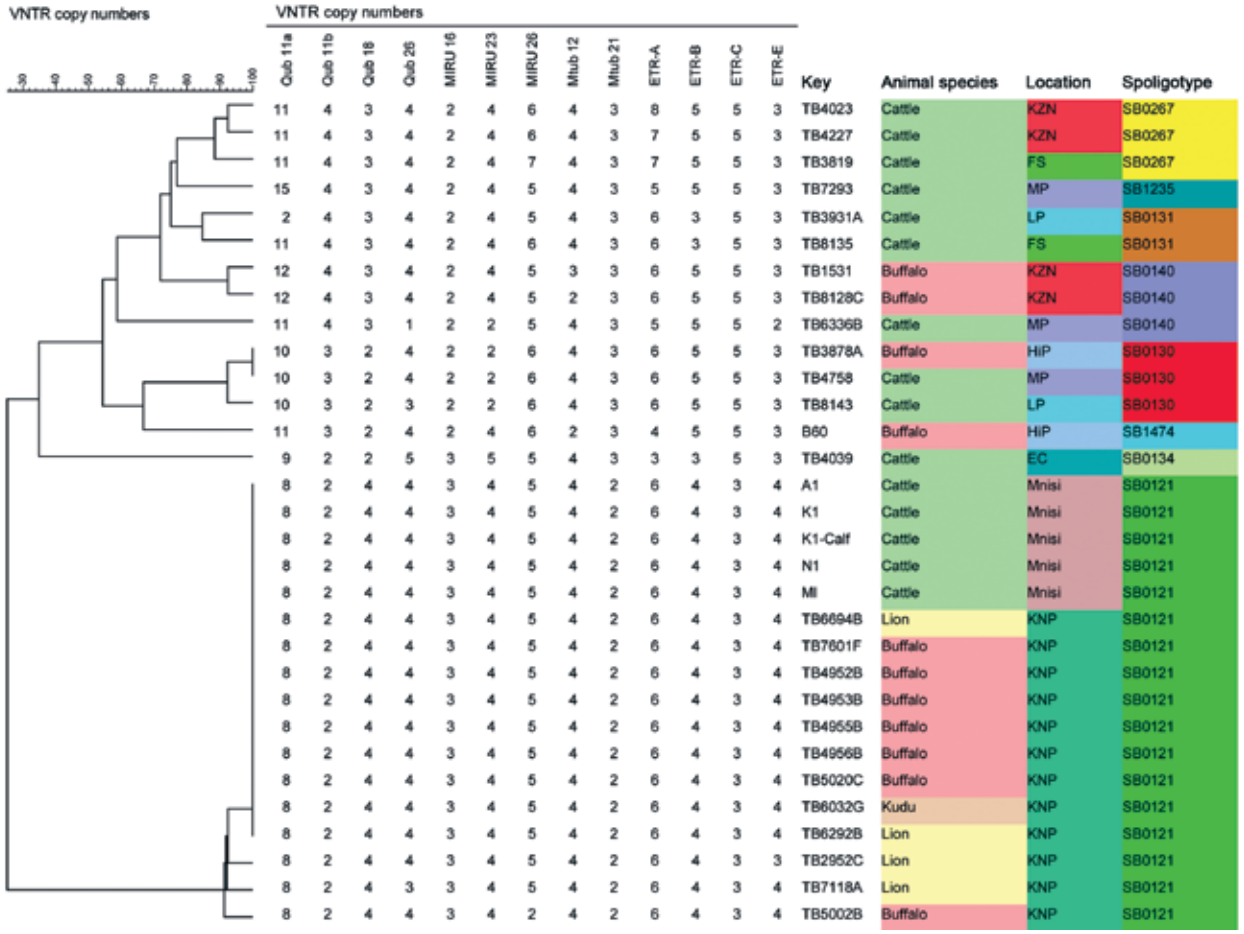

Figure 2. Dendogram depicting the genetic homology between isolates obtained in study of bovine tuberculosis transmission in the Greater Kruger National Park Complex during August 2012–February 2013 and from other outbreaks in South Africa. Colors differentiate the isolates. EC, Eastern Cape; FS, Free State; HiP, Hluhluwe iMofolozi Game Reserve; KNP, Kruger National Park (current study area [Mnisi]); KZN, Kwa-Zulu Natal; LP, Limpopo; MP, Mpumalanga; VNTR, variable number tandem repeat typing.

finding strongly suggests the spillover of *M. bovis* infection from wildlife to neighboring cattle (Figure 2). Alternatively, the KNP outbreak strain could have persisted in the area from which it entered the wildlife population of the GKNPC (*6*) and subsequently could have spread outside the KNP and reached the study area, a distance of ≈180 km. However, during 1996–2012, provincial State Veterinary Services of Mpumalanga tested a total of 96,806 head of cattle in this region of interest, which comprises the veterinary districts of Bushbuckridge (where the study area is located), Nsikazi (bordering the GKNPC in the west), and Nkomazi (south of KNP) using the CIDT as part of its regular bovine tuberculosis surveillance (B.J.A. du Plessis, unpub. data). No bovine tuberculosis reactors were detected in Bushbuckridge or in Nsikazi districts. In 3 unrelated outbreaks during 2009, 2010, and 2011 in the Nkomazi district, 1–3 bovine reactor animals were detected (B.J.A. du Plessis, unpub. data.). All outbreak strains were genotyped, and their spoligotypes and VNTR profiles differed from each other and from the *M. bovis* strain endemic to the GKNPC (results not shown). This information supports the hypothesis that bovine tuberculosis–infected cattle in our current study contracted *M. bovis* from neighboring wildlife in the GKNPC.

In conclusion, our study provides evidence that infected wildlife in the GKNPC constitute a risk factor for bovine tuberculosis infection of neighboring cattle, despite the separation of livestock and wildlife by a well-maintained disease control fence. These findings are of great concern, not only to livestock health and production in communities bordering the GKNPC but also to public health and to human livelihoods because of the zoonotic potential of bovine tuberculosis.

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Prisons as Reservoir for Community Transmission of Tuberculosis, Brazil

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We conducted a population-based study of tuberculosis (TB) cases in Dourados, Brazil, to assess the relationship between incarceration and TB in the general population. Incarceration was associated with TB in an urban population; 54% of *Mycobacterium tuberculosis* strains were related to strains from persons in prisons. TB control in prisons is critical for reducing disease prevalence.

Brazil has the fourth largest incarcerated population in the world and a tuberculosis (TB) incidence that is 20 times higher among prisoners than among the general population (*1,2*). It has been hypothesized that prisons serve as institutional amplifiers for TB, wherein poorly controlled transmission among incarcerated persons is a driver of TB in the broader population (*3,4*). However, few data show for linkages between prison and community epidemics of TB. To address this issue, we conducted a population-based study of TB cases in Dourados, a medium-size city in west–central Brazil, and used case–control and molecular methods to assess the relationship between incarceration and TB in the general population.

The Study

Dourados has a population of ≈177,160 persons, of which 1,500 are inmates of a prison for men. We identified and recruited TB patients reported to the Sistema de Informação de Agravos de Notificação National (Notifiable Diseases Information System) and who resided in Dourados during June 2009–March 2013. We then conducted a case–control study in which 2 control persons without a TB diagnosis were identified and matched for each TB case-patient according to age group and place of residence.

We performed conditional logistic regression to identify significant ($p<0.05$) risk factors for active TB. Variables were included in a multivariable model if they reached a significance level of $p<0.20$ in univariate analysis. *Mycobacterium tuberculosis* isolates were typed by IS*6110* restriction fragment length polymorphism (RFLP) analysis (*5*). RFLP patterns were analyzed by using an IS*6110* RFLP database (RIVM–Bionumerics; Applied Maths, Sint-Martens-Latem, Belgium). A cluster was defined as a group of ≥2 isolates obtained from different patients for which the RFLP patterns were identical with respect to the number and size of bands.

A total of 240 TB cases were reported, of which 60 (25%) and 180 (75%) were in prisoners and community residents, respectively (Figure 1). The annual incidence of TB in the prisoner population was ≈40 times higher than in the community population (1,044 cases/100,000 persons [95% CI 797–1,344 cases/100,000 persons] vs. 26 cases/100,000 persons [95% CI 23–31 cases/100,000 persons]). All 60 prisoners had pulmonary TB and it was confirmed bacteriologically for 54 (90%) persons. Among 180 persons with TB cases in the community population, 133 (74%) had pulmonary TB, 34 (19%) had extrapulmonary TB, and 13 (7%) had both forms; 107 (59%) of the TB cases were confirmed bacteriologically. During the study, 49 (82%) prisoners with TB completed treatment, 2 (3%) were not cured, 3 (5%) died, and 6 (10%) were transferred to other prisons. Prisoners with cases were incarcerated for an average duration of 26 months before diagnosis.

We recruited 61 persons with TB and 122 controls from the community to evaluate risk factors for TB acquisition (Figure 1). Multivariable analysis showed that male sex (adjusted odds ratio [AOR] 6.6, 95% CI 2.4–18.1), monthly income ≤100 US dollars (AOR 3.4, 95% CI 1.1–10.6), alcohol use (AOR 11.5, 95% CI 2.0–67.0), known history of contact with a TB patient (AOR 5.6, 95% CI 1.4–22.0), and prior incarceration (AOR 24.5, 95% CI 2.4–254.6) were independent risk factors for TB (Table). A total of 23% (14/61) of the community cases were in persons previously incarcerated in the Dourados Prison.

We genotyped 97 (86%) of 113 strains isolated from persons with culture-positive *M. tuberculosis* infection, of which 59 and 38 were isolated from community persons and prison patients, respectively (Figure 1). Of these, 79 (81%) isolates were grouped into 17 clusters, and 18 isolates had unique RFLP patterns. Among the 17 cluster types, 10 types included 65 strains from community and prison settings, 6 types included 12 strains exclusively from the community setting, and 1 type included 2 strains

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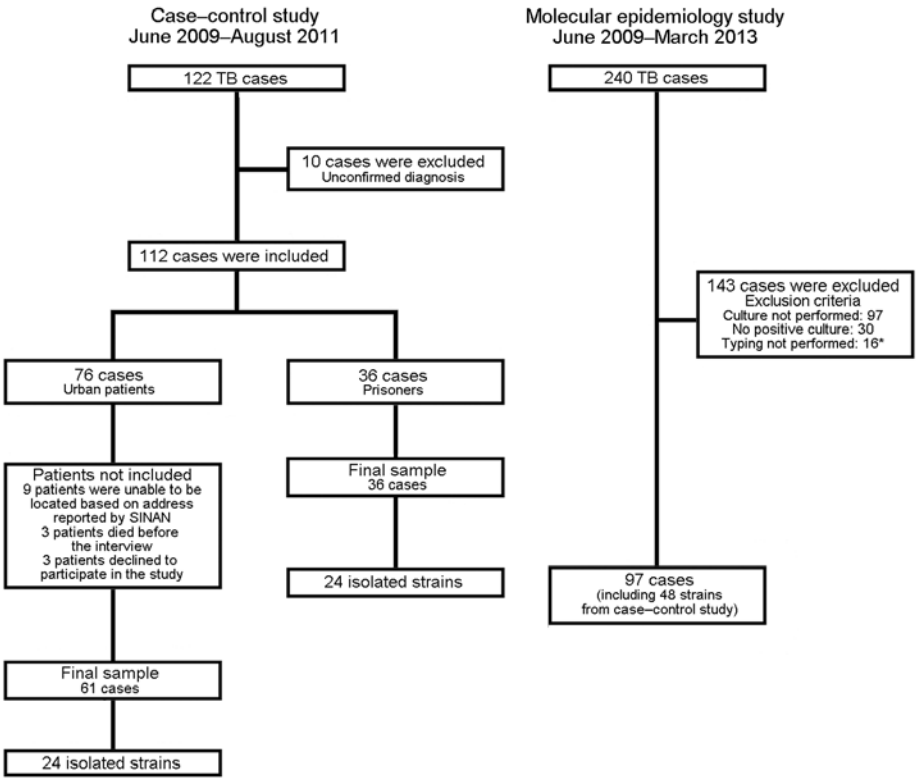

Figure 1. Flowchart for recruitment of patients with tuberculosis (TB) for case–control and molecular studies of prisons as reservoir for community transmission of tuberculosis, Brazil, June 2009–March 2013. *Eleven strains were not reactive after freezing, and 5 strains had <5 bands of IS*6110*. SINAN, Sistema de Informação de Agravos de Notificação (National Notifiable Diseases Information System).

exclusively from the prison setting. Cluster 10, the largest cluster (20 cases), was predominantly found in prisoners, but was also isolated from ex-prisoners and community members without a history of incarceration. Of the 12 community cases with cluster types exclusively found in the community, one was in an ex-prisoner (Figure 2).

Overall, 54% (32/59) of community strains belonged to cluster types that also included prison strains. Among the 32 cluster strains that were circulating in the prison and community, 12 (37%) were isolated from ex-prisoners who were recently released from prison. Ten (83%) of 12 cases occurred within ≤2 years of the inmate's release from prison (Figure 2).

Table. Risk factors for TB in community and prison populations, Dourados, Brazil, June 2009–August 2011*

	Community, n = 183, no. asked/no.responded (%)				Prison, n = 108, no. asked/no. responded (%)		
Variable	TB cases, n = 61	Controls, n = 122	Crude OR (95% CI)	Adjusted OR (95% CI)	TB cases, n = 36	Controls, n = 72	Crude OR (95% CI)
Male sex	41/61 (67)	42/122 (34)	3.9 (2.0–7.5)	6.6 (2.4–18.1)	NA	NA	NA
Income ≤$100†	19/61 (31)	22/122 (18)	2.1 (1.0–4.2)	3.4 (1.1–10.6)	NA	NA	NA
No primary school	15/61 (25)	25/122 (21)	1.3 (0.6–2.6)	NA	11/36 (31)	14/72 (19)	1.82 (0.73–4.57)
Smoked	20/61 (33)	24/122 (20)	2.0 (1.0–4.0)	NA	17/36 (47)	36/72 (50)	0.89 (0.40–1.99)
Alcohol use	14/61 (23)	7/122 (6)	4.9 (1.9–12.9)	11.5 (2.0–67.0)	6/36 (17)	12/72 (17)	1.00 (0.34–2.93)
Drug use	15/61 (25)	2/122 (2)	19.6 (4.3–88.9)	NA	26/36 (72)	48/72 (67)	1.30 (0.54–3.13)
Diabetes	7/61 (12)	13/122 (11)	1.1 (0.4–2.9)	NA	1/36 (3)	1/72 (1)	2.03 (0.12–33.40)
Contact with person with TB	18/61 (30)	15/122 (12)	3.0 (1.4–6.5)	5.6 (1.4–22.0)	23/36 (64)	54/72 (75)	0.59 (0.25–1.40)
Mycobacterium bovis BCG vaccine scar	41/61 (67)	96/122 (79)	0.6 (0.3–1.1)	NA	28/36 (78)	54/72 (75)	1.17 (0.45–3.02)
Prior incarceration	14/61 (23)	1/122 (0.8)	36.0 (4.6–281.8)	24.5 (2.4–254.6)	NA	NA	NA

*TB, tuberculosis; OR, odds ratio; NA, not applicable; BCG, Bacillus Calmette-Guérin.
†Monthly individual income in US dollars.

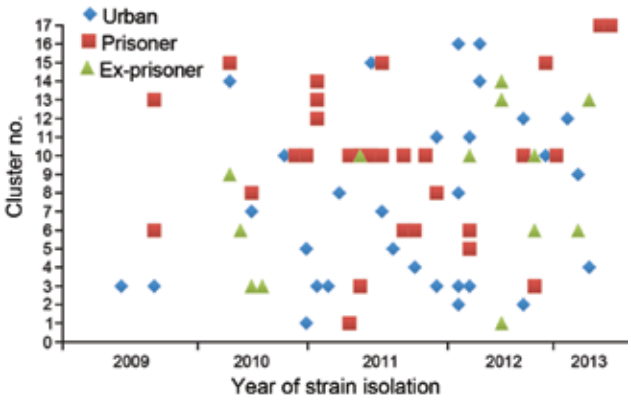

Figure 2. Temporal distribution of *Mycobacterium tuberculosis* strains isolated from the urban population, prisoners, and ex-prisoners in Dourados, Brazil, clustered by IS*6110* restriction fragment length polymorphism analysis, June 2009–March 2013, and stratified by year of isolation and number of the identified cluster.

Conclusions

Prisons have long been recognized as high-risk environments for TB (*6,7*), but there are little data concerning the potential transmission of the disease into community settings. During a 4-year period in a medium-size city in Brazil, 25% of TB cases occurred among prisoners, who represented <1% of the population. Our case–control study showed that that ex-prisoners had 23% more cases of TB than the general population. Among cases in ex-prisoners, 83% (10/12) were diagnosed in the first 2 years after release from prison, which suggests recent infection acquired in the prison setting.

Although exposure to TB might occur after a prisoners's release, we believe that this is less likely because most (71%) ex-prisoners had isolates with the same RFLP pattern as patterns found in prison isolates. Also, 83% (10/12) of ex-prisoners who had an isolate with a similar genetic profile were reported after a case of TB in the prison was reported (Figure 2). Furthermore, we found that baseline tuberculin skin test positivity rates were low (7%) among newly incarcerated inmates, which further supports the assertion that the high rate of TB among prisoners was caused by transmission in the prison setting, rather than by exposure to the disease in the community before incarceration.

The presence of multiple clusters involving prisoners and the general population indicates that TB can spread between these 2 populations. Only 1 previous study reported a link between TB cases in a community setting and those in a jail or prison, but this finding was based on an outbreak that involved only 1 strain (*8*). If one considers the genetic linkages observed in this study and the high rates of disease among prisoners and ex-prisoners, our findings suggest that prisons serve as major reservoirs of TB for the general population.

This study had several limitations. First, we did not evaluate the linkage between cases by investigating close contacts, which limited our ability to establish exact epidemiologic connections between patients. Future contact tracing studies might enhance our understanding of the chain of TB transmission in these settings (*9–11*). Second, we used RFLP to assign clusters, which might overestimate the proximity of genetic or epidemiologic linkages (*12*); future studies involving whole-genome sequencing might help clarify the timing and directionality of disease transmission (*13*). Third, we assessed data for only 1 city and 1 prison. The prison had typical conditions in terms of layout, crowding, and diagnostic resources. However, other studies have found that TB incidence and transmission rates are even higher in other prisons in Brazil (*3,14,15*). Thus, the contribution of spillover infections to the general community may be even greater.

Our data demonstrate that incarceration is a strong risk factor for acquiring TB and that the epidemic of TB in prisons is interlinked with that in the general population. Policies and programs aimed at reducing transmission in prisons and preventing TB among released prisoners should be considered to successfully control TB in the general population. Effective responses will require improving TB diagnostic capacity in prisons, implementing active case detection strategies, such as annual mass screening, testing for latent TB and provision of isoniazid preventive therapy, and developing transitional care and follow-up programs for prisoners released into the community.

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Polycystic Echinococcosis in Pacas, Amazon Region, Peru

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In the Peruvian Amazon, paca meat is consumed by humans. To determine human risk for polycystic echinococcosis, we examined wild pacas from 2 villages; 15 (11.7%) of 128 were infected with *Echinococcus vogeli* tapeworms. High *E. vogeli* prevalence among pacas indicates potential risk for humans living in *E. vogeli*–contaminated areas.

In the Peruvian Amazon, the presence of parasites associated with zoonotic diseases is affected by subsistence hunting, sale of bush meat for human consumption, and increased human contact with wild animals (*1*). One such zoonotic disease, polycystic echinococcosis, is caused by ingestion of food or water contaminated with *Echinococcus vogeli* tapeworm eggs and the subsequent development of larvae (cysts), mainly in the liver (*2*). Although this disease is seriously underreported, >200 cases in humans from 12 countries in Central and South America have been described (*2*–*4*); most confirmed cases were in Colombia and Brazil, and the first case in Peru was reported in 2004 (*2*,*5*).

Bush dogs (*Speothos venaticus*) are the most common definitive hosts for *E. vogeli* cestodes (adult cestode carriers; wild rodents, especially pacas (*Cuniculus paca*), may be the most common intermediate hosts (larvae or metacestode carriers) (*2*). In the Amazon region, pacas are among the most frequently hunted animals, and paca meat is highly commercialized in regional cities (K. Moya. Monitoreo de la comercialización de carne de monte en los mercados de Iquitos y estrategias para su conservación [undergraduate thesis]. Iquitos [Peru]: Universidad Nacional de la Amazonia Peruana; 2011). To verify the presence of *E. vogeli* cysts in wild pacas in 2 communities in the Peruvian Amazon (Nueva Esperanza and Diamante/7 de Julio), we examined paca viscera submitted by hunters.

The Study

During June 2009–August 2013, as part of an ongoing participatory conservation program that involves hunters in community-based wildlife management, hunters collected thoracic and abdominal organs from pacas. The research protocol was approved by the Dirección General de Flora y Fauna Silvestre (0350-2012-AG-DGFFS-DGEFFS) from Peru. No pacas were killed solely for this study.

Viscera from 128 pacas were submitted for examination. Of the 120 pacas for which sex was known, 66 (55%) were female. A total of 120 viscera samples were obtained from pacas in Nueva Esperanza and 8 from pacas in Diamante/7 de Julio.

Organs with cyst-like structures were analyzed at the San Marcos University School of Veterinary Medicine or the Peruvian National Institute of Health. Protoscolices collected from cysts were mounted in Berlese mounting medium. *E. vogeli* protoscolices were identified according to the shape and size of rostellar hooks (*6*). Tissue samples were fixed in 4% formalin before staining with hematoxylin and eosin for histopathologic examination. The Fisher exact test was used to analyze differences between communities, seasonality (wet and dry), and sex of pacas; significance was set at $p<0.05$.

Of the 128 pacas, polycystic echinococcosis was present in 15; overall prevalence was 11.7% (95% CI 6.7–18.6%). In Diamante/7 de Julio, prevalence was 25.0% (2/8; 95% CI 3.2%–65.1%), and in Nueva Esperanza, it was 10.8% (13/120; 95% CI 5.9%–17.8%; $p = 0.24$). The number of infected pacas differed between wet and dry seasons, but the difference was not significant (wet season = 13 infected pacas, dry season = 2; $p = 0.14$). No significant association between infection and sex was found (8 males and 7 females; $p = 0.78$).

In 14 of the infected pacas, only liver cysts were found; in the other paca, cysts were found in liver and lungs (Figure 1). An average of 3.6 ± 8.2 liver cysts were found (1–52 cysts, 0.4–3.5 cm diameter). The only paca with cysts in the lungs had 8 such cysts, 0.2–1.0 cm diameter. The mean ± SD of the total length of large hooks was 41.9 ± 0.4 μm; the mean size of small hooks was 34.3 ± 0.9 μm. For large hooks, the handle length was 15.7 μm and the blade length was 27.6 μm. The number of hooks (large and small) ranged from 42 to 46 (Figure 2).

Histologic examination revealed metacestodal tissue (protoscolices, inner germinal layer, intermediate thick laminated layer, and outer adventitial layer) compressing hepatic parenchyma. The acellular laminated layer appeared convoluted, and eosinophils were seen after staining with hematoxylin and eosin. Intact laminated layers were lined on the inside by the germinal layer, which formed brood capsules

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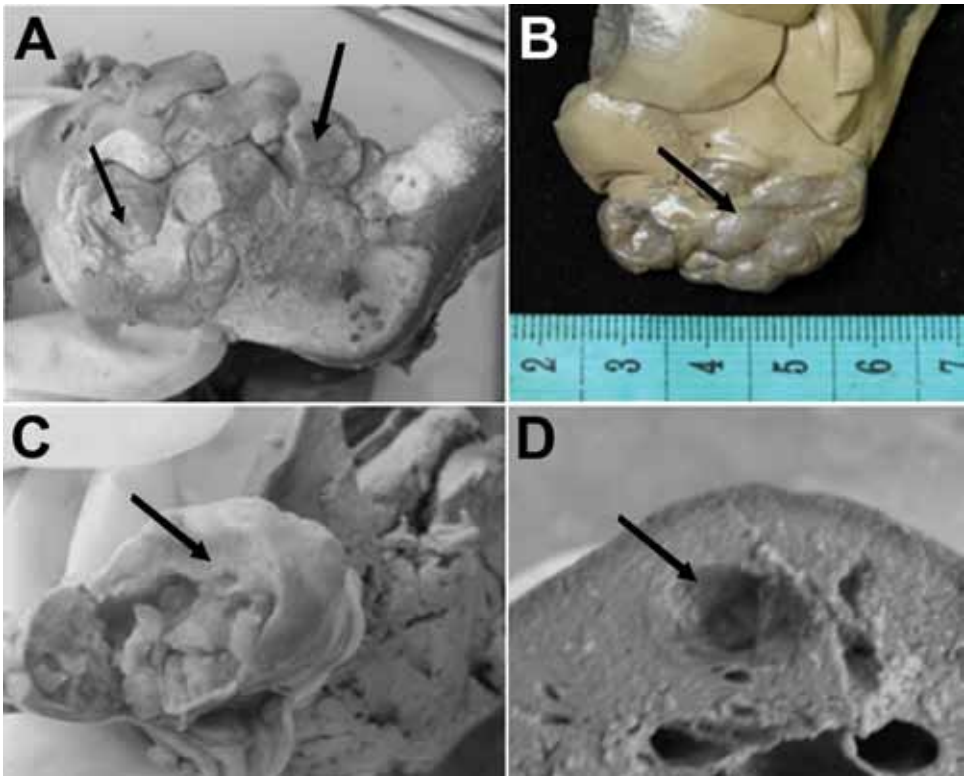

Figure 1. Multiple *Echinococcus vogeli* cysts (larval stage) in the liver of a wild paca. A, B) Vesicles exposed at the hepatic surface (arrows). C, D) Transected larvae in thick sections of liver showing internal structure of vesicles and characteristic distribution of brood capsules (arrows). Ruler in panel B indicates centimeters.

and had free protoscolices. A thick and fibrous layer surrounded the liver cysts with an infiltration of macrophages, neutrophils, eosinophils, plasma cells, and lymphocytes.

Conclusions

The high prevalence of polycystic echinococcosis in pacas in this study is consistent with the paca being a common intermediate host and the possibility that human cultural and dietary habits in the Amazon region might induce a parasite life cycle that involves domestic animals, particularly dogs. Most persons in the Amazon depend on subsistence hunting (*7*). They do not, however, consume the organs from hunted pacas; instead, they typically feed the viscera to their dogs, which might become definitive hosts. This feeding practice results in a high risk for introduction, development, and dissemination of *E. vogeli* cestodes in rural communities; humans might consequentially be infected through contact with feces from infected dogs. Other associated risk factors are poor hygienic conditions, unavailability of clean water, inadequate medical care, and insufficient knowledge about local diseases.

Among humans who receive medical attention, the polycystic echinococcosis fatality rate is 29%; causes of death are surgical accidents during cyst removal and direct parasite consequences (e.g., hepatic failure and its complications) (*2*,*3*). However, reported cases in humans might represent only the tip of the iceberg (*2*). Poor accessibility to rural jungle areas and lack of adequate public health infrastructures inhibit research among humans and wild animals (*8*), probably resulting in underestimation, inaccurate recording, or nondetection of cases (*2*,*3*). This neglected disease may cause chronic conditions that reduce productivity and income earning capacity.

High levels of bush meat commercialization in cities in the Amazon region of Peru (K. Moya, 2011) suggest direct contact among wildlife, humans, and domestic animals (*9*). The study area has not been affected much by humans; most contact between the local population and wildlife occurs during hunting activities. Outdoor activities, such as logging, oil extraction, or road construction, may cause human migration from urban areas to the rainforest, increasing risk for exposure to sylvatic pathogens (*10*) such as *E. vogeli* cestodes. Human movements introduce domestic animals, such as dogs, to forested ecosystems where they can encounter parasites or other infectious disease agents. Ecotourism also increases the likelihood of contact between tourists, wildlife, and potential pathogens (*11*).

This study was limited in that samples were neither random nor numerous, and the number of collected viscera differed between the 2 communities. Collection of paca viscera was part of the ongoing participatory conservation program; although hunters were trained to collect the viscera, sample availability was based on hunters' willingness to participate. However, the infection rate we

Figure 2. Large and small rostellar hooks from protoscolices of *Echinococcus vogeli*. A) Large (top) and small (bottom) hooks. B) Rows of rostellar hooks. Scale bars indicate 40 μm.

found might indicate an emerging public health problem in many countries; for instance, *E. vogeli* infections have been documented in a hunter from French Guyana (*12*), in an indigenous human population in the Amazon region of Venezuela (*13*), and in pacas and bush dogs in Argentina (*4*). The disease may also emerge in other countries, such as the Netherlands, where polycystic echinococcosis in a human was recently diagnosed (*14*).

Our study could be the beginning of more investigations involving wild and domestic animals and humans in the Amazon to determine the incidence and prevalence of polycystic echinococcosis in human populations and the potential role of domestic dogs as carriers of adult *E. vogeli* cestodes (*4*). The true prevalence and public health effects of this parasite must be estimated in communities in the Amazon region, and the search for *E. vogeli* infections should be expanded to other regions and countries.

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Spatiotemporal Analysis of Guaroa Virus Diversity, Evolution, and Spread in South America

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We conducted phylogeographic modeling to determine the introduction and spread of Guaroa virus in South America. The results suggest a recent introduction of this virus into regions of Peru and Bolivia over the past 60–70 years and emphasize the need for increased surveillance in surrounding areas.

Guaroa virus (GROV; family *Bunyaviridae*, genus *Orthobunyavirus*) infection in humans frequently results in febrile illness, and limited serologic surveillance indicates that the virus infects a substantial portion of the rural population in tropical regions of Central and South America (Figure 1, panel A) (*1*,*2*). However, similar to the situation for many arboviruses found in the Neotropics, limited systematic surveillance for GROV in rural communities contributes to a lack of information regarding the ecology and actual effect of GROV on public health. In addition, the very limited resources available for such work results in a critical need for approaches that can help identify where these resources can be put to best use. To further our knowledge of GROV in South America, we conducted a spatiotemporal analysis to estimate when and where the virus was introduced into the region, model its subsequent pattern of spread, and obtain insights into the ecologic and anthropogenic factors that may have affected these processes.

The Study

To generate the large, complete genome dataset needed for phylogeographic analysis, we obtained 12 GROV strains from the American Type Culture Collection (ATTC VR-394, strain CoH352111; Manassas, VA, USA) or from the World Reference Center for Emerging Viruses and Arboviruses (all other GROV strains; University of Texas Medical Branch, Galveston, TX, USA). These viruses had been isolated during 1956–2011 and originate from 4 of the 5 countries in which GROV has been isolated: Columbia, Brazil, Peru, and Bolivia (Figure 1, panel A; online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/3/14-1351-Techapp1.pdf). Clinical specimens used in this study were obtained under the terms of a human use protocol (NMRCD.2000.0006). This protocol and the consent procedure were approved by the Naval Medical Research Center Institutional Review Board in compliance with all US and Peruvian federal regulations governing the protection of human subjects.

Viral RNA was extracted from the isolates by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), and reverse transcription PCR reactions were performed by using the Superscript III Reverse Transcriptase (Life Technologies, Grand Island, NY, USA) and the iProof High-Fidelity PCR Kit (Bio-Rad, Hercules, CA, USA). Coding region sequencing was based on partial sequences of strain BeH22063 (GenBank accession nos. X73466 [S segment], AY380581 [M segment], JN801039 [L segment]) and completed where necessary by using primer walking. Terminal noncoding sequences were obtained by using ligation-anchored PCR, as previously described (*3*). Complete genome sequences were deposited in GenBank (online Technical Appendix Table); primer sequences are available upon request.

We then used the large GROV dataset and one for the closely related Wyeomyia virus (WYOV) group to conduct a phylogeographic analysis (*4*). GROV and WYOV are geographically restricted to Central and South America, but the next most closely related virus clades are not found in the region, suggesting a specific introduction of the GROV/WYOV common ancestor into South America (Figure 1). We calculated Bayesian coalescent phylogenies, incorporating sample times and locations (online Technical Appendix Table), by using BEAST v1.8.0 (http://beast.bio.ed.ac.uk/) with multiple sequence alignments containing nucleoprotein open-reading frame sequences from the GROV and WYOV groups. The resulting trees, along with tables containing the geographic coordinates of the samples, were then input into SPREAD v1.0.6 (*5*) to calculate ancestral locations (online Technical Appendix Figure) and 80% highest posterior density polygons. Graphical map overlays were generated,

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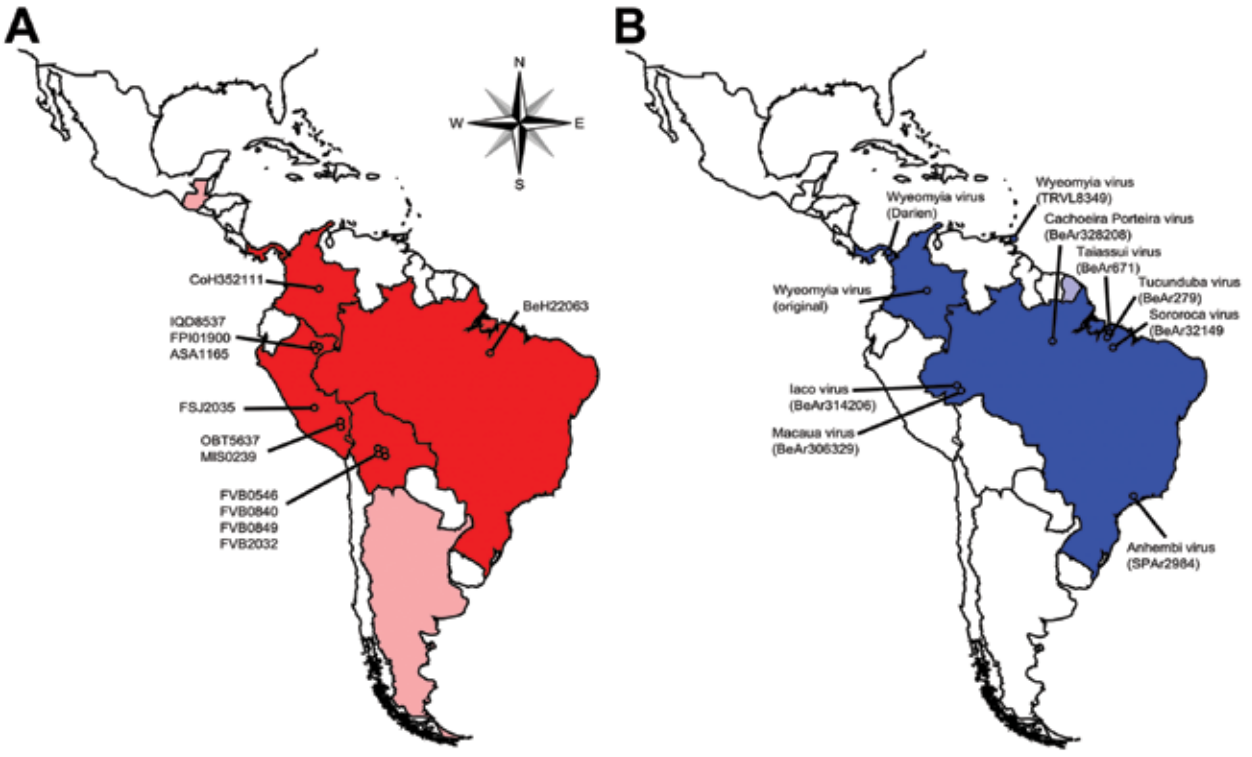

Figure 1. Comparison of the geographic locations from which Guaroa virus (GROV) strains used in this study were isolated (A) and the geographic distribution of the Wyeomyia virus (WYOV) group (B), Central and South America. Countries from which GROVs have been isolated are shown in dark red; countries from which WYOVs have been isolated are shown in dark blue. Light red indicates countries with only serologic evidence of GROV circulation; light blue indicates countries with only serologic evidence of WYOV circulation. Circles indicate the geographic locations from which the virus strains used in the present study were isolated.

viewed by using Google Earth (https://www.google.com/earth), and exported as video files (online Video 1, http://wwwnc.cdc.gov/EID/article/21/3/14-1351-F1.htm and online Video 2, http://wwwnc.cdc.gov/EID/article/21/3/14-1351-F2.htm). Because decimal coordinates were used for sample locations, we performed a continuous phylogeographic analysis (*6*).

Conclusions

The results of our analysis indicate that the GROV/WYOV common ancestor was introduced into South America ≈250 years ago (i.e., about 1764) at a site along the Amazon River; the identified site is within ≈250 km of Manaus, Brazil, which has been a major population center in this area since the early days of European colonization, possibly even longer (*7*) (Figure 2; online Videos 1 and 2). This estimated date corresponds with a tumultuous period in South American history, during which various revolts, insurrections, and wars for independence were taking place. In addition, this era was associated with record levels of slave importation from Africa to facilitate the growing economy of Brazil (*8*). Thus, the GROV/WYOV common ancestor might have been introduced by several different means during the transport of persons, supplies, or both. Furthermore, our estimated date and location of introduction is consistent with that determined for the introduction of yellow fever virus (YFV) into Brazil (*9*), indicating that the geopolitical conditions at this time may in fact have facilitated the introduction and subsequent spread of several different viruses into this area.

From this central introduction point of the GROV/WYOV common ancestor, the GROV group and the WYOV group, which is further made up of 2 distinct lineages (the WYOV and Anhembi virus [ABMV] lineages), exhibit differences in their patterns of spread. The ABMV lineage spread in a predominantly southward direction within Brazil (Figure 2, panel A), and the WYOV lineage spread northward into northern Brazil and, ultimately, into Columbia, Central America, and the Caribbean (Figure 2, panel B). In contrast, although the spread of GROV initially closely followed that of the WYOV lineage into the northern regions of South America, our results indicate that over the past 60–70 years, it spread rapidly spread southward into Peru and, subsequently, into Bolivia (Figure 2, panels C and D; online Videos 1 and 2). Recent spread of GROV into the regions of Peru and Bolivia highlights the need for focused surveillance in these areas to monitor for continued spread of GROV into surrounding areas. This

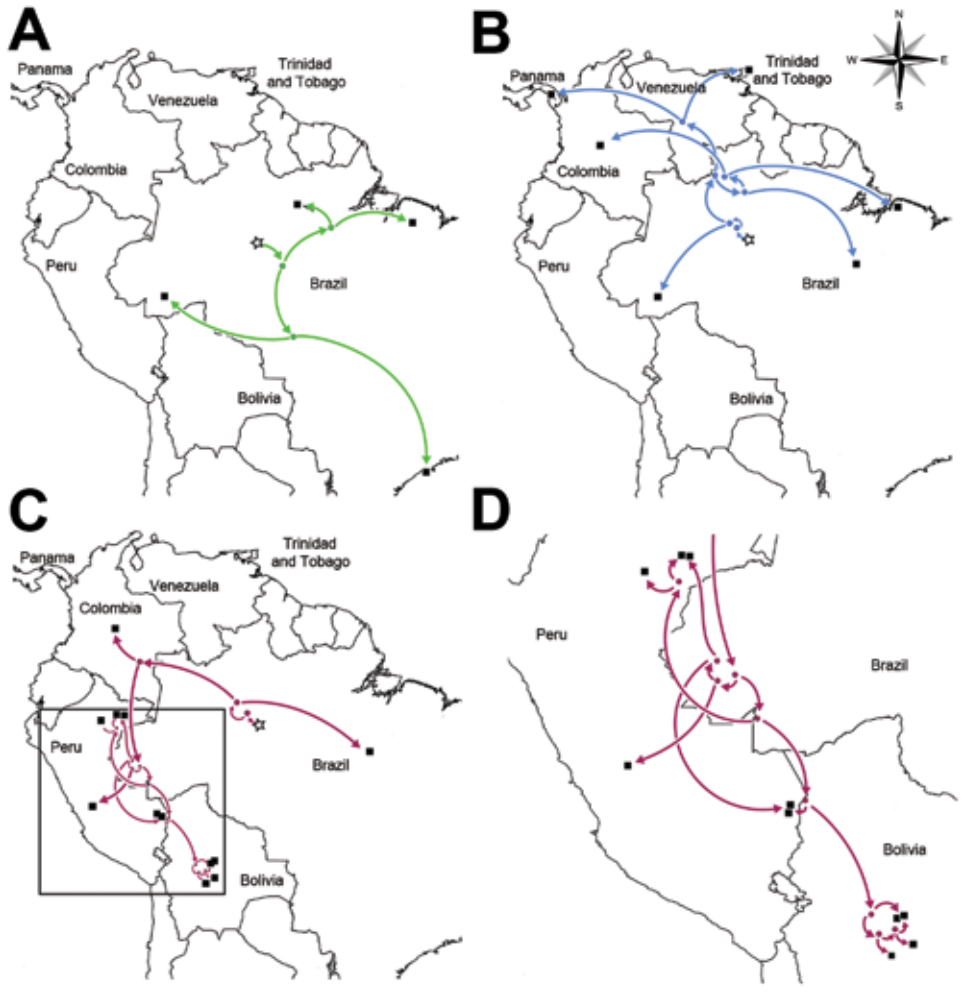

Figure 2. Spread (arrows) of Wyeomyia virus (WYOV) group viruses and Guaroa virus (GROV) in Central and South America. A) Anhembi lineage WYOV group viruses; B) Wyeomyia lineage WYOV group viruses; C) GROVs. D) Enlargement of boxed area in panel C, showing the spread of GROV in Bolivia and Peru, as determined by phylogeographic analysis. Bayesian coalescent phylogenies incorporating sample times and locations (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/3/14-1351-Techapp1.pdf) were calculated for the nucleoprotein open-reading frame dataset by using BEAST v1.8.0 (http://beast.bio.ed.ac.uk/) and then input into SPREAD v1.0.6 (*5*) to calculate ancestral locations and corresponding graphical map overlays. Stars in each panel represent the predicted site of introduction for the GROV/WYOV common ancestor; dots represent the predicted locations associated with all other nodes (online Technical Appendix Figure). Black boxes indicate the locations at which the viruses used in this study were isolated.

finding also appears to be consistent with serologic investigations in this area, which indicate that, whereas the virus has only recently been detected in persons in these regions (*2*), GROV antibody has been detected in serum samples from the population dating back to 1965 (*10*).

The observed spatial patterns are also notable in that they suggest possible mechanisms of virus spread within the endemic region. The northwest/southeast axis of spread seen with the WYOV and ABMV lineages closely reflects what was reported for YFV (*9*), which, given the nonsylvatic nature of YFV transmission in South America, suggests a direct contribution of human, mosquito, or both populations to this pattern of spread. However, this finding may not adequately explain the spread pattern observed for GROV. This pattern might be explained by the involvement of avian reservoirs: ecological data indicate the infection of South American birds with several closely related orthobunyaviruses that are endemic to the New World (*11*). Furthermore, Columbia represents an intersection point between flyways from Brazil and those that run along the western coast of South America (*12*). These observations then raise the possibility that unidentified bird species, including migratory birds, could also be involved in the spread of GROV, and suggest that avian species should be considered a priority for future surveillance efforts within the GROV-endemic region.

Overall, our study has led to the generation of a substantial set of full-length GROV sequences, which we anticipate will aid in future efforts to develop improved diagnostic approaches for this and related viruses, and has enabled us to model the spread of GROV within the virus-endemic area. In doing so, we have identified Peru and Bolivia as regions of recent and active GROV spread that should be considered areas for future virus surveillance efforts. In addition, our data implicate both human-associated factors and possibly bird populations in the spread of this virus within South America.

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Red Deer as Maintenance Host for Bovine Tuberculosis, Alpine Region

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To estimate the prevalence of bovine tuberculosis in the Alpine region, we studied the epidemiology of *Mycobacterium caprae* in wildlife during the 2009–2012 hunting seasons. Free-ranging red deer (*Cervus elaphus*) were a maintenance host in a hot-spot area, mainly located in Austria.

Bovine tuberculosis has one of the broadest host ranges of any known zoonotic pathogens. In addition to cattle, bovine tuberculosis affects many wild animal populations in North America, Europe, Africa, Asia, and New Zealand. Under certain conditions, wildlife play a role as reservoir and source of infection for domestic animals. *Mycobacterium caprae* has been isolated from cattle, domestic goats, domestic pigs, red deer (*Cervus elaphus*), and wild boar (*1*). Evidence is increasing that *M. caprae* is emerging in free-ranging red deer and cattle in the Alps (*2*,*3*).

The Study

To estimate the prevalence of bovine tuberculosis (which is caused by *M. bovis* and *M. caprae*) in wildlife in the Alps, we investigated 1,655 hunted red deer of both sexes and different ages in Austria, Germany, Switzerland (including the Principality of Liechtenstein), and Italy. The deer were hunted specifically for the study by trained hunters. A sampling/hunting plan was calculated in advance that indicated the number of animals needed in each sampling region to calculate prevalence estimates; the number was based on the red deer density of a region. The numbers of animals killed and sampled during 3 consecutive hunting seasons (2009–10, 2010–11, and 2011–12) coordinated nearly perfectly with the sampling plan that had been developed for each sampling area (Table 1). After pathomorphologic examination of carcasses (from Germany, Austria, Italy, Swiss Tessin) or samples (from Swiss St. Gall, Swiss Grisons, and Liechtenstein), we conducted microbiological analysis from sample material. Sample material included both medial retropharyngeal lymph nodes and tracheobronchial, mediastinal, and mesenteric lymph nodes and any other tissue with macroscopically visible lesions (Table 2). For bacteriologic cultivation the sample material was homogenized by using the IKA Ultra Turaxx Tube Drive System (Staufen, Germany), decontaminated with 1% N-acetyl-L-cystein solution and neutralized in phosphate buffer (pH 6.8) as recommended by the World Organisation for Animal Health (*4*). After sedimentation, inoculation was performed on 2 growth media: Stonebrink including PACT (polymyxin B, amphotericin B, carbenicillin, and trimethoprim) and Lowenstein-Jensen with glycerin and PACT (Heipha Diagnostika, Eppelheim, Germany). After 12 weeks' incubation, a total of 82 bacterial cultures from 59 hunted red deer from Austria, Germany, and Italy were isolated (Tables 1, 2). All isolates were identified as *M. caprae* whether by reversed line blotting (Geno Type MTBC, HAIN Lifescience, Nehren, Germany) or by restriction fragment length polymorphism PCR of the *gyrB* gene, as previously described (*5*).

Red deer with macroscopically visible lesions (purulent abscesses varying remarkably in size) were all *M. caprae* positive from ≥1 lymph nodes/tissue, and 4 animals without visible lesions were *M. caprae* positive (Table 2). Microscopic examination showed that lesions had thin-walled fibrous capsules, occasionally with neutrophil granulocytes and calcifications in the necrosis zone, with an increased occurrence of neutrophil granulocytes and calcifications around the necrosis zone, and with a high occurrence of epithelioid and giant cells at the inside capsule wall. The large majority of these lesions were in the lymph nodes, particularly the medial retropharyngeal lymph nodes. Lesions in the lungs, observed in 3 animals, were consistently found in combination with lesions in the tracheobronchial and mediastinal lymph nodes; lesions in the tonsils were found with lesions in the retropharyngeal lymph nodes (Table 2). The medial retropharyngeal lymph nodes and the mesenteric lymph nodes were the primary sites of infection;

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Table 1. Estimated prevalence of *Mycobacterium caprae* in red deer (*Cervus elaphus*), Alpine region, 2009–10, 2010–11, and 2011–12 hunting seasons

Study area	No. animals	No. *M. caprae* positive	Estimated prevalence (95% CI), %
Austria, total	590	55	
Tyrol			
Lechtal I	173	40	23.1 (17.0–30.2)
Lechtal Mitte	98	7	7.1 (2.9–14.2)
Lechtal II	15	1	6.7 (0.1–32.0)
Tannheimertal	32	0	0 (0.0–9.0)
Schwarzwasser	38	0	0 (0.0–7.6)
Vorarlberg			
Region 1	50	0	0 (0.0–5.9)
Region 2	61	1	1.6 (0.0–8.8)
Region 3	47	6	12.8 (4.8–25.8)
Region 4	41	0	0 (0.0–7.1)
Region 5	35	0	0 (0.0–8.3)
Switzerland, total	273	0	
Grisons	88	0	0 (0.0–3.4)
St. Gall	48	0	0 (0.0–6.1)
Tessin	89	0	0 (0.0–3.4)
Liechtenstein	48	0	0 (0.0–6.1)
Italy, total	514	1	
Bergamo/Brescia	77	1	1.3 (0.0–7.1)
Bolzano			
East	23	0	0 (0.0–12.3)
North	29	0	0 (0.0–9.9)
South	10	0	0 (0.0–25.9)
West	60	0	0 (0.0–4.9)
Como/Lecco	61	0	0 (0.0–4.8)
Sondrio	95	0	0 (0.0–3.2)
Trento			
East	41	0	0 (0.0–7.1)
West	53	0	0 (0.0–5.5)
Varese	65	0	0 (0.0–4.6)
Germany, total	278	3	
Region 1	187	1	0.5 (0.0–3.0)
Region 2	91	2	2.2 (0.2–7.8)

most of the deer had lesions in the medial retropharyngeal lymph nodes and in the mesenteric lymph nodes, either exclusively or in combination with other sites (Table 2). Furthermore, the 4 animals without visible lesions were *M. caprae* positive from the medial retropharyngeal lymph nodes, suggesting a very early stage of infection.

M. bovis infections of cattle or deer tonsils led to bacterial colonization in the medial retropharyngeal lymph nodes with subsequent lymphatic spread to pulmonary lymph nodes. Ingestion of the *M. bovis* bacteria led to lymphatic spread from the primary infection, the gut, to mediastinal or tracheobronchial lymph nodes, which explained the lesions in the mesenteric lymph nodes (*6*). *M. bovis* causing tuberculosis is proposed to be a lymphatic disease (*6*); we propose the same for *M. caprae*.

We calculated regional prevalence estimates with 95% CIs (Table 1; Figure 1). Prevalence estimates differed greatly among the sampling areas in Austria, Switzerland, Germany, and Italy. Moreover, a spatial cluster of positive samples (hot spot) around the Austrian sampling area Lechtal I was identified using point pattern analysis. The cluster comprised all positive animals from Lechtal I and the contiguous sampling areas of Lechtal Mitte, Regions 2 and 3 of Vorarlberg, and Region 1 of Bavaria (Germany) ($p<0.001$) (*7–10*). Consequently, this hot spot included 55 of the 59 positive samples detected in this study (Table 1; Figure 2).

Genotyping was performed from *M. caprae* isolates by spoligotyping (*11*) and by mycobacterial interspersed repetitive unit typing and variable number tandem repeat genotyping for 24 loci (*12*). All isolates in the hot-spot area and the 2 other isolates from Austria were of the Lechtal genotype. The single isolate in Italy, located >200 km from the hot spot, was also of the Lechtal genotype, and an association might exist between this case and cattle previously imported from Austria to Italy (*13*). By contrast, 2 isolates from Germany found in the Karwendel Mountains were of the Karwendel genotype. All other deer were negative for *M. caprae* and for *M. bovis*. After the transnational project, in the 2012–13 hunting season, the prevalence in Bavaria 1 (Germany) was 5.3%, indicating an expansion of or shift in the hot spot.

Conclusions

Our data indicate a localized bovine tuberculosis problem in wildlife in the Alps related to wildlife management

Table 2. *Mycobacterium caprae*–positive red deer (*Cervus elaphus*) and occurrence of macroscopically visible lesions in selected lymph nodes and other tissues, Alpine region, 2009–10, 2010–11, and 2011–12 hunting seasons*

	Lymph node†				
No. animals, N = 59	Retropharyngeal	Mediastinal	Tracheobronchial	Mesenteric	Other tissue‡
29	+	NLD	NLD	NLD	NLD
5	NLD	NLD	NLD	+	NLD
4	NLD	NLD	NLD	NLD	NLD
3	+	NLD	NLD	+	NLD
2	NLD	NLD	+	NLD	Lung
1	+	NLD	+	NLD	Lung
1	+	NLD	NLD	+	Tonsil
1	NLD	NLD	NLD	NLD	Parotid lymph node
2	+	+	+	NLD	NLD
2	NLD	NLD	+	+	NLD
1	+	+	+	+	NLD
1	NLD	+	+	+	NLD
1	+	NLD	+	+	NLD
1	+	+	NLD	+	NLD
1	NLD	+	NLD	+	NLD
1	+	NLD	+	NLD	NLD
1	+	+	NLD	NLD	NLD
1	NLD	NLD	+	NLD	NLD
1	NLD	+	NLD	NLD	NLD

*NLD, no lesions detected; +, macroscopically visible lesions.
†No. animals with positive findings: retropharyngeal, 41 deer; mediastinal, 8 deer; tracheobronchial, 12 deer; mesenteric, 16 deer.
‡No. animals with positive findings: 5 deer.

strategies. Supplementary feeding is common in the hot-spot area to prevent migration and to keep red deer populations high (*3*). In the hot-spot area, the overall density is 5.6 animals/km^2, but because of aggregation, which is considered the main cause of bovine tuberculosis maintenance in wildlife, winter habitats of red deer around feeding sites have up to 46.2 animals/km^2 (Statistik Austria, http://www.statistik.at) (*14*). Low (0.5–2.5 and 2–4/km^2) or medium densities (9.7 deer/km^2) with prevalence estimates of 0% were found in Switzerland (Saint Gall, Liechtenstein, and Grisons, respectively) (*14*). Brescia, the only place in Italy where *M. caprae* was found in red deer, is considered a high-density region (16 animals/km^2), but management strategies promoting aggregation are largely absent in Italy and Switzerland (*13*). Furthermore, red deer in the hot-spot area are a reservoir for bovine tuberculosis and are the source of *M. caprae* infections for domestic cattle grazing on Alpine pastures during the summer (*3*). Molecular-epidemiologic studies indicate that *M. caprae* isolates from red deer and cattle are of the same genotype (*2*). Our finding that retropharyngeal lymph nodes and mesenteric lymph nodes were often the primary site of infection suggests oral rather than aerosol transmission. Oral transmission does not require direct contact, either among red deer or between red deer and cattle.

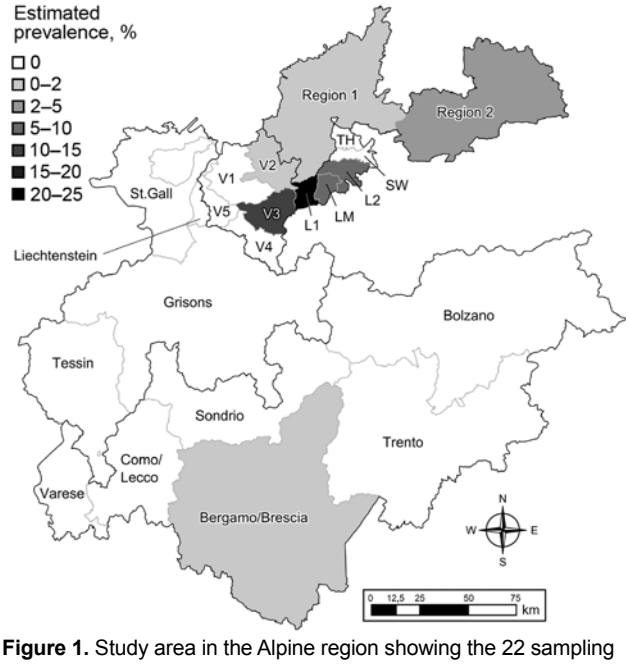

Figure 1. Study area in the Alpine region showing the 22 sampling regions and the estimated prevalences of *Mycobacteria caprae*. Prevalence ranges are classified into 6 intervals, wherein the upper bounds are not included in the interval. Austria: Vorarlberg (V1–V5) and Tyrolean Lech valley: Lechtal I (L1), Lechtal Mitte (LM), Lechtal II (L2), Schwarzwasser (SW),and Tannheimertal (TH). Germany: Bavaria (Region 1 and Region 2). Switzerland: St. Gall, Grisons, Tessin, and Liechtenstein. Italy: Varese, Como/Lecco, Sondrio, Bergamo/Brescia, Trento, Bolzano.

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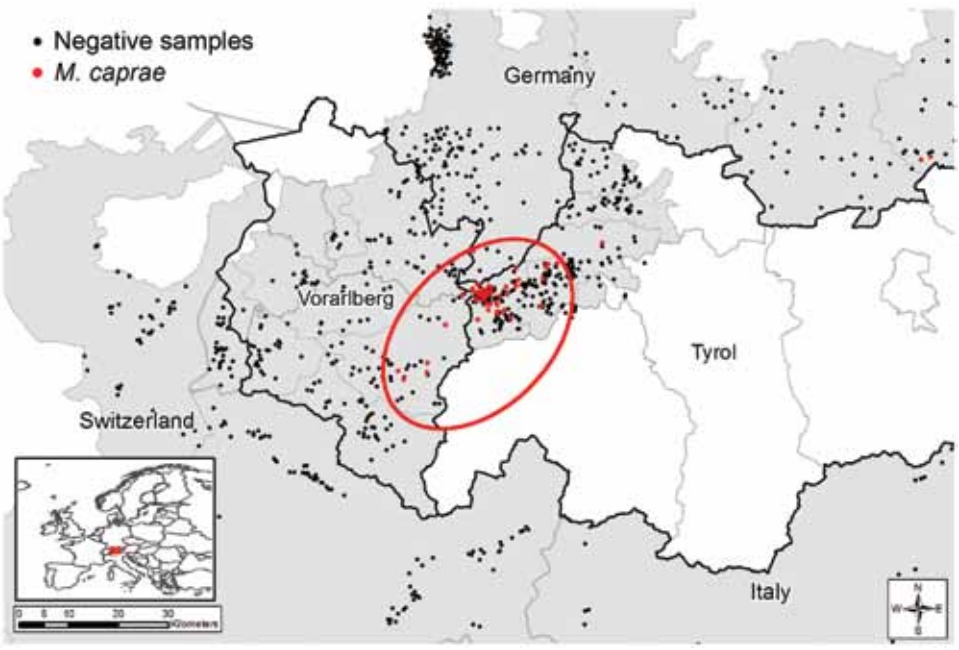

Figure 2. Statistically evident spatial cluster of *Mycobacterium caprae*–positive red deer in the Alpine region, 2009-10, 2010-2011, and 2011-12 hunting seasons. Area in red circle contained significantly more *M. caprae*–positive red deer than the remaining study area ($p<0.001$). Inset shows location of Austria and Germany within Europe (shading).

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Noninvasive Test for Tuberculosis Detection among Primates

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Traditional testing methods have limited epidemiologic studies of tuberculosis among free-living primates. PCR amplification of insertion element IS*6110* of *Mycobacterium tuberculosis* from fecal samples was evaluated as a noninvasive screening test for tuberculosis in primates. Active tuberculosis was detected among inoculated macaques and naturally exposed chimpanzees, demonstrating the utility of this test.

The susceptibility to tuberculosis (TB) of nonhuman primates in captivity is established (*1*,*2*), although the extent of the disease among free-living primates remains unclear. Much of our understanding of primate TB is based on documentation of *Mycobacterium tuberculosis* transmission in captive primates (*1*,*2*), but TB caused by *M. bovis* spillover dominates among populations of free-living monkeys (*3*,*4*). Research demonstrates increases in *M. tuberculosis* complex (MTC) infections among free-ranging macaques in areas of frequent human contact and high human TB prevalence (*5*). The first evidence of TB in a free-living ape was reported in 2009 in West Africa; the infectious agent was identified upon routine necropsy of a chimpanzee as a novel MTC strain closely related to lineage 6 (i.e., *M. africanum* West-Africa type-2) (*6*).

Epidemiologic studies of TB among free-living primates have been limited by existing diagnostic technologies. Diagnosis of disease in primates traditionally relies upon procedures that identify tissue lesions or demonstrate host immune responses or upon culture of the organism (*1*), methods that are generally not feasible for free-living species because of the need for handling and anesthesia. To overcome this challenge, we evaluated a novel approach using molecular detection of MTC-specific DNA in noninvasively collected fecal samples. This approach has shown excellent sensitivity among humans with active pulmonary TB (*7*,*8*). Our objective was to evaluate the performance of PCR amplification of IS *6110* of *M. tuberculosis* in fecal samples (fecal IS*6110* PCR) for noninvasive TB detection in inoculated and naturally exposed primates.

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The Study

Fecal IS*6110* PCR was first evaluated by using samples from primates with known TB infection status. Fecal samples were collected from 41 adult (>4 years) cynomolgus macaques (*Macaca fascicularis*) included in experimental *M. tuberculosis* infection studies and 13 uninfected rhesus macaques (*M. mulatta*) included in diabetes studies. All experiments and protocols were approved by institutional animal care and use committees at the University of Pittsburgh School of Medicine or University of Minnesota.

For concurrent studies, 36 cynomolgus macaques were inoculated with a low or mid dose (≈25 or 50–100 colony-forming units, respectively) *M. tuberculosis* Erdman strain by bronchoscopic instillation, as described (*9*); 5 animals were uninfected controls. samples from 10 macaques that had active disease, 23 animals characterized as latently infected, and 3 infected animals classified as subclinically diseased or "percolators" (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/3/14-0052-Techapp1.pdf) (*9*). Fecal samples were collected from all macaques on a single day, coinciding with varying durations of infection, ranging from 63 to 286 days (Table 1). The online Technical Appendix includes details on disease development and infection status classification.

Fecal IS*6110* PCR was also evaluated in primates under conditions of natural exposure and infection. Fecal samples were collected from 36 juvenile and adult (7–27 y, mean 15 y) chimpanzees (*Pan troglodytes*) managed in 2 sanctuaries and 1 zoo in East Africa. Housing and management are described in the online Technical Appendix. All animals were considered to be clinically healthy during sampling. Fecal PCR results of sanctuary chimpanzees were compared with their most recent tuberculin skin test (TST) responses (*10*). TSTs were performed opportunistically on 27 chimpanzees during routine exams on the same day as fecal collection. For the remaining 9 animals, TST results were available from 9 months before fecal collection for 3 chimpanzees and from 2 years before for 6 chimpanzees. In addition to TST, results from the PrimaTB STAT-PAK (Chembio Diagnostic Systems, Inc., Medford, New York, USA), a field-based serologic assay, were also available for 6 animals.

We extracted DNA from fecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Inc., Valencia,

Table 1. Fecal IS*6110* PCR results for detection of tuberculosis among cynomulgus and rhesus macaques, by infection status, inoculation dose, and time to sampling

Species and infection status	Inoculation dose	No. animals	Time postinoculation for sampling, mo	No. PCR positive
Cynomolgus				
Active	Mid	4	2	2
	Mid	1	5	1
	Mid	1	6	0
	Low	2	7	1
	Low	1	8	1
	Low	1	9	0
Latent	Mid	4	2	0
	Mid	1	5	0
	Mid	4	6	0
	Low	3	7	0
	Low	4	8	0
	Low	4	9	1
	Low	3	10	1
Subclinical	Low	2	7	1
	Low	1	8	0
Uninfected	N/A	5	NA	0
Rhesus				
Uninfected	N/A	13	NA	0

CA, USA). Feces-free negative controls were included in all extraction procedures. Conventional and real-time PCR were used to amplify a portion of the IS*6110* insertion sequence. Primers, master mixes, and thermocycling conditions are included in Table 2. For conventional PCR, amplicons of target size were confirmed as IS*6110* by Sanger sequencing (University of Minnesota Genomics Center, St. Paul, Minnesota, USA). Nuclease-free water (QIAGEN) negative controls were included in all amplification reactions. The online Technical Appendix contains additional methodological details.

Conclusions

Fecal IS*6110* PCR was effective in identifying 5 of 10 inoculated macaques with active disease and 8 of 36 total infected macaques. No uninoculated macaques were positive by results of IS*6110* PCR. Conventional PCR identified 3 actively infected macaques and real-time PCR identified 2 additional active infections. Two latently infected macaques and 1 with subclinical infection were also positive by using IS*6110* PCR. Overall sensitivity for this testing method was 22% (95% Wilson CI 12%–38%) and specificity was 100% (95% Wilson CI 82%–100%). Sensitivity of detection of active infections was estimated at 50% (95% Wilson CI 24%–76%). The latter sensitivity estimate is equivalent to that of gastric aspirate of children with radiographic evidence of pulmonary TB (*11*).

The observed sensitivity of fecal IS*6110* PCR is limited by several factors. Unlike immunologic tests, the success of this approach relies on bacterial shedding in sputum, subsequent swallowing, and excretion in feces; hence, active infection. Thus, most latent infections may go undetected, as observed in this study. Aside from outbreaks, identifying large numbers of actively infected primates for test validation is challenging. We sampled animals in experimental infection studies, but even so, active infections were few. Also, low numbers of organisms are likely shed intermittently in feces; thus, serial testing of multiple fecal samples may improve diagnostic sensitivity. PCR may also be paired with mycobacterial culture of feces for further molecular characterization of infection (*8*). Overall, this study demonstrates that fecal detection of mycobacterial DNA is best suited for identifying actively infected primates, which are crucial in TB transmission.

Table 2. Fecal IS*6110* conventional and real-time PCR master mixes and reaction conditions for investigation of noninvasive tuberculosis detection in primates

PCR type	Primers, 5′ → 3′	Master mix	Reaction conditions
Conventional	Forward: TTCAGGTCGAGTACGCCTTC Reverse: CGAACTCAAGGAGCACATCA	12.5 μL HotStarTaq Master Mix:* 8 μL RNase-free water,* 0.4 μM of each primer, 1.25 μL DMSO, 0.25 μL 1% BSA, 1 μL DNA template. Total volume 25 μL	95°C for 15 min/DNA polymerase activation; 40 cycles: 94°C for 30 s/denaturation, 56°C for 30 s/annealing, 72°C for 1 min/extension. Termination at 72°C for 10 min
Real-time	Forward: AGAAGGCGTACTCGACCTGA Reverse: CCGGATCGATGTGTACTGAG	LightCycler 480 Probes Master:† 0.2 mM of each primer, 0.2 mM of the FAM-labeled IS*6110* probe,† 5 μL DNA template. Total volume 25 μL	95°C for 5 min; 45 cycles: 95°C for 10 s/denaturation; 50°C for 30 s/annealing; 72°C for 1 s/extension. Termination at 65°C–95°C at 2.2°C/s/melting curve analysis

*QIAGEN, Inc., Valencia, CA, USA.
†Roche, Indianapolis, IN, USA.

TST conversion was not observed in any chimpanzees; however, IS*6110* DNA was detected in 3 chimpanzee fecal samples. TST was conducted the same day as fecal sampling for 1 of these animals, 9 months before for 1 animal, and 2 years before for 1 animal. TST is a common TB screening method used in primate sanctuaries but it is limited by sensitivity and specificity (*1*). Although this limitation can be overcome with Bayesian methods to estimate sensitivity and specificity for test validation purposes, the challenge remains in effectively identifying populations of captive primates with TB. Unfortunately, confirmation of infection status by additional diagnostic testing modalities of the 3 fecal PCR-positive chimpanzees has been limited.

Test results for 1 fecal PCR–positive chimpanzee demonstrated an immunological response to *M. tuberculosis* antigen by using the PrimaTB STAT-PAK, but culture of a bronchoalveolar lavage (BAL) sample was unsuccessful. Another chimpanzee, positive by fecal PCR, retested positive the next year by fecal IS*6110* PCR. The body size of this 14-year-old male that was historically TST negative was stunted (e.g., reduced growth) compared with other male chimpanzees of similar age.

These circumstances demonstrate the complexity of TB diagnosis and the challenges surrounding successful validation of TB tests in the natural setting. To reach a more complete understanding of diagnostic performance of fecal IS*6110* PCR in a natural setting where disease prevalence is low, large-scale and long-term testing across many captive primate populations is still needed.

Fecal IS*6110* PCR is a novel approach to the noninvasive detection of TB infection in primates, offering a new opportunity to screen for TB in free-living primates. IS*6110* detection is advantageous for its MTC specificity, which is optimal given the known susceptibility of primates to *M. bovis*, *M. tuberculosis*, and the recently discovered strain known as chimpanzee bacillus. This approach offers new direction for the epidemiologic investigation of tuberculosis in free-living primate populations.

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Vertical Transmission of Bacterial Eye Infections, Angola, 2011–2012

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To determine transmission rates for neonatal conjunctivitis causative microorganisms in Angola, we analyzed 312 endocervical and 255 conjunctival samples from mothers and newborns, respectively, during 2011–2012. Transmission rates were 50% for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* and 10.5% for *Mycoplasma genitalium*. Possible pathogenic effects of *M. genitalium* in children's eyes are unknown.

Ophthalmia neonatorum (neonatal conjunctivitis) can be easily prevented with prophylactic administration of topical antimicrobial drugs, as recommended by global guidelines for the management of sexually transmitted infections (*1*). However, in Angola, routine prenatal and prophylactic care to prevent this disease in newborns is lacking. In 2009, a project aimed at developing a national program for ophthalmia neonatorum prophylaxis in all maternity wards of Angola was started through the Spanish Agency for International Cooperation and Development and with the collaboration of the Angolan Ministry of Health. A previous pilot study in Luanda, Angola, showed that clinical cases of acute conjunctivitis among newborns were frequent; ≈12% of infants were born with bilateral acute conjunctivitis (*2*). However, the absence of microbiology laboratory resources at the study site resulted in the inability to determine the causative pathogens. One of the phases of the program was to analyze the usefulness of molecular biology tests to detect 2 of the most common sexually transmitted pathogens associated with transmission from the mother to the eye of the child—*Chlamydia trachomatis* and *Neisseria gonorrhoeae* (*3*)—and an emerging third pathogen (*4*), *Mycoplasma genitalium*. Our aim with this study was to assess the frequency of these 3 infections in a sample of mothers and their newborns in Angola and to determine the rate of vertical transmission.

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The Study

In this prospective, observational study, from December 2011 through February 2012, pregnant women and their newborns were recruited from 2 obstetric clinical wards at the Augusto N'Gangula Hospital and the Health Center of Samba in Angola. Study participation was voluntary. Specific informed consent was obtained from all participants before specimens were collected. Approval of the study protocol for scientific and ethical aspects was obtained from the Ethical Commission of the School of Medicine of the Agostinho Neto University (Luanda, Angola). The study was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and later amendments.

Included in the study were mothers who were healthy (with the possible exception of genitourinary disease) and their newborns with a gestational time of 37–40 weeks and a weight of ≥2.3 kg. Endocervical samples were obtained consecutively from pregnant women who agreed to participate in the study and were collected after removal of postpartum secretions from the endocervical os. Ocular samples were obtained from both eyes of the newborns by vigorous swabbing across the inferior tarsal conjunctiva. Samples from each eye were then pooled for analysis. After sampling, the newborns were prophylactically given 5% povidone iodine eyedrops.

Samples were collected with flocked swabs in Universal Transport Medium (Copan Italia S.p.A., Brescia, Italy), stored at –70°C, and shipped to the Department of Microbiology at the Hospital Clínico Universitario of Valladolid, Valladolid, Spain. DNA extraction was performed according to routine laboratory standards with the GXT DNA/RNA reagents in a GenoXtract extractor (Hain-Lifescience, Nehren, Germany). A multiplex PCR that co-amplified DNA sequences of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, and an internal control was performed by using the Bio-Rad Dx CT/NG/MG assay (Bio-Rad, Hercules, CA, USA) (*5*). The primers and probe for *C. trachomatis* targeted a sequence of the cryptic plasmid located outside the region deleted in the new variant strain

of *C. trachomatis* (*6*). For *N. gonorrhoeae*, the target was a sequence in the *pilE* gene that rarely yields false-positive results. For *M. genitalium*, the target was a sequence in the *MgPa* gene. To avoid laboratory or sampling errors, we processed the samples from the mothers and newborns separately and included positive and negative controls in the PCRs. Amplification and detection were performed in a 7500Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Statistical analyses were performed by using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria).

A total of 567 samples were analyzed (Table 1). Results of detection by multiplex PCR were considered for prevalence estimates of the 3 infections (Table 2). Transmission rates were 50% for *C. trachomatis* and *N. gonorrhoeae* and 10.5% for *M. genitalium*. For 7 infected mothers (2 *C. trachomatis*–infected and 5 *M. genitalium*–infected), no samples could be obtained from their newborns.

Conclusions

The microorganism most frequently found among mothers was *M. genitalium*, and the microorganism most frequently found among newborns was *C. trachomatis*. Despite the association of *M. genitalium* with sexually transmitted infections in men and women (*4*), we are aware of only 1 other case of conjunctivitis associated with *M. genitalium* (*7*).

Only 1 mother was co-infected by 2 microorganisms, *C. trachomatis* and *M. genitalium*, consistent with the low rate of this co-infection found in other studies (*8*). This mother gave birth to 1 of the 4 *C. trachomatis*–infected newborns. Not answered by this study is the hypothetical role of *M. genitalium* as a co-factor for transmission of other major sexually transmitted pathogens. *M. genitalium* has also been studied as a possible contributor to the pathogenesis of trachoma in a trachoma-endemic area of Tanzania, but no evidence was found regarding its contribution (*9*). Our findings regarding *M. genitalium* infections in this sample need further study because the relatively small number of infected mothers and children can be a confounding factor and because the real prevalence of *M. genitalium* needs to be assessed by larger studies.

The frequency of *C. trachomatis* infection among the study sample (mothers and newborns), 2.1%, was lower than that found in previous studies in different populations in Africa (*10*). The frequency of *N. gonorrhoeae* infection among the study sample, 0.5%, was also lower than that found in other African countries, such as the Central African Republic (3.1%) and South Africa (7.8%) (*11*). The frequency of *M. genitalium* infection among the study mothers, 6.1%, was slightly higher than that found in other studies of asymptomatic women (*8*).

Table 1. Samples collected for analysis of vertical transmission of eye infections, Angola, 2011–2012

Sample type	Augusto N'Gangula	Health Center of Samba	Total
Endocervical	169	143	312
Conjunctival*	130	125	255
Total	299	268	567

*For reasons other than exclusion criteria, samples from 57 newborns were not obtained.

Rates of transmission from the mother to the eye of the child differed markedly for each of the 3 microorganisms studied. The rate of *M. genitalium* transmission was much lower than that for *C. trachomatis* and *N. gonorrhoeae*. Vertical transmission of *M. genitalium* is uncommon; we are aware of only 1 reported case (*12*). Although *M. genitalium* is among the most prevalent pathogens in this sample of mothers in Africa, vertical transmission of this microorganism was less frequent than that of other classical sexually transmitted pathogens like *C. trachomatis* and *N. gonorrhoeae*. However, high prevalence in any maternal population, even with a relative low rate of transmission, could lead to a large number of neonatal infections.

All infected children were from mothers who were also infected. It can be assumed that, as with HIV infection, detection of any of the assayed microorganisms in conjunctival samples of newborns predicts diagnosis in their mothers (*13*). This so-called mirror effect is clinically useful in view of cultural behaviors that would complicate detection of sexually transmitted pathogens in adult women, especially in geographic settings of Muslim practices (*14*).

The major limitation of this study was the difficulty of correctly preserving samples in the hot and humid environment of Angola. To prevent DNA denaturation, samples were frozen immediately after collection and shipped as soon as possible. Unfortunately, those precautions precluded the culturing of samples, so PCR results could not

Table 2. Prevalence of microorganisms among mothers and newborns from Augusto N´Gangula Hospital and Health Center of Samba in Luanda, Angola, 2011–2012

Microorganism, sample type	No. positive/ no. collected	Prevalence, % (95% CI)
Chlamydia trachomatis		
Endocervical	8/312	2.6 (1.3–4.9)
Conjunctival	4/255	1.6 (0.6–3.9)
Total	12/567	2.1 (1.2–3.6)
Neisseria gonorrhoeae		
Endocervical	2/312	0.6 (0.2–2.3)
Conjunctival	1/255	0.4 (<0.1–2.2)
Total	3/567	0.5 (0.2–1.5)
Mycoplasma genitalium		
Endocervical	19/312	6.1 (3.9–9.3)
Conjunctival	2/255	0.8 (0.2–2.8)
Total	21/567	3.7 (2.4–5.6)
All 3 microorganisms		
Endocervical	28/312*	9.0 (6.3–12.6)
Conjunctival	7/255	2.7 (1.3–5.5)
Total	35/567*	6.2 (4.5–8.5)

*One mother was co-infected with *C. trachomatis* and *M. genitalium*.

be compared with culture results. Another disadvantage was the lack of more precise clinical information from participants because of the absence of antenatal care for most.

Our study findings indicate that new molecular techniques will help microbiological diagnosis of neonatal conjunctivitis in Africa. They also show the need for a national program for neonatal conjunctivitis prophylaxis in Angola, taking into account the most frequent causative microorganism.

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Increased Risk for Multidrug-Resistant Tuberculosis in Migratory Workers, Armenia

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To understand use of tuberculosis (TB) services for migrant workers, we conducted a cross-sectional census of 95 migrant workers with TB from Armenia by using medical record reviews and face-to-face interviews. Prolonged time between diagnosis and treatment, treatment interruption, and treatment defaults caused by migrant work might increase the risk for multidrug-resistant TB.

Economic transition after the breakup of the former Soviet Union pushed many Armenians to look for new job opportunities in other countries (*1*–*3*). A total of 15.0% of Armenian families include migrant workers who regularly seek seasonal work in host countries and return on completion of that work (*1,2*). Armenia is a country in the southern Caucasus and has a population of 3 million persons (*4*) who face a reemerging threat from tuberculosis (TB). According to the National TB Control Program, the incidence of TB/100,000 persons increased from 16.6/10,000 persons in 1990 to 62.4/100,00 persons in 2005 (*5*). This threat is exacerbated by increased rates of drug-resistant TB (*6*).

Among newly diagnosed TB cases in Armenia, ≈9.4% are multidrug-resistant TB (MDR TB), and 43.0% of previously treated TB cases become MDR TB cases (*5*–*7*). Armenia reported 7 cases of extensively drug-resistant TB in 2011 (*5*). The purpose of this study was to evaluate how characteristics of migrant workers influence use of TB services in the host country of work and in the country of origin and TB treatment outcomes.

The Study

Study protocols were approved by the American University of Armenia Institutional Review Board. Given the lack of data on TB among migrant workers, we contacted TB physicians working in all 72 outpatient TB centers in Armenia. The attending TB physicians were familiar with the migrant worker status of their patients, and a list of their patients formed a census study frame for all migrant workers with TB. All members of the study population had a diagnosis of TB. A study participant had to be a migrant worker outside Armenia for ≥3 months during 2008–2011, be ≥18 years of age, have Armenian citizenship, and speak Armenian. We collected data from eligible study participants in the census study time frame through face-to-face interviews and abstraction of data from medical records. After considering national data on TB prevalence in Armenia and the number of migrant workers with a TB diagnosis provided by TB physicians and confirmed during the study, we estimated that ≈7.0% of all TB patients treated during 2008–2011 in Armenia were migrant workers.

We attempted to contact all 126 eligible migrant workers in Armenia at the time of the survey (January–March 2012) and recruited 95 study participants (75.0% response rate). Approximately 91.0% of study participants had either a high school or professional technical education, 85.0% were from low-income households, and ≈33.0% were registered in a social support program for persons living in poverty. Median ages of surveyed persons and the population with TB in Armenia in 2012 were similar (46 and 45 years, respectively). The 95 study participants reported 166 visits for seasonal work to other countries during 2008–2011; the Russian Federation was the most common destination (147 visits) (Figure). The type of work in which study participants were involved outside Armenia was construction (65.0%), driving/transportation (11.0%), commerce (8.0%), production (7.0%), services (5.0%), food industry (4.0%), and farming (1.0%). For ≈63.0% of all host country work visits, work was in confined spaces, of which 23.0% had >6 persons working in the same enclosed space. For 7.0% of all visits, respondents reportedly resided in overcrowded conditions.

Medical record reviews of 95 study participants indicated that 28.3% (26/92) of respondents had MDR TB and 5.3% (5/94) had HIV/TB co-infection compared with 5.6% and 3.1%, respectively, for all TB patients in Armenia reported by the National TB Control Program in 2011 (Table 1). All study participants were treated for TB at least once, 73.6% (70/95) were reportedly treated only once, 13.7% (13/95) were treated twice, 12.6% (12/95) were treated ≥3 times, and 44.2% (42/95) of migrant workers were reportedly first given a diagnosis of TB in the host country of work.

The period between first diagnosis and first treatment was reportedly ≈5 times longer (95% CI 1.9–34.8, p = 0.06) for those who were given a diagnosis in the host country of work than for those given a diagnosis in Armenia (Table 2). Approximately 92.5% (86/93) workers reported that they received inpatient hospital care during their first TB

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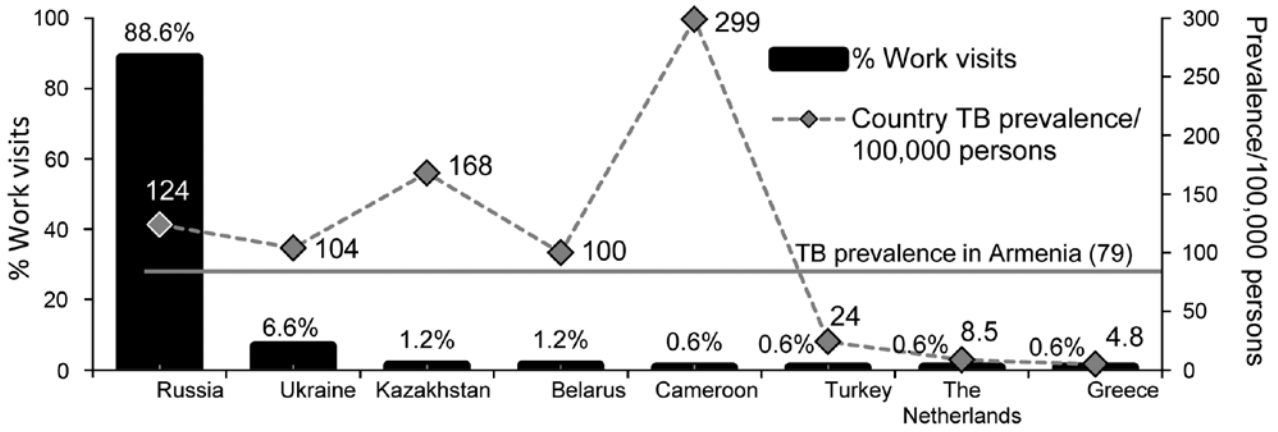

Figure. Percentage of work visits by migrant workers to host countries-of-work and tuberculosis (TB) prevalence/100,000 persons in host countries compared with that in Armenia, 2008–2011 (*7*).

treatment, of whom 5 reportedly did not complete the full course of inpatient treatment. The mean duration for the first inpatient TB treatment for participants who received TB treatment in Armenia was 78 days (range 40–425 days). For those who received their first inpatient treatment in the host country of work, the mean duration was 164 days (range 20–912 days). Those workers who received treatment in the host country of work were 3.9 times more likely to have a failed or defaulted treatment outcome than those who received treatment in Armenia (95% CI 11.4–74.1, p = 0.001). However, only 13.7% (13/95) of respondents received treatment outside Armenia. Of those reporting, 9.3% (8/86) experienced an interruption of prescribed TB treatment for ≥1 day during the inpatient phase of their first treatment (mean duration of interruption 7 days, range 1–30 days).

During the ambulatory phase of their first treatment, 20.4% (19/93) of respondents reported treatment interruptions (mean duration of longest interruption 7.8 days, range 1–60 days). Approximately 80.8% (55/68) of persons who completed their first treatment for TB reported that they completed the treatment (cured or completed treatment with no treatment failure), 11.7% (8/68) reported defaulting (lost-to-follow-up), and 7.4% (5/68) reported as being sputum-smear-positive at the end of treatment (treatment failed) (*8*). Bivariate analysis showed that patients who had incomplete ambulatory treatment had greater odds of having MDR TB than having drug-sensitive TB than did those who completed ambulatory treatment (odds ratio [OR] 4.0, 95% CI 1.6–10.1, p = 0.003).

Multivariate logistic regression showed that persons who did not receive standardized TB treatment with supervision during the ambulatory phase of treatment had 4 times greater odds (OR 4.4, 95% CI 1.3–14.9, p = 0.02) of having ≥2 TB treatments (versus only 1 TB treatment) than persons who received standardized treatment with supervision during the ambulatory phase. After adjusting for confounding, we found that persons who had MDR TB had 2 times greater odds (OR 2.3, 95% CI 1.1–5.0, p = 0.04) of having ≥2 TB treatments (versus only 1 TB treatment) than persons who had TB (Table 3, http://wwwnc.cdc.gov/EID/article/21/3/14-0474-T3.htm).

Conclusions

We found that among migrant workers with a diagnosis of TB, migratory work was associated with higher rates of MDR TB and HIV co-infection, which suggested that migratory work may provide impetus for spread of HIV infection and TB. Treatment for TB that started in the host country of work was usually interrupted because migrant workers wanted to return to Armenia. Some of these workers did not resume treatment for TB in Armenia. Those migrant workers with TB who experienced treatment delays, dropped out of therapy, or had therapy interrupted were likely to increase the period of infectivity and spread TB to other persons.

Table 1. Characteristics of 126 migrant workers and all TB patients in Armenia, 2008–2011*

Characteristic	No. positive/no. tested (%)		Year, no. (%) patients			
	Survey participants, n = 95	Refused to participate, n = 31	2008, n = 2,125	2009, n = 2,006	2010, n = 1,780	2011, n = 1,584
HIV/TB co-infection	5/94 (5.3)	1/30 (3.2)	12 (0.6)†	17 (0.9)†	17 (1.0)†	49 (3.1)
MDR TB	26/92 (28.3)	5/31 (16.1)	77 (3.6)†	134 (6.7)†	154 (8.7)†	88 (5.6)†
Extrapulmonary TB	1/94 (1.1)	4/31 (12.9)†	380 (17.9)†	374 (18.6)†	429 (24.1)†	361 (22.8)†
SS+ at time of diagnosis	47/93 (50.5)	15/31 (48.4)	679 (32.0)†	561 (28.0)†	434 (24.4)†	420 (26.5)†

*MDR TB, multidrug-resistant tuberculosis; SS+, sputum smear positive.
†Significant differences (p≤0.001) vs. noninfected survey participants.

Table 2. Time intervals between first symptoms, diagnosis, and tuberculosis treatment for migrant workers, Armenia, 2008–2011

Interval	Mean (SD) duration, mo		p value
	Armenia	Host country of work	
First symptoms–first diagnosis	3.1 (6.3)	1.7 (3.1)	0.23
First diagnosis–first treatment	0.6 (1.9)	3.1 (9.3)	0.06*
First symptoms–first treatment	3.8 (6.6)	3.8 (8.5)	0.54

*Marginally significant difference (0.1≤p≤0.05).

There is no official referral system between the Armenian National TB Program and their counterparts in host countries of work. On the basis of our study findings, we recommend establishing closer collaboration between health systems of countries supplying migratory workers and host countries for work at governmental and nongovernmental levels to improve treatment completion rates and reduce adverse outcomes. This study could become a basis for further efforts in understanding the mechanisms that impede access of immigrants to treatment and planning target strategies for early detection of TB and treatment in this group of patients. It could also become an example for further large-scale projects worldwide, especially in the Commonwealth of Independent States region to explore the correlation between migrant labor and TB.

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Endemic and Imported Measles Virus–Associated Outbreaks among Adults, Beijing, China, 2013

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In 2013, a resurgence of measles occurred in Beijing, China. The outbreaks occurred among adults and were associated with endemic genotype H1 and imported genotype D8 viruses. Migrant workers were disproportionately represented in the outbreaks; thus, vaccinating such workers against measles may be an effective strategy toward the elimination of this disease.

All 6 World Health Organization (WHO) regions have set goals to eliminate measles (*1*,*2*). In China, a nationwide measles supplementary immunization activity was conducted in 2010, and the incidence of measles in mainland China subsequently reached its lowest reported level in 2012 (6,183 cases, 4.6 cases/million total population). However, in 2013, a nationwide resurgence of measles occurred primarily among young, unvaccinated children (*3*). In contrast to the nationwide resurgence, the measles resurgence in China's capital, Beijing, was primarily among adults ≥15 years of age (65.7% of cases) and occurred in large, clothing wholesale markets.

Beijing has >12.96 million permanent residents and an additional ≈7.73 million floating residents (i.e., internal migrants who move into the city, usually for employment) (*4*). The routine measles vaccination schedule in use in Beijing consists of 3 doses of measles-containing vaccine; the first dose is administered at 8 months, the second at 18 months, and the third at 6 years of age. Also, since 2006, an additional dose has been administered to college students who move to Beijing to study (*5*). In this study, we used genotype analysis to describe the measles outbreaks among adults in Beijing, and we suggest an immunization strategy to help prevent similar outbreaks in the future.

The Study

In early 2013, a resurgence of measles in Beijing was reported to the China Information System for Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing); by the end of the year, a total of 1,233 suspected cases had been reported and investigated. Serum and throat swab samples were collected from 97.3% and 96.8% of the suspected case-patients, respectively. The samples were tested for measles IgM by using the VIRION SERION ELISA Measles Virus IgM test (Virion/Serion, Wurzburg, Germany) or for measles virus genes by using a real-time reverse transcription PCR kit (Jiangsu Bioperfectus Technologies, Jiangsu, China). Of the 1,233 samples, 558 were positive, and 5 additional cases were confirmed by epidemiologic linkage. Thus, a total of 563 measles cases in Beijing were confirmed in 2013; this number represents a 6-fold increase from the number of cases in 2012.

The number of reported cases was highest during March–May. Most cases occurred among a floating population of adult migrant workers and permanent adult residents and their children (Figure); 67.3% of the cases in adults were in migrant workers. Reports of cases increased shortly after the national holidays associated with the spring festival, during which many persons travel to visit relatives.

Among the persons with confirmed measles, 22.8% (128) were <8 months of age, 11.5% (65) were 8 months–14 years of age, and 65.7% (370) were ≥15 years of age and defined as adult patients. The median age of the adult patients was 23.5 years (range 15.0–70.0 years). Vaccination history was unknown for 87.6% (324/370) of the adults. Among the 65 patients in the age group targeted for measles vaccination, 36.9% (24) had not been vaccinated because of contraindications.

We used real-time reverse transcription PCR–positive samples to try to isolate and genotype virus from 468 of the 563 positive samples. Genomic sequencing and phylogenetic analyses were based on N450 nucleotide sequences, as previously described (*6*–*8*). Measles genotype was determined for 45.6% (257/563) of the cases reported from 14 of 16 districts or counties. Among those 257 cases, 84.0% (216) were caused by endemic genotype H1 viruses and 15.2% (39) and 0.78% (2), respectively, were caused by imported genotype D8 and D9 viruses. All 14 districts had confirmed measles cases caused by genotype H1 viruses, and 6 and 2 districts, respectively, had cases caused by genotype D8 and D9 viruses. Of the 257 measles cases with genotype information, 61.6% (133) of those caused by

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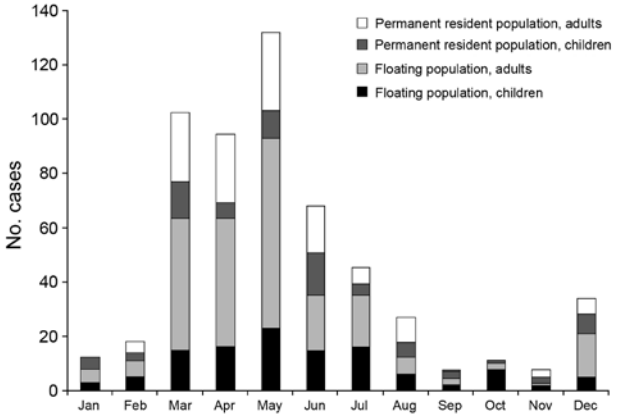

Figure. Number of measles cases among persons by residency status, age, and month of infection onset, Beijing, China, 2013. Floating populations represent internal migrants who move to an area temporarily, usually for employment (e.g., migrant workers).

genotype H1 and 97.4% (38) of those caused by genotype D8 were in adults (Table).

Measles viruses within a transmission chain had identical or nearly identical N450 sequences (*9*). Phylogenetic analysis showed that 216 genotype H1 viruses were associated with 30 different chains of transmission (online Technical Appendix Figure, panels A, B, http://wwwnc.cdc.gov/EID/article/21/3/14-0646-Techapp1.pdf), and 39 genotype D8 viruses were associated a single chain of transmission, although 1 virus differed by only 1 nt (online Technical Appendix Figure, panels A, C). Nucleotide sequences of 43 representative viruses were deposited in GenBank (accession nos. KJ556851–93). A search of the World Health Organization's Measles Nucleotide Surveillance (MeaNS) database (*10*) showed that genomic sequences of genotype D8 viruses from the outbreak shared 99.8%–100% nucleotide identity with genomic sequences of strains from measles patients in Russia, France, Canada, Thailand, Denmark, Germany, and other locations.

Genotype D8 measles viruses were associated with at least 2 outbreaks in different large, wholesale clothing markets. The outbreaks occurred during March–July 2013 and were almost completely confined to adults; only 1 child was infected, possibly because of high coverage of measles-containing vaccine among Beijing children. We were unable to identify the source of the infections. Phylogenetic analysis suggested that the genotype D8 virus might have been imported to Beijing from Europe, America, or another location (*10–13*) and subsequently spread beyond Beijing by virus transmission from infected adults (data not shown). For at least 21 years, genotype H1 measles viruses have been the only endemic measles circulating in China (*6–8*); measles cases caused by all other genotypes have been associated with imported viruses (*7*).

Conclusions

Our findings show that transmission of measles virus among adults contributed to a resurgence of measles in Beijing during 2013. Nonendemic genotype D8 measles viruses were associated with at least 2 outbreaks in different wholesale clothing markets during March–July, 2013. Many persons from domestic and international areas visit wholesale markets every day; thus, such markets are high-risk settings for the transmission and importation of measles viruses (*14*).

Because migrant workers were disproportionately affected in the Beijing outbreaks and because their work settings have high measles transmission potential, we support an outreach strategy to prevent measles among this floating population. These workers usually live with their families and register with the local authorities for government services. Thus, we suggest that the offer of measles vaccine to workers as they register to live and work in the commodity markets might be a reasonable strategy to prevent future measles outbreaks. Serologic surveys can be used to refine such a strategy by assessing immunity within the target population.

The foundation strategy for eliminating measles globally is based on the timely vaccination of young children with 2 valid doses of measles-containing vaccine. However, laboratory-supported surveillance analysis and outbreak investigations are critical to the identification of gaps in immunity among older age groups, which may need to be filled, and to the identification of strategies to prevent similar outbreaks. The fact that more than a third of infected children in the vaccine-targeted age group were unvaccinated because of vaccination contraindications suggests that an evaluation is needed to ensure the use of valid contraindications only.

It is difficult to identify narrow, age-based risk groups to target for vaccination when a high proportion of adults are unvaccinated and may still be susceptible to measles. Unselective vaccination of millions of adults, based solely on their age, is unlikely to be feasible in China. Additional risk factors for measles need to be identified to develop more feasible immunization strategies.

Table. Age distribution of measles case-patients for whom measles virus genotype information was available, Beijing, China, 2013

Age group	H1	No. (%) genotype	
		D8	D9
0–7 mo	31 (14.3)	1 (2.6)	1 (50.0)
8 mo–14 y	52 (24.1)	0	0
≥15 y	133 (61.6)	38 (97.4)	1 (50.0)

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Mycobacterium bovis Infection in Humans and Cats in Same Household, Texas, USA, 2012

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Mycobacterium bovis infection of cats is exceedingly rare in regions where bovine tuberculosis is not endemic. We describe the diagnosis and clinical management of pulmonary *M. bovis* infection in 2 indoor-housed cats and their association with at least 1 *M. bovis*–infected human in Texas, USA, in September 2012.

Tuberculosis in humans and animals results from infection by bacilli within the *Mycobacterium tuberculosis* complex (*1*). Despite ≈99.95% genome sequence identity, *M. bovis* and *M. tuberculosis* exhibit distinct differences in host adaptation and susceptibility (*2*). *M. bovis* is the primary causative agent of bovine tuberculosis and infects a wider range of hosts than *M. tuberculosis*. In domestic cats, tuberculosis is caused primarily by infection with *M. bovis* or *M. microti* (*3*–*5*); *M. tuberculosis* infection is less common (*6*).

Before implementation of bovine tuberculosis control programs and wide-scale pasteurization of milk, alimentary tract disease was the most common form of tuberculosis in cats (*7*); today, lymphadenopathy and cutaneous forms are more common (*4*). Diagnosis is based on clinical examination, imaging, biopsy with histopathologic examination, culture of aspirates or tissues, and specific immune-based blood assays (*4*,*8*,*9*). Intradermal skin tests are generally unreliable for diagnosing tuberculosis in cats (*10*). Client history is critical for determining the possibility for exposure of the cat to the pathogen, and zoonotic aspects should be considered (*11*). We describe the diagnosis and clinical management of pulmonary *M. bovis* infection in 2 indoor-housed cats and their association with at least 1 *M. bovis*–infected human in Texas, USA.

Case Report

In September 2012, a 5-year-old female domestic cat (cat Y) was seen by a veterinarian for dyspnea, tachypnea, hyporexia, and lethargy. She lived indoors with 4 other cats and their female owner. The vague history provided by cat Y's owner indicated that, ≈11 months earlier, her husband had died of tuberculosis only 6 weeks after diagnosis and initiation of directly observed antimycobacterial therapy. At the time the husband's tuberculosis was diagnosed, the woman was Mantoux-test negative; ≈2 months after his death, she converted to skin-test positive but had normal findings on thoracic radiographs. She was subsequently treated with antimycobacterial drugs. The woman also reported that, in June 2012, another cat in the household was euthanized after clinical signs developed that were similar to those of cat Y; no necropsy was performed. Additional pertinent history included relocation of the deceased husband from Mexico to Texas 15 years earlier, frequent contact with recent immigrants from Central America and Mexico, and consumption of unpasteurized Mexican cheeses.

On examination, cat Y had a productive cough with blood-tinged sputum, increased respiratory effort, and dorsal muscle wasting. A complete blood cell count revealed an elevated total leukocyte count, elevated neutrophil count, and mild anemia, consistent with chronic inflammatory disease. Abdominal and thoracic radiographs (Figure 1) showed severe bronchointerstitial consolidating lung disease with poorly defined nodules, tracheobronchial lymphadenopathy, and mild hepatosplenomegaly. Lung aspiration and cultures were performed under general anesthesia. Bloody sputum was noted in the endotracheal tube. Aerobic and anaerobic cultures and acid-fast bacilli (AFB) stains of aspirated samples were negative, and cytologically the samples were identified as mixed-cell inflammation. Given the history and clinical suspicion of tuberculosis, rifampin, marbofloxacin, and clarithromycin were prescribed, along with isolation of cat Y in a separate room in the household. Cat Y responded favorably to empirical treatment with antimycobacterial drugs (Figure 1).

One month after cat Y was initially seen for care, all 5 cats in the household (cat Y, cats A–C, and cat G) were brought for screening thoracic radiographs and serology. Cats A–C and G exhibited no respiratory signs and appeared clinically healthy. Lung radiographs of cat G showed perihilar nodular changes and consolidation consistent with granulomas. The 5 cats were tested by 3 antibody-detection assays, MAPIA, TB STAT-PAK, and DPP VetTB (Chembio Diagnostic Systems, Inc., Medford, NY, USA), known to detect specific antibody in feline *M. bovis* or *M. microti* infection (*8*,*12*). Cats Y and G were seropositive by all 3 immunoassays upon initial

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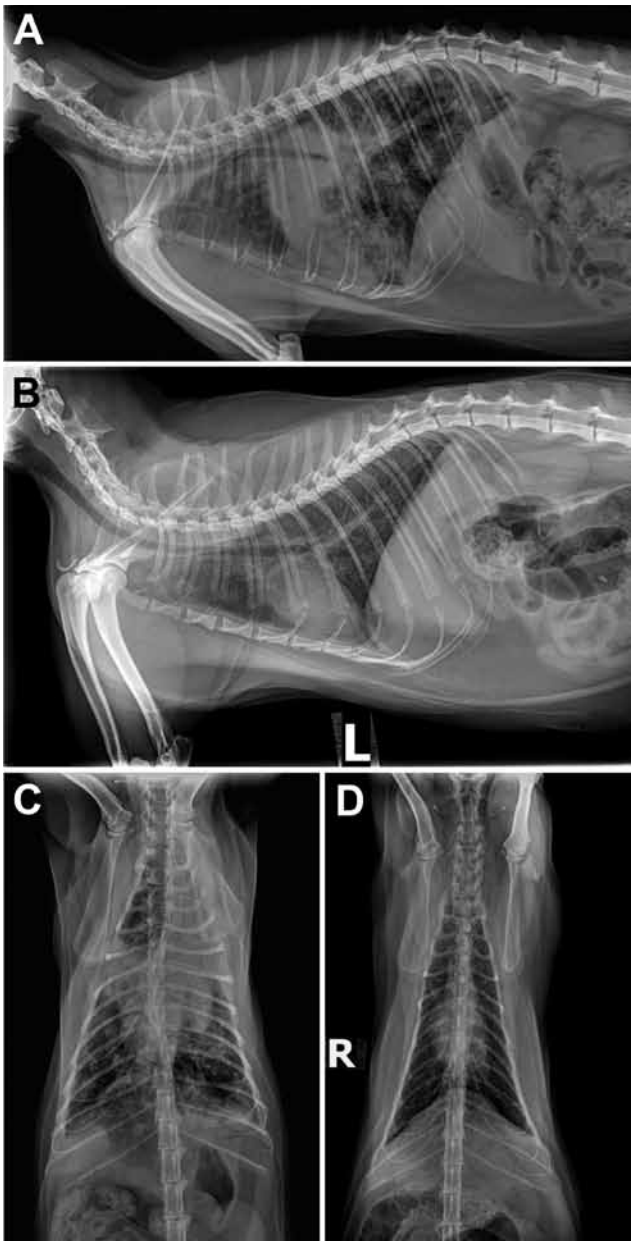

Figure 1. Radiograph images of cat Y showing pulmonary lesions before and after antimycobacterial treatment for *Mycobacterium bovis* infection, Texas, USA, 2012. A) Pretreatment, right lateral thoracic radiograph showing severe coalescing interstitial to alveolar pulmonary infiltrates before treatment. B) Posttreatment, left lateral thoracic radiograph after 2 months of marbofloxacin, rifampin, and a macrolide for 2 months in cat Y and then another 3.5 months of rifampin and marbofloxacin alone. C) Pretreatment, ventrodorsal view showing severe bronchointerstitial disease with poorly defined nodules or complete consolidation in the perihilar region, right middle lung lobe, and cranial segment of the left cranial lung lobe. D) Posttreatment, ventrodorsal thoracic radiographs after 2 months of triple antimycobacterial therapy and then another 3.5 months of rifampin and marbofloxacin alone. Considerable improvement occurred after therapy: the perihilar region cleared but a heavy interstitial marking throughout the lungs remained, most suggestive of fibrosis from scarring or, less likely, from smaller active granulomata.

testing; the other cats (cats A, B, and C) were seronegative (Figure 2). Serum from cats Y and G reacted with MPB70 and MPB83 antigens. Serum from cat G also reacted to ESAT-6 protein and its fusion with CFP10 (Figure 2, panel A). The feline antibody profiles were consistent with the known serologic immunodominance of these antigens in *M. bovis* infection reported for other host species (*13*). As with elephants (*14*), serum antibody levels in cats Y and G gradually declined after 1–3 months of antimycobacterial treatment (Figure 2, panel B). Cats A–C remained antibody negative in all 3 immunoassays during the same period (data not shown).

Given the clinical and serologic findings, the treating veterinarian contacted the appropriate public health authorities, and the owner was instructed to continue isolation of cats Y and G because of the clinical, serologic, and radiographic evidence suggestive of tuberculosis. Efforts to obtain her husband's medical records to aid in the cats' diagnosis had been refused until positive serologic test results for cats Y and G were produced (Figure 2), after which permissions from surviving family members were obtained. A local public health clinic, where the deceased husband had received diagnosis and treatment for pulmonary tuberculosis, provided results of sputum culture. These results showed that *M. bovis* had been isolated from his sputum; however, a previous investigation had not elucidated a definitive source of infection. After the cats' veterinarian received this new information, bronchoalveolar lavages and gastric washes were performed on cats Y and G. AFB were detected in cat G's gastric wash, and *M. bovis* was isolated by culture conducted at the National Veterinary Services Laboratories (Ames, IA, USA). No mycobacterial growth was obtained from cat Y, presumably because of the 7 weeks of antimycobacterial therapy before sampling. Antimycobacterial therapy (rifampin, marbofloxacin, and clarithromycin) was initiated for asymptomatic cat G. Whole-genome sequencing performed at the National Veterinary Services Laboratories revealed that the isolate from the husband and cat G were closely related but not identical; there were 8 single nucleotide polymorphisms (SNPs) between the 2 isolates (Table). As of April 8, 2014, all 5 cats and the owner remained clinically well.

Conclusions

Our findings highlight the possibility of *M. bovis* infection in an unlikely geographic locale (Houston, Texas) to which bovine tuberculosis is not endemic and an unlikely population (cats and humans living in the same household). The owners and their cats had no known contact with cattle or wildlife; however, the deceased husband had relocated to Texas from a country where bovine tuberculosis is considered endemic. The family also had

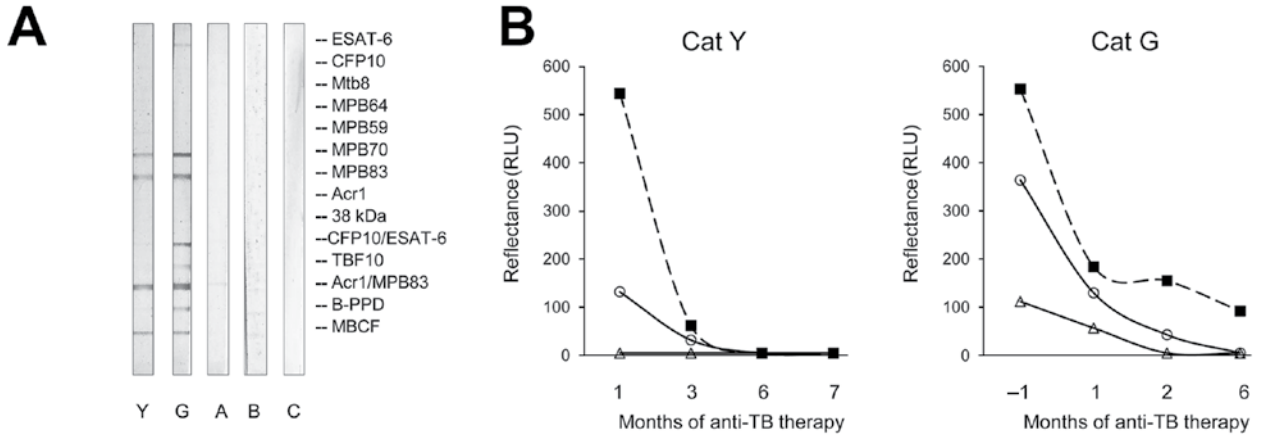

Figure 2. Antibody test results for cats tested for *Mycobacterium bovis* infection, Texas, USA, 2012. A) Antibody reactivity in infected (Y, G) and presumed noninfected (A–C) cats. Images show MAPIA strips processed with serum samples as described previously (*13,14*). Visible bands reflect the presence of IgG to *Mycobacterium bovis* antigens indicated in the right margin. B) Antibody levels in infected cats Y and G during antimycobacterial therapy. Intensity of test bands in TB STAT-PAK (solid squares) and DPP VetTB assay (circles, MPB83 antigen; triangles, ESAT-6/CFP10 antigen) was measured by optical reader. The TB STAT-PAK assay can detect both IgM and IgG; DPP VetTB detects IgG only (all assays from Chembio Diagnostic Systems, Inc., Medford, NY, USA).

frequent visitors and consumed unpasteurized dairy products from bovine tuberculosis–endemic countries. The cat that had died with signs consistent with pulmonary tuberculosis spent extended close contact with the owner, who died of pulmonary *M. bovis* infection. All cats in the household had close and frequent contact with both owners. Whole-genome sequencing indicated that the cat and human *M. bovis* isolates, although closely related, were either 3 or 5 SNPs from sharing a common ancestor. Previous research suggests variable SNP accumulations (0–2) per transmission event, which implies that direct transmission between the owner and cat G is unlikely (*15*). However, whether the isolates recovered reflect all the genotypes within the individual is unknown. A clinically relevant finding was the use of serologic testing to justify additional procedures necessary for isolating *M. bovis* and assisting with treatment monitoring. These findings highlight the complexity of diagnosing *M. bovis* infection in an unanticipated host and setting.

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Table. Single nucleotide polymorphism differences between the isolates from a cat and a human with *Mycobacterium bovis*, Texas, USA, 2012*

Genome position aligned to the reference *M. bovis* AF2122/97	1859572	3639316	586911	2567	2847080	3394685	3603337	80817
Reference call	**C**	**C**	**G**	**C**	**G**	**T**	**T**	**T**
12–9271_TX_Cat_Domestic	**T**	**A**	**A**	C	G	T	T	T
13–0751_TX_Human	C	C	G	**T**	**C**	**C**	**G**	**C**

*The sequences were aligned against the reference genome, *M. bovis* AF2122/97 (reference call). Boldface highlights the different amino acid at each single nucleotide polymorphism between the 2 genotypes.

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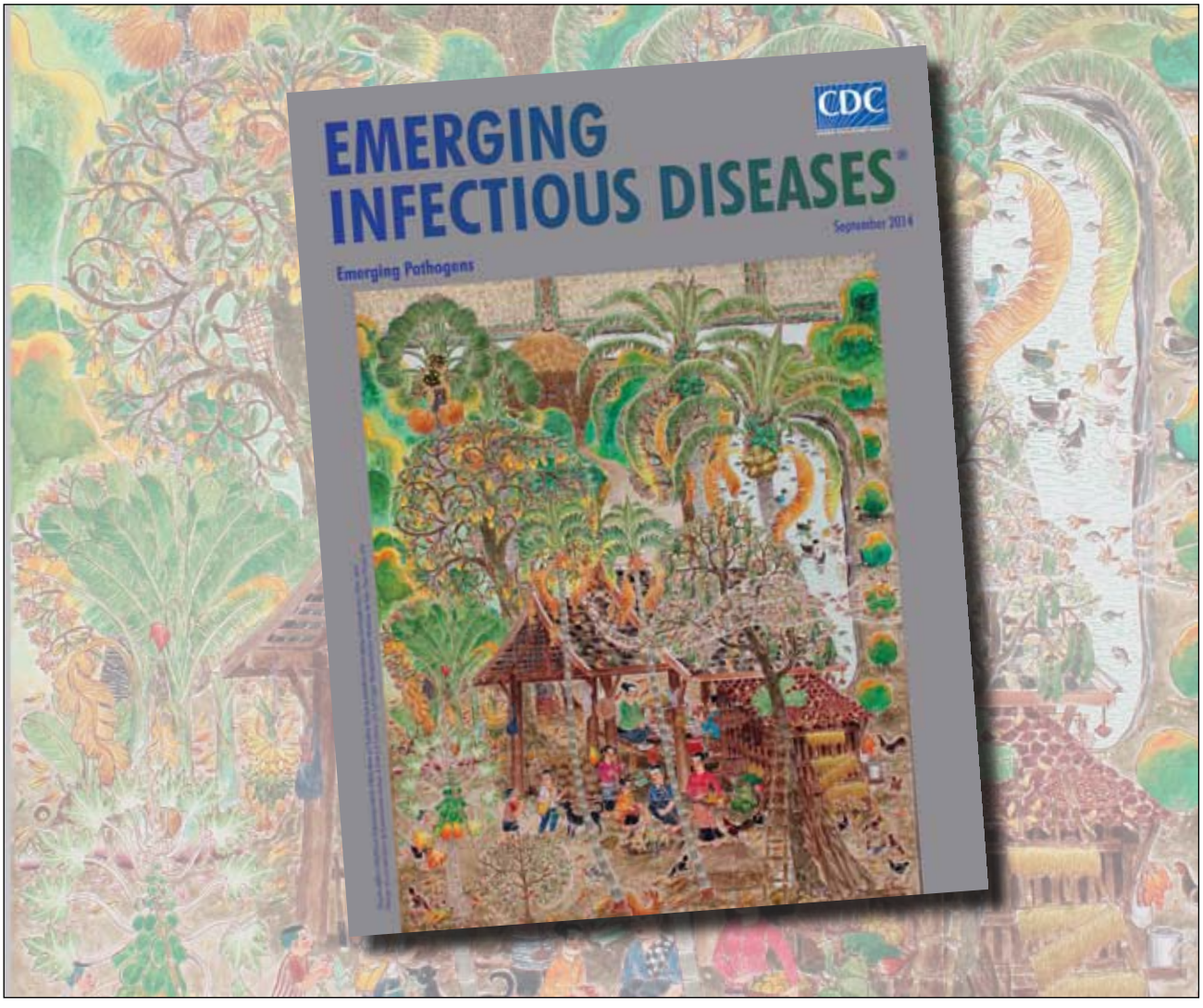

Reemergence of Murine Typhus in Galveston, Texas, USA, 2013

Lucas S. Blanton, Rahat F. Vohra, Donald H. Bouyer, David H. Walker

Twelve patients with murine typhus were identified in Galveston, Texas, USA, in 2013. An isolate from 1 patient was confirmed to be *Rickettsia typhi*. Reemergence of murine typhus in Galveston emphasizes the importance of vector control and awareness of this disease by physicians and public health officials.

Murine typhus, caused by *Rickettsia typhi*, is typically transmitted to humans by *Xenopsylla cheopis*, a flea that infests rats. The disease is endemic to tropical and subtropical seaboard regions throughout the world (*1*). Before the introduction of DDT in 1946 as a means of controlling rat ectoparasites, murine typhus was a frequent cause of illness in Galveston, Texas, USA (*2*). After nearly 8 decades during which no cases were identified in this city (although unreported cases might have occurred), murine typhus was diagnosed in 2 Galveston residents in April (*3*) and October 2012 (L.S. Blanton, unpub. data). These cases prompted this study of murine typhus as a cause of undifferentiated febrile illness in residents of Galveston County who sought care at the University of Texas Medical Branch in Galveston.

The Study

Physicians who were aware of the study alerted our team of investigators to patients ≥18 years of age who had reported fever during February–December 2013. Alternative syndromes that warranted exclusion included skin and soft tissue infections, urinary tract infections, cavitary or lobar pneumonia, and pyogenic intraabdominal processes (e.g., abscesses, appendicitis, cholecystitis, and diverticulitis). We obtained informed consent, medical history, and physical examination to record symptoms, signs, and laboratory data from each patient. We collected blood for use in real-time PCR, shell vial culture, and immunofluorescence assay (IFA) for the diagnosis of murine typhus (*4*–*6*). The conjugate used in IFA assays was Alexa Fluor 488 goat anti-human IgG (γ-chain–specific) at a dilution of 1:400 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). We made extensive attempts to collect blood from enrollees for convalescent IFA testing. Further PCR analysis to amplify portions of the 17-kDa antigen, citrate synthase, and outer membrane protein B (*ompB*) genes were performed on DNA from a cell culture isolate (*7*–*9*). Amplicons were cloned and sequenced (*4*).

A confirmed case of murine typhus was defined as having compatible signs and symptoms and seroconversion (diagnostic cutoff of 1:64) or a 4-fold increase in IgG titer against *R. typhi* from acute- and convalescent-phase serum samples; PCR detection of rickettsial DNA within acute-phase blood; or isolation of *R. typhi* from blood. A probable case was defined as having a single IgG titer of ≥1:256 during acute illness.

In addition to identifying acutely ill patients, we sought to determine the local prevalence of those who had *R. typhi* reactive antibodies. We repurposed serum samples collected from 500 Galveston residents; the samples were scheduled to be discarded after being used for routine clinical testing from outpatient clinics during the summer and fall of 2013. We screened for the presence of *R. typhi* IgG at a titer of 1:128 by IFA as described above. Endpoint titers were established in reactive samples. We performed IFA against spotted fever antigens (*R. rickettsii*) and Western blot analysis using *R. typhi* and *R. rickettsii* antigens to confirm the specificity of samples reactive to *R. typhi* as previously described (*6*,*10*). The University of Texas Medical Branch institutional review board approved these studies.

Eighteen patients who met study criteria were enrolled. Of these, 10 were determined to have murine typhus (7 confirmed and 3 probable) (Table 1). In addition to those identified prospectively, 2 probable cases were identified retrospectively. Serologic testing was the primary diagnostic method (6 of 7 confirmed cases). One case was confirmed by isolation of *R. typhi* from blood. This patient was an alcoholic man, 48 years of age, who sought care 4 days after the onset of fever, chills, and myalgias. Sequences of portions of the citrate synthase (GenBank accession no. KJ648945) and *ompB* (accession no. KJ648946) genes of this isolate revealed 100% homology to *R. typhi* Wilmington strain, and the sequence of the 17-kDa antigen gene (accession no. KJ648944) revealed 1 base pair difference. Real-time PCR screening of DNA extracted from whole blood demonstrated the presence of rickettsial DNA in the patient from whom the *R. typhi* isolate was obtained.

In addition to fever, the 12 patients, all reported headache, 7 (58%) had chills, 6 (50%) reported myalgias, 6 (50%) had rashes, and 9 (75%) had elevated hepatic transaminases (75%). Seven (58%) were hospitalized, and 2 (17%) were admitted to the intensive care unit. Except for

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Table 1. Summary of reciprocal antibody titers in 12 patients with murine typhus, Galveston, Texas, 2013*

Patient no.	Illness duration†	Acute titer (date collected)	Convalescent titer (date collected)	Diagnosis by case definition
1	12 d	256 (Apr 10)	1,024 (Apr 30)	Confirmed
4‡	4 d	NR (May 23)	NA	Confirmed
6	4 d	NR (Jun 16)	128 (Jul 2)	Confirmed
8	7 d	NR (Jul 10)	512 (Aug 8)	Confirmed
9	10 d	NR (Jul 16)	256 (Jul 30)	Confirmed
10	13 d	NR (Jul 19)	256 (Jul 30)	Confirmed
17	6 d	NR (Sep 27)	1,024 (Dec 13)	Confirmed
13	8 d	256 (Jul 29)	NA	Probable
15	8 d	256 (Aug 9)	NA	Probable
16§	3 wk¶	256 (Jul 30)	NA	Probable
19	3 wk	512 (Oct 30)	NA	Probable
20§	8 d	256 (Mar 26)	NA	Probable

*NR, nonreactive at a titer of 1:64; NA, not available.
†Duration of illness before workup and treatment.
‡Confirmed by culture isolate.
§Case identified retrospectively.
¶Patient was treated with levofloxacin 2 weeks before murine typhus workup.

1 of the retrospectively identified cases, all patients were treated empirically for murine typhus (Table 1). Eleven (92%) were treated with either doxycycline or minocycline; minocycline was prescribed for some because of a national shortage of oral doxycycline. All patients had subsequent resolution of illness with no obvious difference in recovery between the 2 drugs. One patient (identified retrospectively) was treated with levofloxacin and reported slow resolution of her symptoms.

The 500 serum samples screened by IFA represent ≈1% of Galveston's population (48,733 by the 2013 census [http://quickfacts.census.gov/qfd/states/48/4828068.html; cited 2014 Dec 27]). The mean age of patients whose samples were tested was 61.5 years. IFA and Western blot testing showed 8 (1.6%) samples to be reactive, which supported typhus group specificity (Table 2). Of those seroreactive, the mean age was 60.9 years, and 5 (62.5%) were women. The geometric mean reciprocal titer was 181.

Conclusions

Galveston is a small city on a barrier island off the upper Texas coast, along the Gulf of Mexico. As with other port cities where rat population numbers are high, the incidence of murine typhus in this city was historically high. However, in 1946, as part of an ectoparasite eradication program, the insecticide DDT was applied to common rat paths; subsequently, the number of ectoparasites spread by rats in human habitats was reduced. As a result, the incidence of murine typhus in Galveston decreased dramatically (*2*) and continued to decline through subsequent decades (*11*). This study describes what is probably the reemergence of murine typhus in this area.

The patients in this study exhibited typical signs and symptoms consistent with murine typhus (*12*). Although we cannot exclude the possibility of a different fleaborne rickettsia (i.e., *R. felis*) infecting some patients, the single rickettsial isolate identified supports *R. typhi* as a causative agent. The seroprevalence supports the occurrence of additional undiagnosed cases. Based solely on the titers of these reactive serum samples, it is not possible to elucidate recent versus distant infections. Sporadic cases may have gone undiagnosed.

Dynamic shifts in the epidemiology and transmission of murine typhus are not unprecedented. Although the rat-to-rat cycle of transmission by fleas is often referred to as an urban cycle, the rural South experienced high rates of murine typhus in the 1940s as a result of a proliferation of rats after a change in crop production from cotton

Table 2. Analysis of serosurvey samples found reactive to *Rickettsia typhi* by IFA and Western blotting, Galveston, Texas, 2013*

	Reciprocal IFA titers		Western blot analysis	
Sample	*R. typhi*	*R. rickettsii*	*R. typhi* protein lysate (OmpB)†	*R. rickettsii* protein lysate (OmpA‡)§
1	256	NR	+	–¶
2	128	NR	+	–
3	128	NR	+	–
4	128	NR	+	–
5	256	NR	+	–
6	128	NR	+	–
7	1024	128	+	–
8	128	NR	+	–

*Omp, outer membrane protein; NR, nonreactive at a titer of 1:128.
†Reactivity to the panrickettsial 135-kDa OmpB.
Reactivity to the spotted fever group 190-kDa OmpA.
§All serum samples reacted with *R. rickettsii* OmpB as described for spotted fever and typhus group OmpB antigens (*10*).
¶Bands corresponding to reactivity to OmpB but not OmpA confirms typhus group specificity.

to peanuts, because rats were attracted to the peanuts as a source of food (*13*). In southern California, opossums infested with *R. typhi*– and *R. felis*–infected cat fleas (*C. felis*) have been associated with a shift of fleaborne rickettsioses from the urban center of Los Angles to suburban areas (*14*). This suburban cycle of transmission involving *C. felis* plays a recognized role in Corpus Christi, Texas, a coastal city ≈220 miles southwest of Galveston (*1*). Additionally, this cycle has been suspected in a recent outbreak of murine typhus in the central Texas city of Austin (*15*).

The recent recognition of murine typhus in Galveston may reflect the reemergence of *R. typhi* in rats; it may also reflect a cycle involving opossums and cats. Additionally, *R. felis* may play a role as a serologically cross-reacting culprit of illness. Further study is required to better understand the ecology and epidemiology of murine typhus as it reemerges in Galveston. Physicians and public health officials should be aware of this reemerging threat. Furthermore, vector control is of utmost importance.

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Severe Fever with Thrombocytopenia Syndrome in Japan and Public Health Communication

Tomoya Saito, Kazuko Fukushima, Kazunori Umeki, Kensuke Nakajima

A fatal case of severe fever with thrombocytopenia syndrome was reported in Japan in 2013. The ensuing process of public communication offers lessons on how to balance public health needs with patient privacy and highlights the importance of multilateral collaborations between scientific and political communities.

Severe fever with thrombocytopenia syndrome (SFTS), caused by SFTS virus (SFTSV), was initially identified in China in 2011 (*1*). SFTS manifests with fever, vomiting, and diarrhea accompanied by clinical signs including low platelet and leukocyte counts; the illness can be fatal. The SFTSV vectors, *Haemaphysalis longicornis* and *Rhipicephalus microplus* ticks, inhabit Japan, but the virus had not been detected in ticks in this country, nor had there been a case report of SFTS from Japan. Thus, the public health risk from SFTS was not recognized until a fatal case of SFTS was confirmed in Japan in early 2013 (*2*,*3*).

The Case

At the end of December 2012, a virologist successfully isolated an unknown virus from a clinical sample from a person in Japan who had died of unknown causes. Whole-genome sequencing showed that the isolate's gene sequences were highly similar to those of SFTSV (*4*). The SFTS diagnosis was confirmed by the Japan National Institute of Infectious Diseases (NIID) on January 29, 2013.

On January 30, a rapid communication on the website of the Infectious Agents Surveillance Report (IASR) described this case as the first case of SFTS in Japan (*2*). In addition, health officials from Yamaguchi Prefecture, where the patient resided, and from the Ministry of Health, Labour and Welfare (MHLW) announced the identification at a press conference. On the same day, the MHLW issued a notice requesting that medical doctors voluntarily report suspected cases that fulfilled the interim SFTS case definition (Table). From that date through March 31, a total of 23 suspected cases were reported, blood samples were submitted, and a retrospective study was conducted (*5*). NIID testing confirmed 11 SFTS cases (median patient age 71 years, range 50–84 years), including a case from 2005. Officials from prefectures in which cases were confirmed made public announcements for most of these cases, and the MHLW issued a press release to convey the information nationwide. Clinical details of retrospective cases were reported on the IASR website (*6,7*).

Effective March 4, 2013, the MHLW revised the Order for Enforcement of the Infectious Disease Control Act to include SFTS as a class IV infectious disease. These diseases are notifiable, but mandatory hospitalization or restrictions of a patient's activities are not warranted. After that date, the MHLW began to report only the case numbers in each prefecture in the Infectious Disease Weekly Report. New infections were reported starting in April 2013. Descriptions of clinical manifestations for new case-patients were also shared promptly on the IASR website (*8*–*10*).

NIID developed a reverse transcription PCR to detect the Japanese strain of SFTSV, and PCR primers and reagents were distributed from NIID to 79 local public health laboratories by the end of March 2013 to establish country-wide diagnostic capacity. Laboratory results were initially confirmed by NIID, acting in a reference capacity for local laboratories (*11*).

To investigate the epidemiology, pathology, life cycle, countermeasures, and geographic distribution of SFTSV in Japan, the government established a 3-year research project in May 2013. Japan has 47 indigenous tick species, but the specific vectors of SFTSV and their habitats, proportion of virus carriers, and interactions with wild animals are unknown. Systems were developed to detect the SFTSV gene in ticks and SFTS antibodies in animal blood samples (*8*). Results of an interim investigation of ticks and of blood samples from hunting dogs, wild deer, and wild boar suggest that, despite the limited reports of human cases from the western part of Japan, the geographic distribution of virus is more extensive than previously understood (*12,13*). *H. longicornis* and *Amblyomma testudinarium* ticks were identified as SFTSV vectors in Japan; however, other species may also be carriers of the virus (*13*).

The initial case of SFTS in Japan attracted substantial public attention, creating the challenge of balancing public health needs with the protection of patient privacy. One of the basic philosophies of the Infectious Disease Control Act is the proactive disclosure of information on the situation, trends, cause, and disclosure of information necessary for prevention and treatment of infectious diseases. At the same time, the Minister of Health, Labour and Welfare and

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Table. Interim case definition for retrospective or prospective voluntary case reports of suspected severe fever with thrombocytopenia syndrome, Japan*

Criteria
Fever >38°C
Gastrointestinal tract symptoms (e.g., nausea, vomiting, abdominal pain, diarrhea, melena)
Thrombocytopenia, $<100 \times 10^9$ platelets/L
Leucopenia, $<4 \times 10^9$ leukocytes/L
Elevated levels of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase
Absence of other causes
Death or admission to an intensive care unit because of symptoms

*All criteria required for case confirmation.

the governors of local governments must remain mindful of the protection of personal information when disclosing information about cases (Paragraph 2, Article 16, Infectious Disease Control Act). The amount of personal identifying information to disclose must be evaluated on a case-by-case basis and must be consistent with the philosophy of the Act. Some public health plans, such as the Smallpox Preparedness Guideline (*14*), include a press release template, which includes disclosure of sex, age, and municipal area of residence. However, the risk communication guidelines in the Guidelines for Pandemic and New Infectious Diseases do not specify the items of patient information to be disclosed, although, in principle, the area of patient's residence at a municipal level should be disclosed to inform the public of the area(s) in which human-to-human spread is a risk (*15*).

Disclosure of personal information for the initial SFTS case-patient was limited to sex, adult status, and prefecture of residence; this information was limited to respect the wishes of the bereaved family. Age, municipal area of residence, and date of death were not disclosed because of the risk that these variables would result in patient identification. However, the amount of information disclosed about the initial case-patient was criticized at the House of Representatives Budget Committee meeting on April 2, 2013. A House of Representatives member claimed that more detailed information about the patient's area of residence was needed so that the geographic area of risk could be identified and residents could be alerted. The Minister responded by saying that the risk for SFTSV infection was not limited to the area surrounding the patient's residence and that tick bite precautions should be practiced throughout the country.

The case information disclosure policy was tempered as public attention waned; after the first 5 reported cases, the patient's age category was disclosed. Overall, the proportion of publicized SFTSV case reports, including the area of residence of patients at a municipal level, increased from 4/12 (30%) for cases occurring before 2012 to 24/40 (60%) for cases occurring during 2013.

Conclusions

A total of 40 SFTS cases were reported in Japan during 2013, which suggests that underdiagnosis might have occurred before the index case was identified. The success of the initial diagnosis of SFTS in the country was the result of a persistent investigation of a death by unknown causes conducted by hospital clinicians who were not aware that the patient had a history of a tick bite. However, the clinicians consulted an animal viral disease expert for assistance in isolating the causative agent because the clinical picture was a virus-associated hemophagocytic syndrome (Dr. Takahashi and Dr. Ishido, pers. comm.).

Our report shows that multilateral collaborations among investigators, including medical infectious disease specialists, epidemiologists, pathologists, virologists, entomologists, and veterinarians, were required for a timely response to this emerging vector-borne disease. Cooperation from professional organizations such as Dainihon Ryoyukai, a hunters' organization, was crucial for obtaining blood samples from wild animals. Maximizing the use of local government resources is essential for a prompt national investigation (e.g., tick collection and blood sample collection from wild animals). Further collaborative investigation is expected to lead to a better understanding of SFTS and SFTSV in Japan.

Dr. Saito was a deputy director at the Tuberculosis and Infectious Diseases Control Division, Health Service Bureau, Ministry of Health, Labour and Welfare of Japan at the time of this study. He is currently a chief senior researcher in the Department of Health Crisis Management, National Institute of Public Health, Japan. His primary research interest is biosecurity and public health emergency preparedness.

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Novel Mutations in K13 Propeller Gene of Artemisinin-Resistant *Plasmodium falciparum*

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We looked for mutations in the *Plasmodium falciparum* K13 propeller gene of an artemisinin-resistant parasite on islands in Lake Victoria, Kenya, where transmission in 2012–2013 was high. The 4 new types of nonsynonymous, and 5 of synonymous, mutations we detected among 539 samples analyzed provide clues to understanding artemisinin-resistant parasites.

The worldwide spread of malaria parasites with resistance to antimalarial drugs has been a serious concern over the past few decades. During the 2000s, *Plasmodium falciparum* parasites acquired resistance to key drugs, such as chloroquine and sulfadoxine–pyrimethamine, in many malaria-endemic countries, including Kenya (*1*). Artemisinin-based combination therapy (ACT) has been introduced in most malaria-endemic countries and is the first-line therapy. However, the first clinical cases of artemisinin resistance in western Cambodia were reported in 2008 (*2*), and *P. falciparum* with reduced in vivo susceptibility to artesunate in western Cambodia was reported in 2009 (*3*,*4*). On the basis of these findings, genome-wide analyses of artemisinin-resistant P. falciparum isolates found strong correlations between a mutant allele in the K13 propeller, in vitro parasite survival rates, and in vivo parasite clearance rates; these correlations indicate that mutations in the K13 propeller (especially C580Y, R539T, and Y439H) are important determinants of artemisinin resistance (*5*,*6*). Analysis of parasites from several Cambodian provinces indicated that K13 propeller mutations are rarely observed in samples from provinces without documented resistance but are prevalent in provinces with reported resistance (*6*,*7*).

In Kenya, ACT was introduced as first-line therapy for *P. falciparum* malaria in 2004 (*1*). However, various types of antimalarial drugs—including chroloquine, sulfadoxine–pyrimethamine, and artemether/lumefantrine—are available for purchase at pharmacies without physicians' prescriptions. In this study, we describe some K13 propeller mutations of *P. falciparum* parasites from western Kenya.

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The Study

The research was conducted at 4 islands on Lake Victoria (Kibuogi, Ngodhe, Takawiri, Mfangano) and 1 shoreline community of Mbita District (Ungoye) in western Kenya (Figure). In this area, the $PfPR_{2–10}$ (community *P. falciparum* parasite rate standardized to the 2- to 10-year age group) was reported in 2009 to be >40% (*8*). Although in some area of Kenya malaria has decreased, its prevalence remains high in the Lake Victoria basin because of the lake environment (*8*–*10*). In 2009, a total of 50%–70% of households owned insecticide-treated bed nets (*11*), which substantially reduce the risk for transmission of malaria parasites by providing barriers against mosquitoes. Although malaria is annually more prevalent in the 2 wet seasons (March–June and October–November) (*9*), in the study sites, it is highly endemic throughout the year.

Filter paper blood spots were collected from participants (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/3/14-0898-Techapp1.pdf) during population-wide cross-sectional malaria surveys conducted in February and August 2012 and August 2013 at the 5 study sites. We obtained ethics approval from the Kenyatta National Hospital and the University of Nairobi. All study participants provided informed consent.

Parasitic DNA was extracted from the filter paper (*12*), and *P. falciparum* DNA was detected by a mitochondrial DNA-based PCR (*13*). Sequencing of the K13 propeller gene was attempted on the diagnostic PCR-positive specimens (online Technical Appendix). The prevalence of *P. falciparum*, as determined by PCR, in the rainy and dry seasons was 7.2%–26.2% and 6.5%–15.5% on the 3 small islands (Kibuogi, Ngodhe, and Takawiri), 47.3% and 31.4% on Mfangano Island, and 38.4% and 41.9%–64.0% in Ungoye, respectively (Table 1).

We successfully analyzed 539 samples for the K13 propeller gene. Nine new types of point mutations were identified among these samples (Table 2). Participants infected with parasites harboring a mutation on the K13 propeller gene are listed in online Technical Appendix Table 2. The sequences reported in this study have been deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB936059–AB936067).

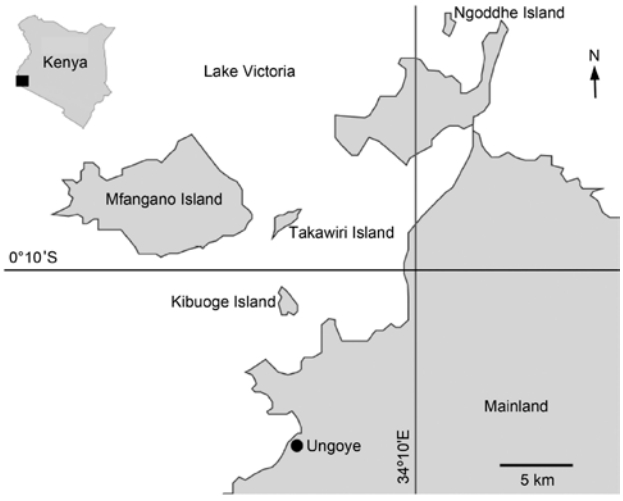

Figure. Study sites for investigation of K13 propeller gene in *Plasmodium falciparum*, Mbita District, Kenya, 2012–2013. Inset shows location of study area in Kenya.

Table 1. Prevalence of *Plasmodium falciparum* and analysis of K13 propeller gene, Mbita District, Kenya, 2012–2013

Time	Study site	Total no. samples	PCR positive, no (%)*	K13 propeller gene analyzed, no.
2012 Feb	Kibuogi	130	34 (26.2)	21
	Ngodhe	250	18 (7.2)	5
	Takawiri	250	34 (13.6)	19
	Mfangano	427	202 (47.3)	138
	Ungoye	250	96 (38.4)	69
2012 Aug	Kibuogi	195	17 (8.7)	10
	Ngodhe	232	36 (15.5)	18
	Takawiri	230	15 (6.5)	9
	Mfangano	706	222 (31.4)	145
	Ungoye	248	104 (41.9)	65
2013 Aug	Ungoye	250	160 (64)	40

*PCR detected *Plasmodium falciparum* only.

Conclusions

We detected 4 novel nonsynonymous and 5 novel synonymous mutations in the highly conserved K13 propeller gene of *P. falciparum* parasites from western Kenya. Ariey et al. noted that the frequency of mutant alleles strongly correlated with the prevalence of day 3 positivity after ACT treatment in humans in Cambodia and that those mutations reflected positive selection (*6*). That study found 17 mutant alleles in the K13 propeller gene. Among them, C580Y, R539T, and Y493H were prevalent and strongly related to in vivo delayed parasite clearance. In our study, all the mutations found differed from those reported in Cambodia, and mutant alleles were not always observed in the proceeding seasons, so some mutations could be occasionally introduced. Most of those mutations are not suitable for the life cycle of parasites, and only a few suitable for survival under the conditions of artemisinin selection pressure could be selected.

We observed no identical mutations at >2 of these 5 study sites. Furthermore, only 1 type of mutation, A578S from Mfangano Island, was detected during 2 seasons, whereas other mutations were not observed in the next season, half a year later. Any family relations were not identified among the 4 participants harboring A578S mutation in February 2012 at Mfangano Island. Point mutations can occasionally occur on the *P. falciparum* K13 propeller gene as a standing variation, but most of the isolates that recently acquired the mutation may disappear because of some fitness disadvantage or the effect of a random genetic drift (*14*).

Malaria parasites grow and multiply at 2 different biologic stages in humans and mosquitoes. Therefore, isolates with new mutations must adapt to both circumstances. We detected the mutant allele A578S in the K13 propeller gene in 2 consecutive seasons on Mfangano Island. This mutation, which modifies amino acids from being hydrophobic to hydrophilic, is close to the prevalent single nucleotide polymorphism C580Y from Cambodia that is thought to be necessary in protein–protein interactions, which could affect artemisinin susceptibility. The genotype analyses of the parasites from this island are critical to understanding the role of this mutation and ACT efficiency in this geographic area.

Our K13 propeller sequence analysis of *P. falciparum* parasites from a malaria-endemic area in Kenya did not detect the predicted artemisinin-resistant genotypes, but we observed some temporal substitutions. A limitation of our study was that the sample size was insufficient to specifically provide an understanding of this result. The accumulation of data from this region and from other

Table 2. Observed mutations in the K13 propeller gene in *Plasmodium falciparum,* Mbita District, Kenya, 2012–2013

Mutation	Amino acid change and location	Genetic change	Study site (no. isolates)		
			2012 Feb	2012 Aug	2013 Aug
Nonsynonymous	M442V	ATG →GTG		Mfangano (1)	
	N554S	AAT →AGT	Ungoye (1)		
	A569S	GCA →TCA			Ungoye (1)
	A578S	GCT →TCT	Mfangano (4)	Mfangano (1)	
Synonymous	C439C	TGC→TGT	Ungoye (2)		
	S477S	TCT →TCG	Takawiri (1)		
	Y500Y	TAT→TAC		Mfangano (1)	
	N531N	AAT →AAC			Ungoye (1)
	G538G	GGT→GGA	Mfangano (3)		

malaria-endemic areas will increase our understanding of the relationship between the K13 propeller gene and artemisinin resistance. Monitoring these molecular markers and the efficacy of antimalarial drugs is critical for increasing understanding of artemisinin resistance and predicting its spread. This study identified clues that are essential in understanding artemisinin-resistant parasites.

Acknowledgments

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Comparison of Porcine Epidemic Diarrhea Viruses from Germany and the United States, 2014

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Since 2013, highly virulent porcine epidemic diarrhea virus has caused considerable economic losses in the United States. To determine the relation of US strains to those recently causing disease in Germany, we compared genomes and found that the strain from Germany is closely related to variants in the United States.

Porcine epidemic diarrhea (PED) is an acute and highly contagious enteric disease of swine that results in severe enteritis, diarrhea, vomiting, and dehydration (*1*). Porcine epidemic diarrhea virus (PEDV), the causative agent, is an enveloped, positive single-stranded RNA virus that belongs to the family *Coronaviridae*, genus *Alphacoronavirus* (*2*).

The disease was first recognized in Europe in 1971 and has thereafter caused high economic losses, particularly in Asia. In May 2013, a highly virulent PEDV variant emerged in the United States; explosive epidemics on swine farms affected pigs of all ages, resulting in a mortality rate of up to 95% among suckling pigs (*3*). Since then, outbreaks have occurred in 30 US states (*4*), causing very high economic losses, and the disease threatens to spread. The involved viruses cluster together with isolates from China from 2011 and 2012 (*5*). Apart from these highly virulent strains, a PEDV variant from the United States (strain OH851) that affected sows instead of younger animals and caused milder disease was recently described (*6*).

The effect of PED in the United States has unsettled pig farmers and veterinarians worldwide; studies have been recently initiated to elucidate the situation in Europe. Despite the history of PED outbreaks in Europe, little is known about currently circulating virus strains and their effect; information about the phylogeny of recent strains and their relation to the outbreak strain in the United States is lacking.

We report a case of PED that occurred on a swine-fattening farm in Germany in May 2014. The causative virus was fully characterized by using conventional methods and next-generation sequencing. We analyzed the resulting full-length genomes and compared them with those of the strains circulating in the United States and Asia to elucidate possible relationships.

The Study

In May 2014, a pig fattening farm in southern Germany (Federal State of Baden-Wuerttemberg) that continuously houses 1,400 fattening pigs reported watery diarrhea in pigs in all age groups (feeders to slaughter animals). The first cases occurred after new feeder pigs from a large piglet producer were brought onto the farm. The incoming animals showed diarrhea 2 days after arrival. Within 1 day thereafter, the disease had spread to pigs in all other age groups. Clinical signs were present for at least 1 week; ≈20 pigs died. Fecal samples from diseased pigs were submitted to the regional laboratory for diagnosis, and coronaviruses were detected by electron microscopy (Figure 1). Additionally, 3 pigs with catarrhal enteritis were euthanized; postmortem examination at the regional laboratory confirmed coronavirus infection in all 3 animals.

Subsequently, PED was diagnosed in a private laboratory (IVD GmbH, Hannover, Germany) by use of a published multiplex reverse transcription quantitative PCR (*7*). Selected positive samples were submitted to the Friedrich-Loeffler-Institut, Isle of Riems, Germany, for confirmation and further virus characterization. At this institution, 2 fecal samples with high genome load, as determined by 2 independent, published (*8*,*9*) reverse transcription quantitative PCRs selective for PEDV nucleocapsid (N) and spike (S) genes, were chosen for routine virologic testing and next-generation sequencing. Sequencing and data analyses were performed as previously described (*10*). The phylogenetic tree, based on full-length genomes, was constructed by using PhyML (*11*) in the Geneious software suite (http://www.geneious.com/) with a generalized time reversible substitution model, and the tree was supported by 1,000 bootstrapping replicates.

Author affiliations: Friedrich-Loeffler-Institut, Greifswald–Insel Riems, Germany (D. Hanke, M. Jenckel, A. Petrov, S. Blome, A. Pohlmann, H. Schirrmeier, M. Beer, D. Höper); Ludwig-Maximilians-University Munich, Oberschleißheim, Germany (M. Ritzmann, J. Stadler); Chemical and Veterinary Investigations Office Stuttgart, Fellbach, Germany (V. Akimkin)

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[1]These authors contributed equally to this article.

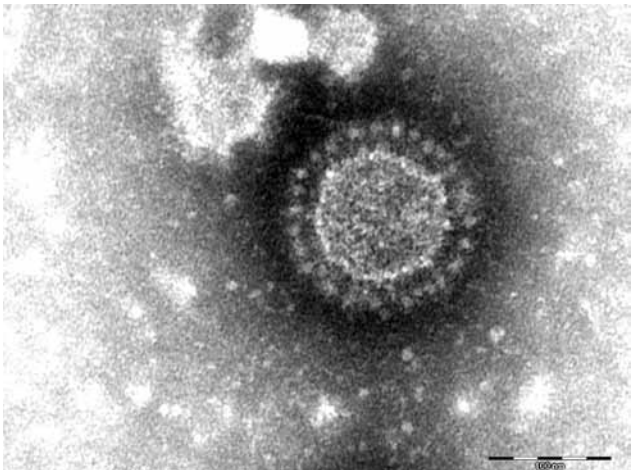

Figure 1. Porcine epidemic diarrhea virus particles seen by negative-stain electron microscopy of fecal samples. Negative staining with 1% phosphotungstic acid. Scale bar indicates 100 nm.

Virus isolates were obtained after inoculation of cells of different permanent cell lines (pig kidney [PK]-15 and Vero) with clinical material from the pigs. Sequencing of nucleic acids isolated directly from diagnostic samples PEDV/GER/L00719/2014 and PEDV/GER/L00721/2014 resulted in 2 viral genomes (Table) encompassing all typical PEDV coding sequences. Each sequence encodes a large replicase polyprotein, a spike (S) protein, an alphacoronavirus-specific accessory membrane protein, an envelope protein, a membrane protein, and a nucleocapsid (N) protein.

Comparative analyses of the full genomes showed that the strains share a very high (99.5%) identity with the new variant OH851 (GenBank accession no. KJ399978) recently reported from the United States (*6*). A more comprehensive comparison of 21 full-length PEDV genomes from different years and locations revealed lower similarities (≈98.7%) with currently circulating highly virulent strains from the United States and from China (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/21/3/14-1165-Techapp1.pdf). In contrast, the new isolates from Germany, PEDV/GER/L00719/2014 and PEDV/GER/L00721/2014, are less similar (97.1%) to the isolate from Europe, CV777, which dates back to the late 1970s (*12*).

The nucleotide alignment of the obtained PEDV genomes and the available references from the database revealed a region with high variability of the first 1,200 nt in the 5′ portion of the S protein–coding sequences (online Technical Appendix Figure, panel B). The N terminal S1 domain of the coronavirus S protein is necessary for virus attachment by interaction with host cell receptors (*13*) and might therefore be highly mutable.

Although in-depth analysis of the deep-sequencing data for PEDV/GER/L00721/2014 revealed a genetically homogenous population, this analysis for PEDV/GER/L00719/2014 uncovered a mixed viral population with a total of 8 single-nucleotide variants. One nonsynonymous single-nucleotide variant (variant position G19042U, amino acid substitution S6348I) was detected in the polyprotein coding sequences. Of note, 7 single-nucleotide variants are located in the aforementioned variable region in the S protein coding sequences, 5 of which are nonsynonymous (Table), thereby confirming the high variability in the N terminal part of the S protein.

Table. Sequencing results of porcine epidemic diarrhea virus isolated from pigs in Germany, 2014*

Isolate	Mapped reads	Average depth	Total length	GenBank accession no.	Variant position (full genome)	Variant position (in CDS)	Codon change	Amino acid substitution	Variant frequency, %
L00719	30,723	146	28,028	LM645058	19,334	G19042U polyprotein	agu→auu	S6348I	48
					20,880	U247G S protein	ucu→gcu	S83A†	41
					21,017	U384A S protein	aau→aaa	N128K‡	43
					21,232	C599U S protein	gcu→guu	A200V§	14
					21,328	G695U S protein	agu→auu	S232I¶	41
					22,527	C1894U S protein	cuu→uuu	L632F#	13
					22,844	C2211U S protein	gac→gat	Silent	46
					22,970	U2337C S protein	auu→auc	Silent	44
L00721	19,274	104	28,028	LM645057	None	None	None	None	None

*CDS, coding sequences; S protein, spike protein. Blank cells indicate not applicable.
†aa 83A also found in strain OH851 (GenBank accession no. KJ399978).
‡aa 128K found in all compared reference sequences.
§aa 200V not detected in reference sequences.
¶aa 232I detected in recent highly virulent strains (GenBank accession nos. KJ778615, KF272920, KF468753, KJ584361, KJ408801, KF650374, KF468752, KF267450, KC210147, JN825712, KC210145).
#aa 632F detected in reference strain KF267450 only.

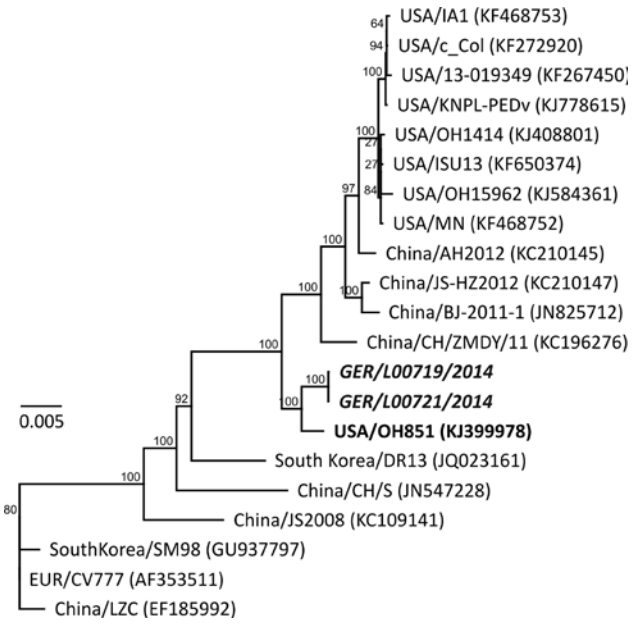

Figure 2. Phylogenetic analysis based on 21 full-length porcine epidemic diarrhea virus (PEDV) genomes. The new strains from Germany (PEDV/GER/L00719/2014 and PEDV/GER/L00721/2014, in boldface and italics) and the new 2014 PEDV variant from the United States (OH851, in boldface) were included and compared with current circulating strains from the United States and China. The tree was constructed by using PhyML (11). Numbers above branches indicate proportions calculated from 1,000 bootstrap replicates: The scale bar represents nucleotide substitutions per site.

Because quite extensive differences (≈50 aa) were found between the recent N terminal S protein region of the isolates from Germany and the highly virulent PEDV strains from the United States and China, the isolates from Germany described in this article seem to not be directly linked to the highly virulent PEDV strains circulating in the United States (Figure 2). In contrast, the recent isolates from Germany and strain OH851 share not only high identity over the entire genome, including the highly variable 5′ region of the S protein coding sequences, but also their clinical phenotype observed under field conditions.

Conclusions

PEDV infection was confirmed in a pig herd in Germany in 2014. Comparative analyses of full-length sequences revealed that the isolates from these pigs in Germany show very high nucleotide similarity with strain OH851 found in the United States in 2014. However, differences exist that distinguish the strains from Germany from the highly virulent PEDV strains that caused the major losses in the United States. Given the fact that PEDV surveillance has been lacking in Germany, we cannot exclude the possibility that the strains described here have already been circulating in Europe for a longer time or were indeed recently introduced from the United States or Asia to Europe. Therefore, our report provides useful information about recent PEDV strains in Europe, but a comprehensive evaluation is still difficult because of a lack of data about additional strains. Future studies should therefore concentrate on analysis of additional PEDV from different years and locations.

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Mr. Hanke and Mrs. Jenckel are doctoral students at the Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Isle of Riems, Germany. Their research focuses on the rational use of next-generation sequencing in metagenomic analyses and molecular epidemiology.

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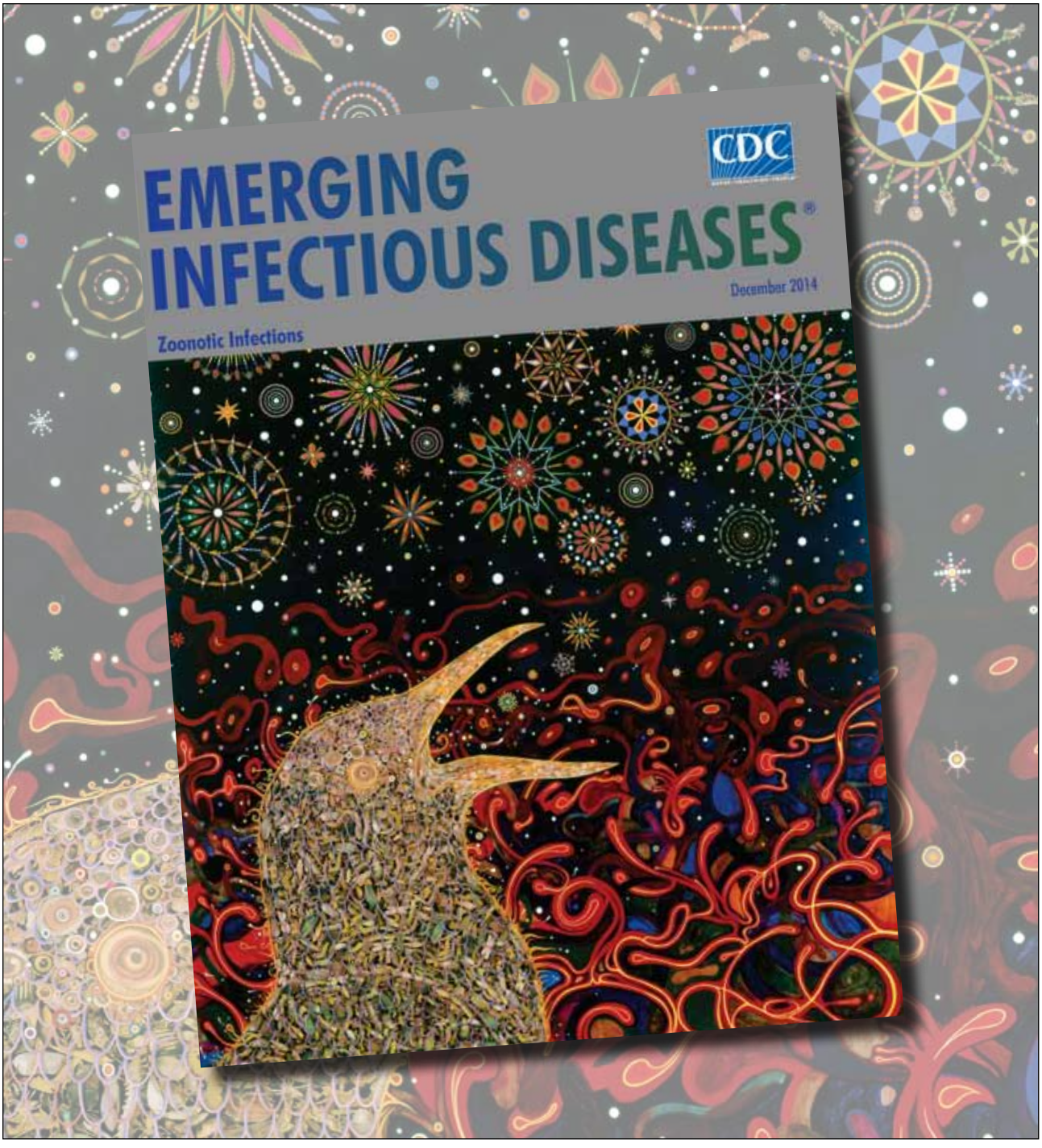

Buruli Ulcer in Traveler from Suriname, South America, to the Netherlands

William R. Faber, Bouke de Jong, Henry J.C. de Vries, Jim E. Zeegelaar, Françoise Portaels

We report Buruli ulcer in a man in the Netherlands. Phenotyping of samples indicate the Buruli pathogen was acquired in Suriname and activated by trauma on return to the Netherlands. Awareness of this disease by clinicians in non–Buruli ulcer–endemic areas is critical for identification.

Buruli ulcer (BU) is a potentially disabling affliction of inhabitants of tropical wetlands (*1*) caused by *Mycobacterium ulcerans* and characterized by necrosis of skin, subcutaneous tissue, and sometimes bone. Major foci are in West and Central Africa and minor endemic foci in Australia, Southeast Asia, Mexico, and South America.

BU is rare in South America; 15 cases were reported in Peru since 1996 (*1*; H. Guerra, pers. comm.), and 242 cases were reported during 1969–2011 in French Guiana (*2*), which is bordered by Suriname to the west. Mycobacterial ulcers have been seen in Suriname in the past (R.F.M. Lai, A. Fat, L. Sabajo, pers. comm.), but these cases were not confirmed by laboratory tests. We report a case of BU in a patient from Suriname diagnosed in the Netherlands.

The Case-Patient

In February 2013, a 70-year-old man sought care at the University of Amsterdam Department of Dermatology for a neuropathic ulcer on his left foot. It was determined that he had a neuropathic ulcer, a complication of nerve damage caused by leprosy, for which he was treated successfully. Data from his medical history were recovered and reexamined at this time.

He was a native of Suriname, where he lived until he moved to the Netherlands 1977. He had multiple instances of treatment for skin infections, beginning with a diagnosis of leprosy (Hansen disease, caused by the mycobacterium *M. leprae*) when he was 10 years of age.

He was treated continuously with dapsone during 1952–1978. In 1977, he moved to Groningen, the Netherlands, and clofazimine was added to his treatment for leprosy for 6 months. In February 1982, he moved to Amsterdam and sought routine follow-up care for leprosy at the University of Amsterdam Department of Dermatology, and quiescent borderline lepromatous leprosy was diagnosed. He was treated during February 1982–February 1983 with a triple therapy consisting of rifampin, dapsone, and clofazimine.

In 1984, the man traveled to Paramaribo and Coronie in Suriname, where he stayed for 4 weeks during the spring season. During that time, he fished in creeks. In December 1984, he sought treatment again at the University of Amsterdam Department of Dermatology for a painful red swelling on the dorsal side of the right wrist after he had scratched it on a rough wall 5 weeks earlier, in mid-November. Beginning in February 1985, the patient had signs of infection of the wound, including conspicuous edematous swelling of the hand. Radiographic imaging showed no signs of osteomyelitis. The patient was treated with flucloxacillin; the symptoms subsided. However, during April 26–May 11, 1985, the patient experienced recurrent infection with abscess formation. A biopsy was taken, and an acid-fast bacilli (AFB) smear and culture for nontuberculous mycobacteria were done. The histopathology report indicated scarring dermis and mixed cell infiltrate, leucocytoclastic vasculitis, fibrinoid changes, and some diffusely spread histiocytes. AFB staining was negative. On June 3, partly dry wounds, 1 deep ulcer, fistula formation, and spontaneous drainage of another lesion were documented. The wound was cultured, and amoxicillin and clavulinic acid 4 times daily for 3 weeks was prescribed; after that time, the patient's treatment for related conditions consisted of local wound care. After 6 weeks' incubation, the mycobacterial culture was positive at 30°C but negative at 37°C and 45°C. In August, ulcers were still present, and multiple nodules at the underarm and an enlarged lymph node at the elbow were palpable. Infection with *M. ulcerans* was suspected clinically.

On January 20, 1986, the isolate was sent to the Institute of Tropical Medicine of Antwerp and was identified as *M. ulcerans* ITM 842 according to phenotypic characteristics (*3*). The isolate was further analyzed by several genotypic methods (*3–6*). By April 7, 1986, the ulcers were nearly completely closed.

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Conclusions

We describe a patient infected with *M. ulcerans* strain ITM 842. Phenotypic characteristics of isolates from Suriname and French Guiana are identical, whereas isolates originating in Africa, Australia, and Asia have different phenotypic characteristics (*3*). Variable-number tandem-repeat genotypic analysis had been shown to discriminate the Suriname isolate from French Guiana isolates and from mycolactone-producing *M. marinum* (*4*,*5*). Results confirmed that this isolate was different from *M. ulcerans* isolates from other geographic origins, albeit closely related to *M. ulcerans* strains from French Guiana by genetic characteristics (*4*,*5*). These results suggest that the patient acquired his infection in Suriname.

The incubation period of primary BU is estimated to be 2–3 months, but latency of the disease can span years. Persons who were in an area to which BU is endemic months to years earlier can manifest BU in a traumatized body site (*6,7*).

Co-infections with leprosy and BU have been described, but rarely (*8*). During 1952–1977, the patient was treated with dapsone monotherapy. Additionally, he received clofazimine for 6 months upon his arrival in the Netherlands. Subsequently, during February 1982–February 1983, he was treated with a triple therapy consisting of rifampin, dapsone, and clofazimine. Treatment of BU patients with dapsone has not been properly evaluated in humans (*9*), and clofazimine is not indicated in the treatment of patients with BU (*10*); however, rifampin is highly bactericidal against this organism (*11*).

If the patient was infected with *M. ulcerans* before his arrival in the Netherlands in 1977, it is unlikely that the dapsone and clofazimine that he received for treatment of leprosy could have controlled the infection. Whether or not he had been infected with *M. ulcerans* when he first arrived in the Netherlands, treatment for 1 year with rifampin and dapsone before he returned to Suriname for 4 weeks in 1984 would have been expected to have killed any *M. ulcerans*. Thus, the most likely hypothesis is that he acquired BU infection during the 4 weeks he spent in Suriname in 1984, and that the scratch obtained against a rough wall in November 1984 in the Netherlands reactivated a latent infection. It is unlikely that the rough wall was contaminated by *M. ulcerans*, because BU is not considered prevalent in Europe.

There are many similarities between this case and that reported by Lindo and Daniels (*7*). Both patients manifested symptoms of BU after having left a disease-endemic area, and in both patients, the lesion developed at the site of a trauma that occurred in a non–disease-endemic country.

Correct laboratory confirmation remains essential to confirm the discovery of new foci of BU, and the use of genetic mapping can provide additional information about the geographical location in which patients were infected. The importance of laboratory confirmation of BU has been emphasized by the World Health Organization's manual for the laboratory diagnosis of BU (*12*).The study of imported cases, and in particular this case from Suriname, has enabled us to acquire more knowledge regarding latency and reactivation of the disease, and the existence of new foci. As in the report by Meyers et al (*13*), this case report emphasizes the role of trauma to the skin in the delayed manifestation of this disease caused by *M. ulcerans*.

Reports from European countries, Canada, America, and Australia describe travelers visiting friends or relatives in BU-endemic countries who manifested BU upon return to a non–disease-endemic area (*14*,*15*). Thus, BU may be diagnosed in patients late, or not diagnosed, in non–disease-endemic countries, where health care professionals are usually not familiar with the condition or its causal organism. Delayed diagnosis often leads to severe disabilities. In countries where BU is uncommon, the clinician's consideration of the patient's recent travel history and awareness of cutaneous and bone lesions of BU are needed for accurate testing, diagnosis, and treatment of the patient.

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Moxifloxacin Prophylaxis against MDR TB, New York, New York, USA

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Contacts of persons infected with multidrug-resistant tuberculosis (MDR TB) have few prophylaxis options. Of 50 contacts of HIV- and MDR TB–positive persons who were treated with moxifloxacin, 30 completed treatment and 3 discontinued treatment because of gastrointestinal symptoms. Moxifloxacin was generally well-tolerated; further research of its efficacy against MDR TB is needed.

Limited data exist on safety of prophylaxis for contacts to persons with multidrug-resistant tuberculosis (MDR TB). All MDR TB strains are resistant to at least isoniazid and rifampin, precluding the use of these drugs for MDR TB prophylaxis. Current local, national, and international guidelines suggest using antibiotics to which the strain from the index case-patient is susceptible (*1*–*6*; Table 1); however, no randomized controlled trial has been conducted to support this recommendation. Global spread of MDR TB necessitates identification of treatment options with acceptable safety and tolerability for persons infected with drug-resistant strains. Choosing appropriate treatment for HIV-positive persons exposed to TB is even more crucial considering the increased risk among these persons for progression from TB infection to active disease (*7*–*9*).

In 2005, two TB outbreaks occurred in New York City (NYC) among HIV-positive persons with 2 distinct MDR TB strains. In both outbreaks, contacts were defined as 1) residents of a building on the floor on which a case-patient resided or visited during the infectious period and 2) health care staff members who provided direct care to case-patients. Eligible contacts were treated with moxifloxacin to prevent progression from TB infection to disease. We present the 9-year follow-up from these exposures and the outcomes of the treated contacts.

The Investigation

The first outbreak we investigated occurred in a facility that provided housing and harm-reduction services to a predominantly HIV-positive, homeless, and drug-using population (site A). The first TB case-patient identified was a 53-year-old HIV-positive man residing there, in whom pulmonary TB was diagnosed by a positive (4+) acid-fast bacilli (AFB) sputum smear and positive culture. His chest radiograph showed extensive bilateral infiltrates and a large pulmonary cavity; he died 4 days after initiating treatment. Subsequent drug-susceptibility results indicated the strain was resistant to isoniazid, rifampin, ethambutol, pyrazinamide, streptomycin, rifabutin, and kanamycin. Within 3 months, TB was diagnosed in 2 additional HIV-positive residents of site A; genotype and drug-resistance phenotype matched those of the index case-patient. A contact investigation and active case finding were initiated at site A, and 3 additional MDR TB cases with matching genotype were identified.

Of 105 close contacts identified, 84 (80%) were HIV-positive, 16 (15%) were HIV-negative, and 5 (5%) had unknown HIV status (Table 2). Among the 21 contacts not known to be HIV-positive, 1 person had a positive tuberculin skin test (TST) result, had normal chest radiograph results, and started moxifloxacin prophylaxis; however, the patient was lost to follow-up after 2 months. Among the 84 HIV-positive contacts, TST results of 2 were positive and that of 1 other contact was positive after a negative result documented 3 years before. Fifty-one (61%) HIV-positive contacts were lost to follow-up or refused evaluation or prophylaxis. Before being tested, 1 (1%) contact died as a result of HIV-related causes. Of the remaining 32 (38%) HIV-positive persons, 26 (81%) started moxifloxacin prophylaxis; 16 (62%) completed treatment, 5 (19%) were lost to follow-up within 2 months (including the 3 who tested TST positive), 3 (12%) were discharged from treatment because of adverse reactions, and 2 (8%) were either medically discharged for unknown reasons or refused to continue treatment.

The second outbreak occurred at a long-term care facility housing HIV-positive, previously homeless persons (site B). The index case-patient was a 49-year-old HIV-positive man for whom smear-positive (2+), culture-positive pulmonary TB was diagnosed. A TB strain resistant to isoniazid, rifampin, and rifabutin was identified; the patient died 1 month later. Contact investigation and active case finding were initiated at site B. Within 6 months of the index case-patient's diagnosis, 5 additional TB cases were identified in 4 HIV-positive residents and 1 HIV-negative staff member. On the basis of genotype, the strain the index case-patient was diagnosed with matched

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Table 1. Current recommendations of international public health agencies for managing multidrug-resistant tuberculosis infection among contacts of infected persons

Recommendation
Treatment with isoniazid alone if high likelihood that the contact had prior exposure to and infection caused by a drug-susceptible case (*2,4*)
Treatment with >2 antimicrobial drugs to which the index case is susceptible, including pyrazinamide and ethambutol (*1,2,4,5*)
Treatment with pyrazinamide or ethambutol and a fluoroquinolone (*1,2,4*)
Clinical monitoring for 2 years for signs or symptoms of active disease (*2,3*)

the strain of the 3 residents and staff member. All isolates also had the same drug resistance phenotype. The other HIV-positive resident had a clinical diagnosis of TB meningitis (no culture results available).

In the site B outbreak, 136 close contacts were identified (Table 2): 83 (61%) HIV-positive residents and 53 (39%) staff members with unknown HIV status. Of the 53 staff members, 22 (42%) were previously TST-positive but had normal chest radiograph results during this evaluation; 25 (47%) tested negative, and 6 (11%) were not evaluated. No staff members were eligible for prophylaxis. Of the 83 HIV-positive residents, 3 (4%) had positive TST results; 2 had documented negative results within the year before their positive result, strengthening evidence of TB transmission in site B.

Considering the drug susceptibility pattern of this strain, a combination of moxifloxacin and pyrazinamide was recommended for all HIV-positive contacts once active disease was ruled out. Among exposed residents, 40 (48%) either died of non–TB-related causes or were lost to follow-up before completing TB evaluation, and 12 (14%) either refused treatment or were not started on treatment because of physician decision. Of the remainder, 24 initiated moxifloxacin and pyrazinamide treatment; 14 (58%) completed treatment, and 10 (42%) refused or were lost-to-follow up after a median 3 (range 1–5) months of treatment. The 2 contacts whose TST results were converted were placed on alternative regimens.

To determine whether TB symptoms subsequently developed in any contact in either outbreak, we compared them to cases identified in the NYC TB registry. As of March 2014, after a maximum of 8.5 years of follow-up at site A and 9 years at site B, 1 contact, a resident at site B who completed 1 month of moxifloxacin and pyrazinamide treatment in 2006 had TB disease caused by a different drug-susceptible strain develop during 2009.

Conclusions

Globally, an estimated 480,000 persons were infected with MDR TB in 2013 (World Health Organization Global Tuberculosis Report 2013, http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf). Although the current priority is accurate diagnosis and treatment of persons with active disease, preventing MDR TB in infected contacts is also crucial. Programs with capacity for contact investigation are faced with the question of what prophylaxis to recommend for persons who have contact with persons who are positive for MDR TB because data are scarce on efficacy and safety of treatment options for these persons.

Currently recommended treatment regimens (Table 1) are not without risk. Studies demonstrate serious adverse effects associated with the use of pyrazinamide in combination with either ethambutol (*10*) or a fluoroquinolone, including ofloxacin (*11*,*12*) and levofloxacin (*13*,*14*). However, a recent study of contacts treated with moxifloxacin- or levofloxacin-based prophylaxis found no serious adverse events and fewer cases of disease among those treated than those untreated (*15*).

Of the 50 contacts initiating moxifloxacin-based prophylaxis in the 2 outbreaks, 30 (60%) completed treatment. Therapy was generally well-tolerated; 3 contacts discontinued treatment because of gastrointestinal symptoms, (nausea, vomiting, diarrhea). None of the contacts manifested TB symptoms regardless of treatment status. Those contacts at greatest risk for development of disease may have done so during the outbreak investigation.

Because of the lack of safety information for moxifloxacin-based prophylaxis, we reported these findings despite key limitations. These outbreak investigations were conducted as part of routine TB control activities; thus, treatment regimens were not standardized across the study populations, and contacts were not actively followed. However, these outbreaks demonstrate known exposure to infectious MDR TB case-patients with strong evidence of transmission to a high-risk HIV-positive population. Because of robust disease reporting and broad surveillance coverage, we are able to report on outcomes in NYC within ≤9 years of follow-up time. This study demonstrates that moxifloxacin-based regimens can be used to treat HIV-positive persons exposed to MDR TB with few serious adverse effects, although we were unable to assess efficacy of the regimen. More research is needed to evaluate the effectiveness of prophylaxis for MDR TB.

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Table 2. Multidrug resistant tuberculosis contact investigation results at 2 sites, by HIV status and country of birth, New York, New York, USA*

Site	US-born, no. (%)	Foreign-born, no. (%)	Unknown country of birth, no. (%)	Total, no. (%)
Site A				
No. contacts	87	6	12	105
HIV-positive	68 (78)	4 (67)	12 (100)	84 (80)
Eligible for testing†	56 (82)	1 (25)	12 (100)	69 (82)
Tested	22 (39)	1 (100)	2 (17)	25 (36)
TST positive	3 (14)	0 (0)	0 (0)	3 (12)
Initiated treatment	24 (35)	3 (75)	2 (17)	29 (35)
Treated with moxifloxacin	21 (88)	3 (100)	2 (100)	26 (90)
Completed treatment	13 (62)	2 (67)	1 (50)	16 (62)
Treated with alternate regimen	3 (13)	0 (0)	0	3 (10)
Did not initiate treatment	44 (65)	1 (25)	10 (83)	55 (65)
Lost to follow-up	28 (64)	1 (100)	10 (100)	39 (71)
Refused evaluation or treatment	12 (27)	0 (0)	0	12 (22)
Died before testing or treatment	1 (2)	0 (0)	0	1 (2)
Physician decision to not treat	3 (7)	0 (0)	0	3 (5)
HIV-negative	14 (16)	2 (33)	0	16 (15)
Eligible for testing*	11 (79)	1 (50)	0	12 (75)
Tested	3 (27)	1 (100)	0	4 (33)
TST positive	0 (0)	1 (100)	0	1 (25)
HIV status unknown	5 (6)	0 (0)	0	5 (5)
Eligible for testing*	3 (60)	0	0	3 60)
Tested	0	0	0	0
TST positive	0	0	0	0
Site B				
No. contacts	47	1	88	136
HIV-positive	47 (100)	1 (100)	35 (40)	83 (61)
Eligible for testing*	45 (96)	0	31 (89)	76 (92)
Tested	20 (44)	NA	20 (65)	40 (53)
TST-positive	3 (15)	0	0 (0)	3 (8)
Initiated treatment	16 (34)	1 (100)	14 (40)	31(37)
Treated with pyrazinamide/moxifloxacin	12 (75)	1 (100)	11 (79)	24 (77)
Completed treatment	9 (75)	1 (100)	4 (36)	14 (58)
Treated with alternate regimen	4 (25)	0	3 (21)	7 (23)
Did not initiate treatment	31 (66)	0	21 (60)	52 (63)
Lost to follow-up	15 (48)	NA	11 (52)	26 (50)
Refused evaluation or treatment	2 (6)	NA	1 (5)	3 (6)
Died before testing or treatment	12 (39)	NA	2 (10)	14 (27)
Physician decision to not treat	2 (6)	NA	7 (33)	9 (17)
HIV status unknown	0	0	53 (60)	53 (39)
Eligible for testing*	NA	NA	28 (53)	28 (53)
Tested	NA	NA	25 (89)	25 (89)
TST positive	NA	NA	0 (0)	0

*Site A was a facility that provided housing and harm-reduction services to a predominantly HIV-positive, homeless, and drug-using population. Site B was a long-term care facility housing HIV-positive, homeless persons. TST, tuberculin skin test.
†Any person who had a history of positive TST result was considered ineligible for testing.

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Rapid Detection of ESBL-Producing *Enterobacteriaceae* in Blood Cultures

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We rapidly identified extended-spectrum β-lactamase (ESBL) producers prospectively among 245 gram-negative bacilli–positive cultured blood specimens using the Rapid ESBL Nordmann/Dortet/Poirel test and direct bacterial identification using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. This combination identified ESBL-producing *Enterobacteriaceae* within 30 min and had high predictive values.

An essential parameter for improving the outcome of sepsis is early implementation of appropriate antibiotic therapy (*1*–*5*). Recently, using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) technology directly with blood cultures was found to help guide clinical management of bacteremia caused by gram-negative bacteria (GNB) (*6*). Resistance to broad-spectrum cephalosporins is spreading rapidly among *Enterobacteriaceae*, mostly related to acquisition of extended-spectrum β-lactamases (ESBLs) (*7*). ESBL-producing *Enterobacteriaceae* (ESBL-E) are usually resistant to most b-lactams except cephamycins and carbapenems.

Using PCR-based molecular techniques on positive blood cultures has been proposed for rapid identification of ESBLs (*8*); however, trained personnel and expensive material are required for their use. In addition, for the TEM- and SHV-type enzymes, detailed gene sequence analysis is required for differentiating narrow-spectrum β-lactamases from ESBLs.

Rapid identification of ESBL producers is possible by using the ESBL Nordmann/Dortet/Poirel (NDP) test (*9*), which is based on the biochemical detection of the hydrolysis of the b-lactam ring of cefotaxime (a broad-spectrum cephalosporin). Presence of these bacteria has previously been evaluated with cultured bacteria and with spiked blood cultures (*9*).

In this study, we evaluated the ESBL NDP test prospectively in clinical settings directly from blood cultures. Identification of the bacterial species was done concomitantly from blood cultures by using enhanced MALDI-TOF procedures.

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The Study

During November 2012–May 2013, we studied a single blood culture positive for GNB from each of 245 patients hospitalized at the Bicêtre hospital, a 950-bed hospital located in a suburb of Paris. Positivity of blood cultures was detected by using the BacT/Alert system (bioMérieux, La Balme-les-Grottes, France). After obtaining Gram stain results, we tested the blood cultures positive for GNB directly for 1) ESBL-E by using the ESBL NDP test, and 2) species identification by using the MALDI-TOF MS technique.

We adapted the protocol of the ESBL NDP test for detection of the ESBL-E from blood cultures (*9*) (Online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/3/14-1277-Techapp1.pdf). The detailed MALDI-TOF MS protocol using the VITEK MS system (bioMérieux) is described in Detailed Methods in the Technical Appendix.

We performed antibiotic susceptibility testing (AST) by the disk diffusion method using bacterial colonies grown from blood cultures according to the Clinical Laboratory Standards Institute (CLSI) recommendations (*10*). The same MALDI-TOF technology and the API Gram negative Identification product (bioMérieux) were used for confirmatory identification of bacteria. AST results, obtained 48 h after blood cultures were identified as positive, were interpreted according to the CLSI breakpoints, as updated in 2014 (*10*). MIC of cefotaxime, ceftazidime, and cefepime were determined on Muller-Hinton (MH) agar and MH agar supplemented with 4 μg/mL of tazobactam (final concentration).

We used the double-disk synergy test (DDST) for the phenotypic detection of ESBL producers (*11*), according to the CLSI recommendations. For each sample, 1 disk contained cefotaxime, ceftazidime, or cefepime, and a second disk contained ticarcillin and clavulanate (*10*). The DDST was also performed on MH agar plates (bioMérieux) containing cloxacillin (150 mg/L) to inhibit cephalosporinase activity of natural producers of those inducible cephalosporinases. Because the DDST was performed in parallel to the AST, results were obtained 48 h later. The DDST was considered to be the reference standard for the detection of ESBL-E.

We also used molecular biology techniques to identify the ESBL genes. We used PCR to amplify DNA, which we then sequenced using bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ primers (*9*).

During the study, 245 blood specimens that were collected from patients hospitalized in any unit of the hospital

were cultured and grew GNB (Table 1). The 245 cases of bacteremia were attributed to *Enterobacteriaceae* (211, 86.1%), nonfermentative GNB (31, 12.7%), and anaerobic GNB (3, 1.2%) (Table 2). Three blood cultures (1.2%) were positive for 2 enterobacterial species (Table *2*). *Escherichia coli* was the predominant enterobacterial species (118/211, 55.9%); the next most prevalent were *Klebsiella pneumoniae* (37/211, 17.5%) and *Enterobacter cloacae* (20/211, 9.5%). *Pseudomonas aeruginosa* (24/31, 77.4%) was the predominant non-fermentative GNB (Table 2). Anaerobic GNB belonged to the *Bacteroides fragilis* group (*Bacillus fragilis* and *Bacillus vulgatus*).

We identified bacteria directly from blood culture using the MALDI-TOF technique for 237 (96.7%) isolates; results corresponded to bacterial identification after culture (Table 2). *Salmonella* spp. (n = 5) were correctly identified at the genus level (Table 1). For the 3 positive blood cultures that contained 2 enterobacterial species (Table 2), results were noninterpretable.

ESBL-E producers (n = 47) represented 22.3% of *Enterobacteriaceae*. Among the 47 ESBL-E, 30 *E. coli*, 13 *K. pneumoniae*, 3 *E. cloacae*, and 1 *Citrobacter freundii* were identified from patients who were infected in the community or the hospital (detailed data not shown) (Table 1; Table 3, http://wwwnc.cdc.gov/EID/article/21/3/14-1277-T3.htm). Most of the ESBLs were of the CTX-M-type (49/51, 96.1%); CTX-M-15 was predominant (35/51, 68.6%). The proportion of ESBL producers were 35.1%, 25.4%, and 15% among *K. pneumoniae*, *E. coli*, and *E. cloacae*, respectively (Table 3). The ESBL NDP test perfectly identified the 47 ESBL-E pathogens (Tables 1, 3). Accordingly, a 100% correlation between intermediated susceptibility of resistance to cefotaxime and positivity of the ESBL NDP test was observed, whereas this correlation was of 76.6% and 74.4%, respectively, when ceftazidime and cefepime susceptibility results were used for this same comparison (Table 3). The ESBL NDP test gave negative results for 164 specimens that were negative for ESBL-E (Table 2). The ESBL NDP test revealed a cefotaxime-hydrolyzing enzyme that was not inhibited by tazobactam for 1 *K. pneumoniae* isolate that produced an acquired cephalosporinase, 3 of the 5 *E. cloacae* that overproduced chromosome-encoded AmpC, and 2 *Bacteroides* spp. strains (data not shown).

The ESBL NDP test used with blood cultures had a sensitivity of 100% (95% CI: 92.4%–100%), a specificity of 100% (95% CI: 97.7%–100%), a positive predictive value of 100% (95% CI: 99.2%–100%) and a negative predictive value of 100% (95% CI: 97.8%–100%) for the detection of ESBL-E.

Conclusions

Detection of ESBLs that are the main source of cephalosporin resistance in *Enterobacteriaceae* still relies on antibiotic susceptibility testing, results of which usually take 24–48 h. We show that the ESBL NDP test directly performed on positive blood cultures is a reliable technique to identify ESBL-E within 30 min. Although these results are promising, they should be further confirmed in other countries where the prevalence and the epidemiology of ESBL-E might be different. A strong correlation between intermediate susceptibility or resistance to cefotaxime and positivity of the ESBL NDP test was observed (Table 3). Similar correlation between resistance to cefotaxime and ESBL production in *Enterobacteriaceae* was obtained in the United States (*12*). A concomitant use of the Carba NP test (*13*) directly from blood culture will also identify carbapenemase producers (such as *K. pneumoniae* carbapenemase producers) that also confer clavulanic-acid–inhibited resistance to cephalosporins.

Table 1. Origin of gram-negative bacilli identified in blood samples drawn from hospitalized patients and cultured by using rapid detection methods for bacterial blood cultures*

Hospital department	No. gram-negative bacilli	No. (%) *Enterobacteriaceae*					No. (%) nonfermenting bacilli
		Total	*Escherichia coli*	Other	ESBL-negative	ESBL-positive	
Cardiology	5	4 (80)	2 (50)	2 (50)	4 (100)	0	1 (20)
Digestive surgery	9	8 (89)	6 (75)	2 (25)	6 (75)	2 (25)	1 (11)
Orthopedic surgery	3	3 (100)	2 (67)	1 (33)	3 (100)	0	0
Endocrinology	2	2 (100)	0	2 (100)	2 (100)	0	0
Gerontology	9	7 (78)	3 (43)	4 (57)	7 (100)	0	2 (22)
Gynecology-obstetric	3	2 (67)	0	2 (100)	2 (100)	0	1 (33)
Hepato-gastroenterology	20	18 (90)	8 (44)	10 (56)	15 (83)	3 (17)	2 (10)
Emergency	40	37 (93)	30 (81)	7 (19)	32 (86)	5 (14)	3 (8)
Infectious diseases	28	25 (89)	16 (64)	9 (36)	17 (68)	8 (32)	3 (11)
Nephrology	28	21 (75)	8 (38)	13 (62)	11 (52)	10 (48)	7 (25)
Neurology	8	8 (100)	5 (63)	3 (38)	8 (100)	0	0
Pediatric unit	2	2 (100)	2 (100)	0	2 (100)	0	0
Pneumology	4	3 (75)	2 (67)	1 (33)	2 (67)	1 (33)	1 (25)
Intensive care unit	67	57 (85)	24 (42)	33 (58)	42 (74)	15 (26)	10 (15)
Rhumatology	3	2 (67)	2 (100)	0	1 (50)	1 (50)	1 (33)
Urology	14	12 (86)	8 (67)	4 (33)	10 (83)	2 (17)	2 (14)
Total	245	211 (86)	118 (56)	93 (44)	164 (78)	47 (22)	34 (14)

*ESBL, extended spectrum β-lactamase.

Table 2. Results of MALDI-TOF and ESBL NDP testing of 245 blood cultures positive for gram-negative bacilli and definitive identification and antibiotic resistance phenotype obtained with cultured bacteria*

30 min			24–48 h	
MALDI-TOF identification	No.	ESBL NDP test	Definitive identification	β-Lactam resistance phenotype a
Enterobacteriaceae				
Escherichia coli	38	–	*E. coli*	Wild-type
	47	–	*E. coli*	Penicillinase
	1	–	*E. coli*	IRT
	1	–	*E. coli*	Low-level cephalosporinase
	1	–	*E. coli*	Overexpressed cephalosporinase
	30	+	*E. coli*	ESBL
Proteus mirabilis	3	–	*P. mirabilis*	Penicillinase
Salmonella spp.	1	–	*Salmonella* Typhimurium	Wild-type
	1	–	*Salmonella* Enteritidis	Wild-type
	1	–	*Salmonella* Paratyphi A	Wild-type
	1	–	*Salmonella* Typhi	Wild-type
	1	–	*Salmonella* Kentucky	Penicillinase
Klebsiella pneumoniae	23	–	*K. pneumoniae*	Wild-type
	1	–	*K. pneumoniae*	Acquired cephalosporinase
	13	+	*K. pneumoniae*	ESBL
K. oxytoca	5	–	*K. oxytoca*	Wild-type
Citrobacter koseri	2	–	*C. koseri*	Wild-type
Enterobacter cloacae	12	–	*E. cloacae*	Wild-type
	5	–	*E. cloacae*	Overexpressed cephalosporinase
	3	+	*E. cloacae*	ESBL
Enterobacter aerogenes	5	–	*E. aerogenes*	Wild-type
Citrobacter braaki	1	–	*C. braaki*	Wild-type
C. freundii	1	+	*C. freundii*	ESBL
Morganella morganii	4	–	*M. morganii*	Wild-type
Providencia rettgeri	2	–	*P. rettgeri*	Wild-type
Proteus vulgaris	1	–	*P. vulgaris*	Wild-type
Serratia marcescens	4	–	*S. marcescens*	Wild-type
None	1	–	*E. coli* + *Proteus mirabilis*	Penicillinase + Penicillinase
	2	–	*K. pneumoniae* + *S. marcescens*	Wild-type + Penicillinase
Nonfermenting bacilli				
Pseudomonas aeruginosa	24	–	*Pseudomonas aeruginosa*	Ceftazidime S
Acinetobacter baumannii	2	–	*A. baumannii*	Ceftazidime S
A. junii	1	–	*A. junii*	Ceftazidime S
Stenotrophomonas maltophilia	2	–	*Stenotrophomonas maltophilia*	Ceftazidime S
Roseomonas gilardii	1	–	*Roseomonas gilardii*	Ceftazidime S
Sphingobacterium multivorum	1	–	*Sphingobacterium multivorum*	Ceftazidime S
Anaerobic gram-negative bacilli				
Bacteroides fragilis	1	–	*B. fragilis*	Wild-type
	1	–	*B. fragilis*	Cefotaxime R
B. vulgatus	1	–	*B. vulgatus*	Cefotaxime R

*MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NDP, Nordmann/Dortet/Poirel test; ESBL, extended spectrum β-lactamase; –, negative test result; IRT, inhibitor-resistant TEM β-lactamase; +, positive test result.

This inexpensive ESBL NDP test might be implemented worldwide. It may optimize rapid choices of antibiotics for treating bloodstream infections. It may also contribute to avoidance of overuse of carbapenems. Finally, a rapid detection of ESBL-E coupled with bacterial species identification will enhance identification of ESBL in species likely to be the source of nosocomial outbreaks (*K. pneumoniae*, *Enterobacter* spp.) and facilitate implementation of a rapid strategy for containment (*14*).

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An international patent form for the ESBL NDP test has been filed on behalf of INSERM Transfert (Paris, France).

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Characteristics of Tuberculosis Cases that Started Outbreaks in the United States, 2002–2011

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A review of 26 tuberculosis outbreaks in the United States (2002–2011) showed that initial source case-patients had long infectious periods (median 10 months) and were characterized by substance abuse, incarceration, and homelessness. Improved timeliness of diagnosis and thorough contact investigations for such cases may reduce the risk for outbreaks.

Tuberculosis (TB) is an airborne disease caused by the bacterium *Mycobacterium tuberculosis* that usually affects the lungs. Most TB cases do not start outbreaks. Contact investigations undertaken after a person receives a diagnosis of infectious TB ideally should identify and treat infected contacts before the infection progresses to disease (*1*). Genotyping data in the United States provide reassurance that most cases do not result from recent transmission (*2*).

However, when TB outbreaks do occur, they can put tremendous strain on local public health resources (*3,4*). A necessary component of all outbreaks is that they must begin with a source case. Recognizing the characteristics of such patients soon after the TB diagnosis could help focus interventions to interrupt transmission and reduce the risk for an outbreak (*5*). We describe a nonrandom sample of TB source cases that started outbreaks in the United States.

The Study

We identified outbreak source cases through a review of investigation reports of TB outbreaks written by Centers for Disease Control and Prevention (CDC) staff during 2002–2011. In a previous publication, we described how CDC teams that assist public health partners with TB outbreaks write standardized reports about field investigations, which include primary data collection and patient re-interviews (*3*). For this review, we included US-based outbreaks that 1) had ≥3 culture-confirmed cases that had epidemiologic links and TB strains with matching genotypes and 2) had the initial source case in the transmission chain, as ascertained by that investigation, identified in the written report. At least 2 co-authors independently reviewed each outbreak report to abstract patient demographic, clinical, and social characteristics. When this dual data entry process showed discrepancies, the authors met to review the report and achieve consensus.

Our main interest was the initial source case; that is, the TB case that began a chain of *M. tuberculosis* transmission that would become locally recognized as an outbreak. Outbreak duration was calculated beginning on the treatment start date for the first reported case and continuing through the treatment start date for the last case as noted at the time of the investigation. The infectious period for pulmonary TB cases was assumed to begin 3 months before TB symptom onset and to end with the initiation of TB treatment (*1*).

Of the 65 TB outbreaks that CDC helped investigate during 2002–2011, a total of 26 met the inclusion criteria. The most common reason for exclusion was not meeting the threshold of ≥3 culture-confirmed cases that had epidemiologic links and matching genotypes (19 outbreaks); 11 adequately large outbreaks or longstanding genotype clusters were excluded because an initial source case could not be unambiguously identified. Other reasons for exclusion included an international setting (6 outbreaks) or missing data (3 outbreaks).

In 20 of the 26 TB outbreaks, the source case-patient was also the first patient in the outbreak to come to the attention of public health authorities (i.e., also the index case-patient). In the other 6 outbreaks, a delay in the diagnosis of the source case meant that another outbreak case was identified before the source case (median delay 2 months, maximum 7 months).

Characteristics of the 26 source cases are provided in the Table. Case-patients ranged in age from 18 to 62 years; most were US-born men, and the distribution of race/ethnicity was similar to that seen in national TB surveillance (*6*). Case-patients had long infectious periods (median 10 months, range 3–36 months); after seeking medical attention for TB symptoms, these patients often experienced delays in TB diagnosis and thus delays in treatment initiation. All patients had pulmonary TB that was smear positive for acid-fast bacilli. Most patients reported excess alcohol or illicit drug use, half had been incarcerated at some point in the past, and nearly half had

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Table. Characteristics of source case-patients for 26 investigated tuberculosis outbreaks, United States, 2002–2011

Characteristic	No. (%)
Demographics	
US-born	19 (73)
Male sex	23 (88)
Race/ethnicity	
White non-Hispanic	7 (27)
Black non-Hispanic	13 (50)
Hispanic	6 (23)
Clinical and laboratory characteristics	
Sputum smear positive for acid-fast bacilli	26 (100)
Cavitary tuberculosis on chest radiograph	21 (81)
HIV co-infection	2 (8*)
Mycobacterium tuberculosis lineage†	
EuroAmerican	18 (69)
East Asian	4 (15)
Social risk factors for tuberculosis	
Excess alcohol use	16 (62)
Illicit drug use	14 (54)
Homelessness within previous year	11 (42)
Incarceration at diagnosis	4 (15)
Incarceration ever	13 (50)
Reasons for prolonged infectious period‡	
Delay in seeking care after symptom onset	8 (31)
Delayed diagnosis once sought care	15 (58)
Noncompliance during treatment	7 (27)
Method of case detection	
Self-reported symptoms led to diagnosis	21 (81)
Tuberculosis contact investigation	1 (4)
Other screening	1 (4)
Unknown	3 (12)

*Of 24 patients for whom HIV test results were available.
†Genotype lineage was not determined for 4 outbreaks that occurred in 2002–2003, before spoligotyping was routine.
‡Causes not always documented and not mutually exclusive.

been homeless in the year before diagnosis. Most cases came to public health attention because the patient sought care for TB symptoms rather than through contact investigations.

Of the 26 outbreaks, 9 were limited to 1 generation of spread from the source case-patient to that person's direct contacts; the other 17 cases had further waves of transmission beyond the source case-patient. At the time of the investigations, median outbreak duration was 13 months (range 4–151 months), and a total of 242 cases (median 8 per outbreak) were diagnosed. The 4 outbreaks in which the source case-patient was incarcerated at diagnosis ranged in size from 7 to 9 cases. For the 11 outbreaks in which the source case-patient had recently been homeless, the median outbreak size at time of investigation was 9 cases, with outbreak size ranging from 3 to 27, the latter being the largest outbreak in our review.

Conclusions

In this nonrandom sample of 26 TB outbreaks in the United States during 2002–2011, we found that characteristics common among TB cases that started outbreaks included pulmonary TB smear-positive for acid-fast bacilli, patient substance abuse, and prolonged infectious periods. The largest outbreaks involved source case-patients who were incarcerated or had been homeless.

Persons with TB can spread the disease until it is correctly diagnosed and treated (*7*). In TB control, the focus is typically on the individual patient, health care provider, and public health factors that contributed to a delayed diagnosis, inadequate isolation or treatment, or otherwise suboptimal response to an individual TB case (*8*). As the frequency of TB cases continues to decline in the United States, however, so does provider experience with its diagnosis, which raises the possibility that the recent trend toward more cases of pulmonary TB being diagnosed in later disease stages might be a related consequence (*9*).

When contact investigations are incomplete because of limited resources or hard-to-reach populations, TB outbreaks can spread (*3,4*). Substance abuse, incarceration, and homelessness, social risk factors that are common among the TB source cases in our review but that greatly complicate contact investigations, have been shown to increase the likelihood of genotype cluster growth (*10,11*) and outbreak development (*3*).

This retrospective review of secondary data sources has several limitations. The beginning of an outbreak can be difficult to determine; the concept of an initial source case is an artificial construct if one considers that every source must have had its own progenitor. In addition, the definition of the outbreak's duration and size was determined on the basis of the timing of the CDC field investigation. The generalizability of the characteristics of these source cases is uncertain.

However, a strength of this analysis is that a group of experienced TB investigators considered all the information available to determine which of the known cases involved in an outbreak most likely represented the source. An additional advantage over many population-based genotype studies is that epidemiologic links among patients were ascertained, enabling confirmation that cases in the same genotype cluster were indeed part of the same chain of transmission. We also knew direction of transmission, enabling us to establish source cases without having to make assumptions on the basis of the timing of diagnoses (*12*). In addition, we had information from patient medical records about TB symptom onset, which enabled us to examine variables related to infectious periods and delayed diagnosis.

TB contact investigations for all persons diagnosed with pulmonary TB with acid-fast bacilli smear-positive test results are a well-known public health priority (*1*). This review underscores the particular importance of prompt and thorough investigations for TB cases confirmed by positive smear for acid-fast bacilli in which patients have experienced substance abuse, incarceration, or homelessness. Public health departments should work with local health

care providers to address barriers to accessing care faced by marginalized populations and in recognizing and diagnosing TB once symptomatic patients seek medical attention.

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Reassortant Highly Pathogenic Influenza A(H5N6) Virus in Laos

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In March 2014, avian influenza in poultry in Laos was caused by an emergent influenza A(H5N6) virus. Genetic analysis indicated that the virus had originated from reassortment of influenza A(H5N1) clade 2.3.2.1b, variant clade 2.3.4, and influenza A(H6N6) viruses that circulate broadly in duck populations in southern and eastern China.

Asian lineage influenza A(H5N1) viruses continue to cause serious disease in poultry and sporadic human infections (*1*). This disease was reported in 2004 in poultry in Laos that were infected with clade 1 influenza A(H5N1) virus and subsequently in poultry infected with clade 2.3.4 and 2.3.2 viruses in 2006 and 2008, respectively (*2*,*3*). Interclade reassortant influenza A(H5N1) virus genotypes homologous to viruses circulating in southern China and Vietnam have also been detected, which indicated previous transboundary virus transfers. However, influenza A(H5N1) virus in poultry has not been reported in Laos since mid-2010 (*4*). We report highly pathogenic avian influenza (HPAI) in poultry in Laos in March 2014 that was caused by an emergent reassortant influenza A(H5N6) virus, apparently imported by live poultry from China.

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The Study

Virus isolations were performed under Biosafety Level 3 containment. Animal trials were conducted after approval of the Australian Animal Health Laboratory Animal Ethics and Institutional Biosafety Committees.

After reports of disease in village poultry in Nan District, Luang Prabang Province, and Xayabouly District, Xayabouly Province (Figure 1), the Lao Provincial Agriculture and Forestry Office visited 2 villages during March 12–14, 2014, and collected samples from dead and sick birds for diagnosis. These birds were positive for avian influenza A virus (H5 subtype) by real-time reverse transcription PCR (RT-PCR) (*5*). Results were reported to the World Organisation for Animal Health on March 31, 2014 (*4*).

Infected poultry in both locations were 2 to 3–day-old chicks and ducklings imported from Jinghong in Yunnan Province, China, to a smallholder distributor in Luang

Figure 1. Locations of cases of highly pathogenic avian influenza in poultry caused by influenza A(H5N6) virus in Laos, March 2014. Dark gray shading indicates the 2 districts (Muang Nan and Muang Xayabouly) situated at the boundaries of Luang Prabang and Xayabouly Provinces, where villages with infected poultry were located. Affected birds were associated with regular consignments of mixed poultry transported from Jinghong and elsewhere in Yunnan Province, China.

Prabang on March 1. Consignments from this batch were delivered to the villages a week later, and birds at both locations showed clinical signs of influenza and died suddenly <24 h after arrival.

Respective pooled organ samples from a chicken and a duck from each village were sent to the Australian Animal Health Laboratory for analysis. These 4 samples were confirmed as positive for avian influenza virus (subtype H5) by RT-PCR but negative for neuraminidase (NA) subtype N1 and were subjected to virus propagation in 9 to 11–day-old specific pathogen–free chicken eggs.

Influenza genome sequencing was performed by using a MiSeq sequencer (Illumina, San Diego, CA, USA) and amplified avian influenza virus DNA libraries from a chicken sample from Luang Prabang and a duck sample from Xayabouly (*6*). Sequencing showed that an average of

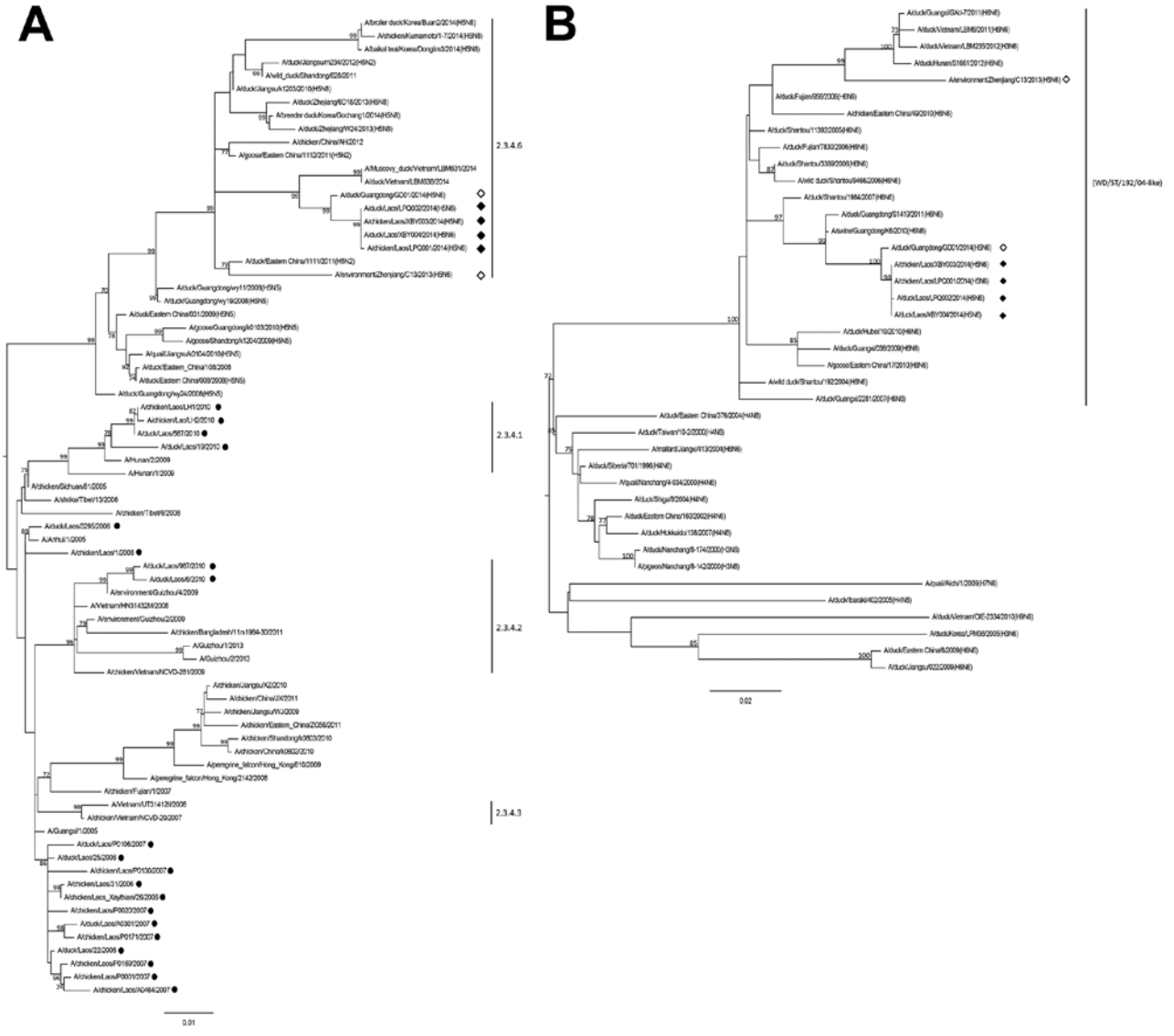

Figure 2. Phylogenetic analyses of influenza A(H5N6) viruses detected in Laos, March 2014, on the basis of the hemagglutinin (HA) and N6 neuraminidase (NA) genes. A) HA subtree showing relationships of emergent influenza A(H5N6) viruses with clade 2.3.4 H5 avian influenza viruses and B) NA subtree showing relationships with Asian lineage N6 avian influenza viruses. Vertical lines denote H5 subtype virus clades on the HA tree and the WD/ST/192/04 (A/wild duck/Shantou/192/2004)-like N6 gene pool on the NA tree. Proposed clade 2.3.4.6 has not been formally recognized by the World Health Organization/World Organisation for Animal Health/Food and Agricultural Organization of the United Nations H5N1 Evolution Working Group. Black diamonds indicate viruses identified in this study, white diamonds indicate Asian influenza A(H5N6) viruses identified in other studies, and black circles indicate viruses previously identified in Laos. All viruses are subtype H5N1 unless otherwise indicated. Bootstrap values ≥70% from 1,000 replicates are indicated at relevant nodes, and scale bars indicate nucleotide substitutions per site. The full HA and NA trees are provided in the online Technical Appendix Figure, panels A and B (http://wwwnc.cdc.gov/EID/article/21/3/14-1488-Techapp1.pdf).

98.6% reads mapped the virus genome with 210–481,069 coverage depth along different segments.

Hemagglutinin (HA) and NA genes were amplified from the 4 virus isolates by using RT-PCR and sequenced by using the Sanger method. Full-length HA and NA sequences from these isolates and 6 internal gene sequences from the 2 representative isolates shared 99%–100% nt identity, which indicated 1 influenza A(H5N6) virus genotype. Twenty consensus virus gene sequences were characterized and deposited in GenBank under accession nos. KM496962–KM496981.

Virus replicated in chicken eggs (titers >9 $\log_{10}$ 50% egg infectious doses). The 4 influenza A(H5N6) virus isolates were designated A/chicken/Laos/LPQ001/2014(H5N6), A/duck/Laos/LPQ002/2014(H5N6), A/chicken/Laos/XBY003/2014(H5N6), and A/duck/Laos/XBY004/2014(H5N6). Virus pathogenicity was evaluated by inoculation of six 4-week-old specific pathogen–free chickens with 6 $\log_{10}$ 50% egg infectious doses of A/duck/Laos/XBY004/2014(H5N6) from egg allantoic fluid by the oral–nasal–ocular route.

Clinical signs, including facial swelling, hunching, fluffed feathers, depression, and huddling behavior, were observed in birds at 28 hours postinoculation. All birds were euthanized by 44 hours postinoculation for ethical reasons. The short incubation period, rapid progression of fulminant disease, and abundant viral antigen in multiple tissue and cell types were consistent with HPAI.

Analysis of each genome segment indicated that the Laos influenza A(H5N6) virus (LAO/14) is a novel triple reassortant. All genome segments of LAO/14 had highest (99%) GenBank sequence matches with corresponding genes of A/duck/Guangdong/GD01/2014(H5N6) (GD01/14), a virus independently identified in March 2014. We performed maximum-likelihood phylogenetic analysis on the 8 gene segments of LAO/14 by using the MEGA6 program (*7*) and avian influenza virus (subtypes H5 or N6) sequences from GenBank.

Table 1. Amino acid substitutions in translated mature HA1 proteins of influenza A(H5N6) virus from Laos and H5 clade 2.3.4 reference viruses*

Mature HA1 position (H5 numbering)†	H5 subtype virus clade‡						
	DK/LAO/XBY4 (H5N6) 2.3.4.6	DK/GD/GD01 (H5N6) 2.3.4.6	WD/SD/628 (H5N1) 2.3.4.6	CK/LAO/XNY26 (H5N1) 2.3.4	JWE/HK/1038 (H5N1) 2.3.4	DK/LAO/3295 (H5N1) 2.3.4	ANH/1 (H5N1) 2.3.4
40	R	R	K	K	K	K	K
45	N	N	N	D	D	D	D
53	K	K	K	R	R	R	R
72	R	R	R	N	N	N	N
82	R	R	R	K	K	K	K
95	L	L	L	F	F	F	F
114	T	T	I	I	I	I	I
115	L	L	L	Q	Q	Q	Q
123	P	P	P	S	S	S	S
124	N	N	D	D	D	D	D
127	T	T	T	A	A	A	A
129	L	L	L	S	S	S	S
133	A	A	A	S	S	S	S
140	M	M	A	T	T	T	T
151	T	T	I	I	I	I	I
155	D	N	D	N	N	N	N
156	A	A	A	T	K	T	T
162	M	M	I	R	R	R	R
169	R	R	R	Q	Q	Q	Q
183	N	N	N	D	D	D	D
189	N	N	N	K	K	K	K
192	K	K	K	Q	Q	Q	Q
198	V	V	V	I	I	I	I
210	E	V	V	V	V	V	V
218	Q	Q	Q	K	K	K	K
223	R	R	R	S	S	S	S
240	H	H	H	N	N	N	N
263	T	T	T	A	A	A	A
265	M	M	M	M	M	I	V
269	M	M	V	V	V	V	V
273	H	H	H	N	N	N	N

*HA, hemagglutinin.
†Sites associated with antigenicity are indicated in boldface (not comprehensive) (*11*).
‡Virus names: DK/LAO/XBY4, A/duck/Laos/XBY004/2014; DK/GD/GD01, A/duck/Guangdong/GD01/2014; WD/SD/628, A/wild duck/Shandong/628/2011; CK/LAO/XNY26, A/chicken/Laos/Xaythiani-26/2006; JWE/HK/1038, A/Japanese white-eye/Hong Kong/1038/2006; DK/LAO/3295, A/duck/Laos/3295/2006; ANH/1, A/Anhui/1/2005. Clade designations provided by the World Health Organization/World Organisation for Animal Health/Food and Agricultural Organization of the United Nations H5N1 Evolution Working Group unified nomenclature system for highly pathogenic avian influenza (H5N1) viruses (http://www.who.int/influenza/gisrs_laboratory/h5n1_nomenclature/en/). Proposed clade 2.3.4.6 has not been formally recognized.

Table 2. Hemagglutination inhibition assay of influenza A(H5N6) virus from Laos with chicken and ferret antisera against reference influenza A(H5N1) viruses*

Antigen	H5 subtype clade†	Reference chicken antiserum‡ CK/VNM/8 (H5N1) 1	CK/IND/BBV M204 (H5N1) 2.1.3	PH/VNM/ 3773 (H5N1) 2.3.2.1c	CK/LAO/ XNY26 (H5N1) 2.3.4	CK/MYM/ 295 (H5N1) 2.3.2.1a	DK/LAO/ XBY4 (H5N6) 2.3.4.6	CK NEG
Reference								
CK/VNM/8	1	**640**	20	40	80	80	80	<10
CK/IND/BBVM204	2.1.3	10	**1,280**	20	10	20	20	<10
PH/VNM/3773	2.3.2.1c	80	80	**640**	80	320	320	<10
CK/LAO/XNY26	2.3.4	160	80	40	**320**	160	80	<10
CK/MYM/295	2.3.2.1a	80	40	160	20	**320**	20	<10
Test								
CK/LAO/LPQ1	2.3.4.6	<10	<10	<10	<10	<10	320	<10
DK/LAO/LPQ2	2.3.4.6	<10	<10	<10	<10	<10	320	<10
CK/LAO/XBY3	2.3.4.6	<10	<10	<10	<10	<10	320	<10
DK/LAO/XBY4	2.3.4.6	40	<10	10	<10	<10	**640**	<10

		Reference ferret antiserum‡ JWE/HK/ 1038 (H5N1) 2.3.4	DK/LAO/329 5 (H5N1) 2.3.4	ANH/1 (H5N1) 2.3.4	CM/HK/ 5052 (H5N1) 2.3.2.1	BS/HK/ 1161 (H5N1) 2.3.2.1b	HUB/1 (H5N1) 2.3.2.1a	DK/VNM/ 2848 (H5N1) 2.3.2.1c
Reference								
JWE/HK/1038	2.3.4	**80**	80	80	<40	<40	ND	ND
DK/LAO/3295	2.3.4	<40	**160**	80	<40	<40	<40	<40
ANH/1	2.3.4	<40	320	**320**	<40	<40	<40	<40
CM/HK/5052	2.3.2.1	<40	40	80	**160**	<40	<40	80
BS/HK/1161	2.3.2.1b	<40	<40	80	80	**320**	<40	80
HUB/1	2.3.2.1a	<40	<40	40	80	<40	**80**	40
DK/VNM/2848	2.3.2.1c	<40	<40	<40	40	160	<40	**160**
Test								
CK/LAO/LPQ1	2.3.4.6	<40	<40	<40	<40	<40	<40	<40
DK/LAO/LPQ2	2.3.4.6	<40	<40	<40	<40	<40	<40	<40
CK/LAO/XBY3	2.3.4.6	<40	<40	<40	<40	<40	<40	<40
DK/LAO/XBY4	2.3.4.6	<40	<40	40	<40	<40	<40	<40

*Virus names: CK/VNM/8, A/chicken/Vietnam/8/2004; CK/IND/BBVM204, A/chicken/Indonesia/ BBVM204/2007; PH/VNM/3773, A/pheasant/Vietnam/3773/2013; CK/LAO/XNY26, A/chicken/Laos/Xaythiani-26/2006; CK/MYM/295, A/chicken/Myanmar/295/2010; JWE/HK/1038, A/Japanese white-eye/Hong Kong/1038/2006; DK/LAO/3295, A/duck/Laos/3295/2006; ANH/1, A/Anhui/1/2005; CM/HK/5052, A/common magpie/Hong Kong/5052/2007; BS/HK/1161, A/barn swallow/Hong Kong/1161/2010; HUB/1, A/Hubei/1/2010; DK/VNM/2848, A/duck/Vietnam/NCVD-2848/2013; CK/LAO/LPQ1, A/chicken/Laos/LPQ001/2014; DK/LAO/LPQ2, A/duck/Laos/LPQ002/2014; CK/LAO/XBY3, A/chicken/Laos/XBY003/2014; DK/LAO/XBY4, A/duck/Laos/XBY004/2014; CK NEG, negative control chicken serum.
†Clade designations provided by the World Health Organization/World Organisation for Animal Health/Food and Agricultural Organization of the United Nations H5N1 Evolution Working Group unified nomenclature system for highly pathogenic avian influenza (H5N1) viruses (http://www.who.int/influenza/gisrs_laboratory/h5n1_nomenclature/en/). Proposed clade 2.3.4.6 has not been formally recognized.
‡Values are titers. Homologous titers of reference virus antigen to its corresponding antiserum are indicated in boldface.

HA gene phylogeny confirmed that LAO/14 and GD01/14 were closely related and belonged to clade 2.3.4.6, which was proposed for H5 subtype HPAI viruses with N1, N2, and N8 subtypes detected in poultry in China since 2010 (*8*) and in Vietnam in 2014 (Figure 2). The progenitor influenza A(H5N6) virus reassortant might have derived its HA gene from A/wild duck/Shandong/628/2011(H5N1)–like viruses in eastern China (Figure 2). Another virus, A/environment/Zhenjiang/C13/2013(H5N6), which has a similar genotype but independently reassorted gene lineages, had also been identified in Jiangsu Province (Figure 2; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/3/14-1488-Techapp1.pdf). The same clade 2.3.4 H5 subtype virus donor pool resulted in a reassortant influenza A(H5N8) virus that has caused influenza outbreaks in poultry in South Korea since January 2014 (*9*).

Mature HA proteins of LAO/14 and GD01/14 have amino acids H103, N182, G221, Q222, and G224 (H5 numbering), which indicates that a preference for avian-like α2,3-sialic acid receptor binding is probably retained (*10*,*11*). Influenza A(H5N6) viruses have the HA cleavage sequence PLRERRRKR/GLF that is common in clade 2.3.4 HPAI viruses. Additional HA1 sites that might contribute to receptor binding and antigenic properties of LAO/14 are shown in Table 1.

The LAO/14 NA gene likely originated from group II lineage influenza A(H6N6) viruses that are established in domestic ducks in China (*12*) and have the highest (98%) nt identities with influenza A(H6N6) viruses isolated from domestic pig and live market poultry (Figure 2). LAO/14 and GD/14 influenza A(H5N6) viruses have the 11-aa deletion in the NA stalk region (positions 59–69; N6 numbering) found in influenza A(H6N6) viruses in China (*12*).

Key known NA and matrix 2 inhibitor resistance markers were not observed in LAO/14 (*2*).

Influenza A(H5N6) viruses have an internal gene backbone from clade 2.3.2.1b influenza A(H5N1) virus, which is also found in domestic ducks from south-central and eastern China (*13*,*14*). The 6 internal genes of LAO/14 had highest (98%–99%) sequence matches with those of A/duck/Hunan/S4220/2011(H5N1) or A/duck/Zhejiang/2248/2011(H5N1). The polymerase basic 2 E627K mutation linked to mammalian host adaptation was not present in influenza A(H5N6) viruses (*10*). Phylogenetic trees of virus internal genes are shown in the online Technical Appendix Figure, panels C–H. The influenza A(H5N6) virus from Zhenjiang, Jiangsu Province, China, had a nontruncated N6 NA and divergent A(H5N1) polymerase basic 2 gene lineage, which supports an independent reassortment origin.

Hemagglutination by LAO/14 was generally uninhibited by chicken or ferret antisera against reference influenza A(H5N1) virus, including antisera to a clade 2.3.4 virus from Laos (Table 2), by hemagglutination inhibition test. Antigenic divergence of LAO/14 from clade 2.3.4 viruses was supported by accumulation of 31-aa substitutions in their mature HA1 (Table 1). However, chicken antiserum against A/duck/Laos/XBY004/2014(H5N6) showed broader cross-reactivity with some viruses of other influenza A(H5N1) clades, which might indicate some conservation of epitopes.

Conclusions

After an absence of 4 years, HPAI in poultry in Laos was shown to be caused by an emergent reassortant influenza A(H5N6) virus. Genetic evidence indicates that this virus probably originated from domestic poultry in China. The common progenitor of LAO/14 and GD01/14 appears to have originated from reassortment of H5 clade 2.3.2.1b, H5 clade 2.3.4.6, and influenza A(H6N6) viruses that circulate in ducks in southern and eastern China. Coincidentally, the first fatal human infection with an influenza A(H5N6) virus in Sichuan Province was reported in May 2014 (*15*). Infection with influenza A(H5N6) virus was confirmed in poultry in Sichuan Province at that time (*4*), although the relationship of this virus with LAO/14 is unclear.

Influenza A(H5N6) viruses might already be widely distributed; poultry in Vietnam have been affected since April 2014 (*4*). LAO/14 was antigenically distant to clade 2.3.4 viruses, which raises concerns about effectiveness of current poultry vaccines against this virus, as well as vaccine candidate selection for prepandemic preparedness.

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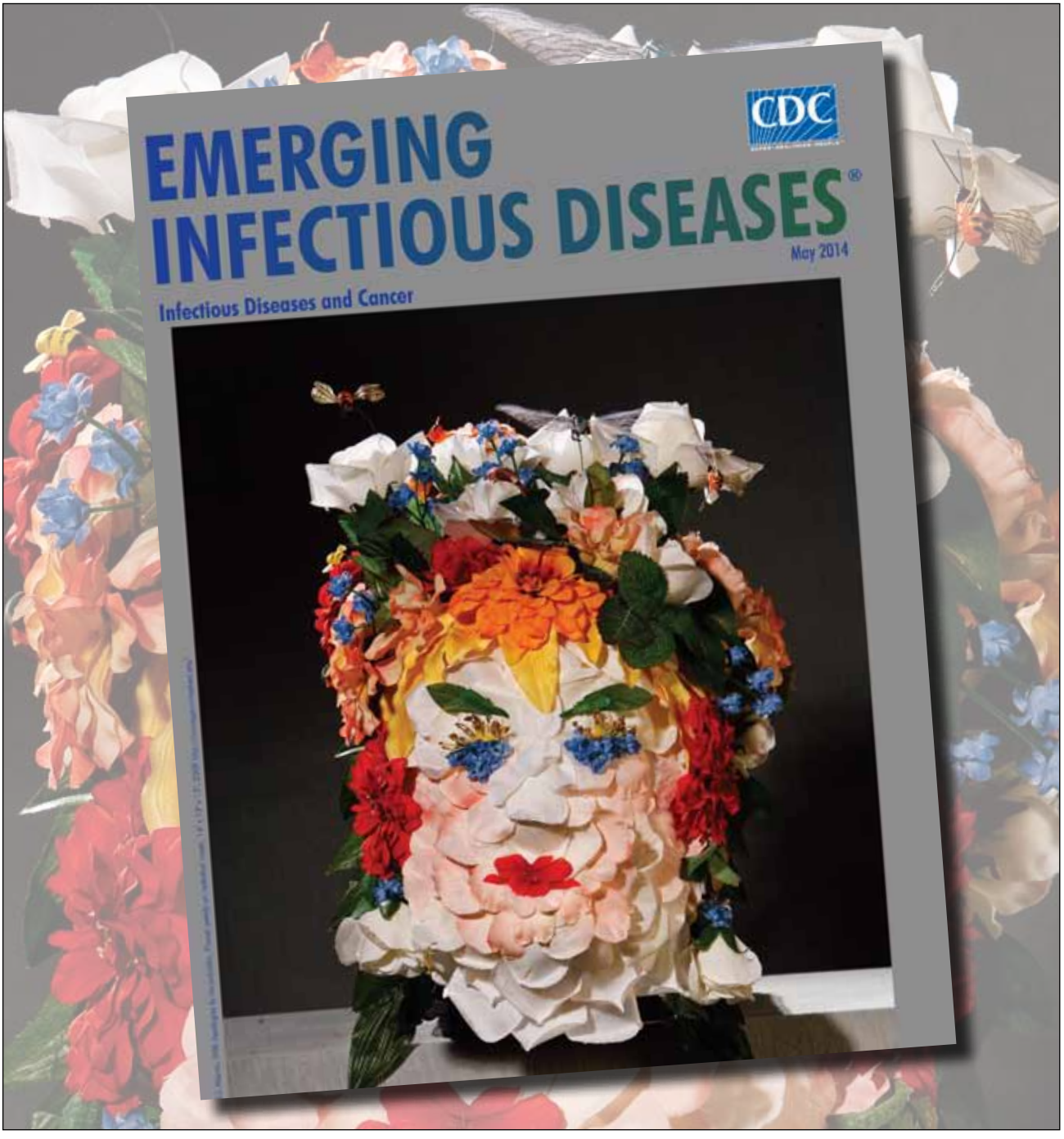

Autochthonous Dengue Fever, Tokyo, Japan, 2014

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After 70 years with no confirmed autochthonous cases of dengue fever in Japan, 19 cases were reported during August–September 2014. Dengue virus serotype 1 was detected in 18 patients. Phylogenetic analysis of the envelope protein genome sequence from 3 patients revealed 100% identity with the strain from the first patient (2014) in Japan.

Although ≈200 imported cases of dengue fever have recently been reported in Japan (*1*), an autochthonous case had not been confirmed there for 70 years (*2*). However, on August 26, 2014, an autochthonous case of dengue fever in a patient with no history of overseas travel was reported in Tokyo, and as of October 31, 2014, a total of 160 autochthonous cases in Japan had been confirmed (*3*).

The Cases

We report 19 cases of confirmed autochthonous dengue fever treated at the National Center for Global Health and Medicine in Tokyo, Japan, during August 26–September 22, 2014 (Tables 1, 2; online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/3/14-1662-Techapp1.pdf). Because the National Center for Global Health and Medicine is located close to the epicenter of this outbreak, 19 (12%) of the 160 cases of this outbreak were confirmed at this Center. Informed consent for participation in this study was obtained from all 19 patients. Of these 19 patients, the median age was 33.0 years (range 6–64 years), and 10 (55.6%) were men. None of the patients had any underlying illness except for hypertension (2 patients) and asthma (1 patient). One patient had a history of having contracted dengue fever while in the Philippines in 2006. None of the patients had traveled overseas during the 3 months before the outbreak of dengue virus type 1 (DENV-1) in Japan.

Places of exposures were assessed for all patients; 15 patients had recently visited Yoyogi Park and were bitten by mosquitoes while there; the remaining 4 patients had visited Shinjuku Central Park, Meiji Jingu Shrine, Meijiingu Gaien, and Ueno Park. All of these parks have been reported as affected regions in this outbreak (*3*) (Figure 1). The day of exposure was estimated for 9 patients for whom the day of visitation and mosquito bites while in the parks could be confirmed. Among these 9 patients, the median incubation period was 6 (range 3–9) days. For the other 10 patients, the incubation period was not determined because they had visited the parks over several days or because they lived near these parks. The dates of symptom onset ranged from August 12, 2014, through September 22, 2014; peak incidence occurred in the beginning of September.

Of the 19 patients, 16 were admitted to the National Center for Global Health and Medicine and discharged without sequelae; the other 3 received outpatient treatment and recovered. The patient with a history of dengue fever (patient 19 in the online Technical Appendix Table) experienced fever lasting 7 days, pleural effusion, spontaneous petechiae, and thrombocytopenia (15×10^3 cells/μL on day 8 after illness onset); dengue hemorrhagic fever was diagnosed for this patient by using the World Health Organization guidelines (*4*). On the day of illness onset for this patient, serum was positive for DENV nonstructural protein 1 (NS1) antigen and IgG but negative for DENV IgM. These results demonstrated that this DENV infection was secondary. Epidemiologic studies have also shown that the risk for dengue hemorrhagic fever is significantly higher

Table 1. Clinical characteristics of 19 patients with dengue fever, Tokyo, Japan, August 26, 2014–September 22, 2014

Sign or symptom	Patients, no. (%)
Fever*	19 (100)
Headache	17 (89.5)
Arthralgia	7 (36.8)
Myalgia	7 (36.8)
Nausea	5 (26.3)
Vomiting	2 (10.5)
Diarrhea	1 (5.3)
Rash at first visit	4 (21.1)
Rash during course of illness	15 (78.9)
Sore throat	2 (10.5)
Cough	4 (21.1)
Sputum	1 (5.3)

*Median duration of fever >38°C = 7 (range 4–11) days.

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Table 2. Laboratory findings for 19 patients with dengue fever, Tokyo, Japan, August 26, 2014–September 22, 2014

Laboratory finding at first visit	Reference range	Patient median (interquartile range)
Leukocytes, cells/mm³	3,500-8,500	2,600 (2,385–3,540)
Hematocrit, %	M 40–50, F 35–45	41.8 (38.5–42.9)
Platelets, $\times 10^3/\mu L$	150–350	115 (79–150)
Aspartate transaminase, IU/L	13–33	35 (22–41)
Alanine transaminase, IU/L	8–42	20 (14–26)
Lactate dehydrogenase, IU/L	119–229	227 (166–261)
C-reactive protein, mg/L	0–0.3	6.1 (2.2–16.1)

for patients with secondary rather than primary DENV infection (*5*).

Of the 19 cases, 18 were confirmed as DENV-1 infection by real-time PCR (TaqMan; Life Technologies, Grand Island, NY, USA) (*6*), and samples were positive for NS1 antigen (Platelia Dengue NS1 Antigen assay; Bio-Rad Laboratories, Marnes-la-Coquette, France). The remaining case (case 11) was confirmed positive for IgM and IgG against DENV by dengue IgM ELISA (Focus Diagnostics, Inc., Cypress, CA, USA) and dengue IgG ELISA (Vircell, Granada, Spain), respectively. The serotype of the DENV in the other 18 patients was confirmed to be serotype 1. Nucleotide sequences were determined by using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA). Phylogenetic analysis of the DENV envelope (E) protein genome sequence obtained from serum from patient 2 (GenBank accession no. LC006123) demonstrated that the E protein shared 100% homology with the sequence of a DENV-1 strain from the first patient in this outbreak in Japan (GenBank accession no. LC002828). The sequence from patient 2 shared 99.7% identity with the sequence of a DENV strain isolated in Guangzhou, China, in 2013 (GenBank accession no. KJ545459) and 99.3% identity with the sequence of a DENV strain isolated in Indonesia in 2010 (GenBank accession no. JN415489) (Figure 2). The sequence of the E protein from another 2 patients (patients 4 and 10) shared 100% homology with that of patient 2.

Conclusions

Our results suggest that a single strain may have caused most of the DENV cases in Tokyo. A similar outbreak of dengue fever had been reported in Ningbo, China (68 cases) (*7*), and in Hawaii, USA (122 cases) (*8*).

DENV is transmitted mainly through the bite of the *Aedes aegypti* mosquito, which is distributed in tropical and subtropical regions. In Japan, the distribution of *Ae. aegypti* mosquitoes is limited, and as of 2013, these mosquitoes had been found only at the Narita International Airport (*9*), which is located ≈60 km from the site of the DENV outbreak in Tokyo. In contrast, the distribution of *Ae. Albopictus* mosquitoes, another vector of DENV, extends from western regions to northern regions of

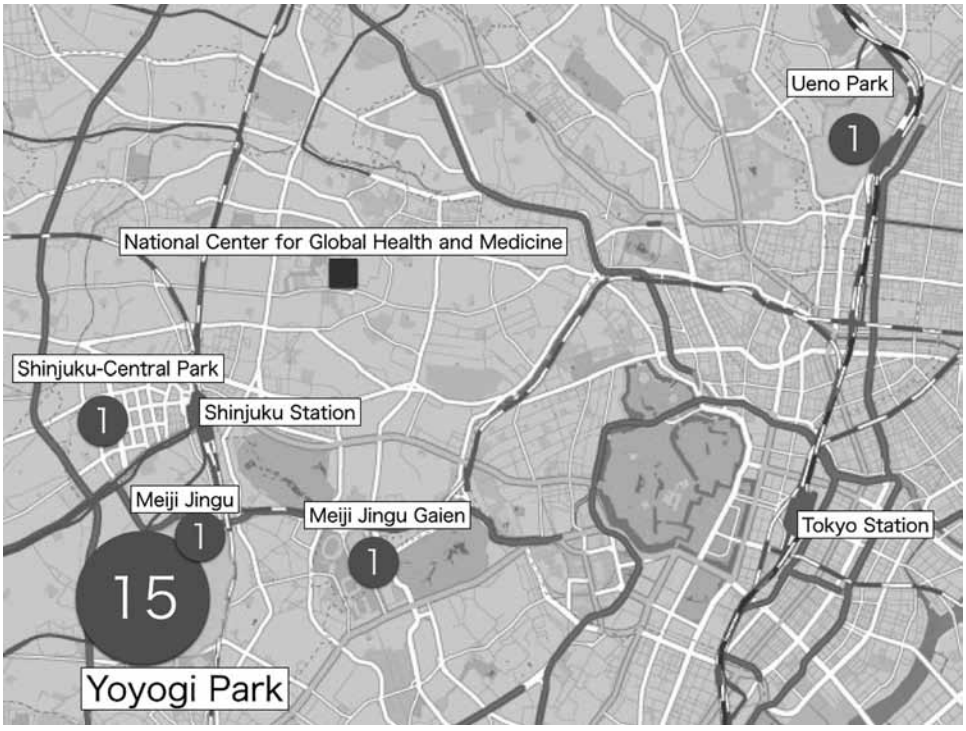

Figure 1. Locations of presumptive exposure to dengue virus mosquito vectors for 19 patients, Tokyo, Japan, August 26–September 22, 2014. Numbers in circles indicate numbers of cases contracted at each location.

Japan, including Tokyo. *Ae. albopictus* mosquitoes are also expanding into the northern regions of the main island because of global warming (*10*). Tokyo is one of the most heavily populated cities in the world, and Yoyogi Park is located at the center of the Shinjuku-Shibuya area in Tokyo.

The population density of *Ae. albopictus* mosquitoes in Tokyo is higher than that in suburban areas (*11*). It is possible that high human and mosquito population densities contributed to this outbreak. All 19 patients had been bitten by a mosquito while in Tokyo, mainly in Yoyogi Park, where most of the 160 patients with autochthonous dengue cases had also been (*12*).

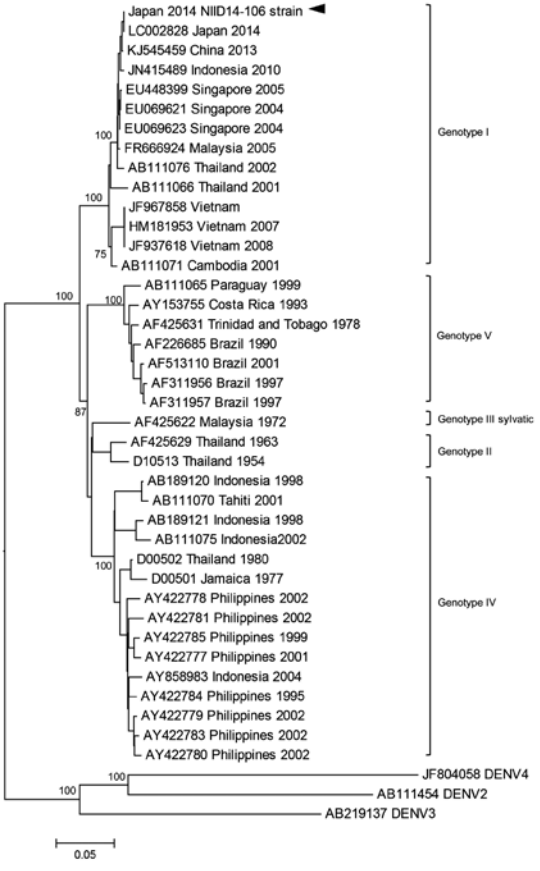

Figure 2. Phylogenetic analysis of a dengue virus (DENV) sequence derived from a patient with confirmed autochthonous dengue fever (patient 2), Tokyo, Japan, contracted during August 26–September 22, 2014. Phylogenetic tree is based on the envelope protein genome sequence of selected dengue virus type-1 (DENV-1) strains. DENV-2, DENV-3, and DENV-4 serotypes were used as outgroups. Percentages of successful bootstrap replication are indicated at the nodes. DENV-1 genotypes are indicated on the right. The DENV-1 National Institute of Infectious Diseases (NIID) strain 14-106 (GenBank accession no. LC006123) is indicated with an arrowhead. Virus strains are indicated by GenBank accession number, place, and date of isolation. Scale bar indicates number of nucleotide substitutions per site.

Recently, a case of dengue fever imported to England from Japan was found to be associated with this outbreak (*13*). Previous investigators speculated that the virus may have been spread from infected visitors by mosquitoes in the park (*13*). The outbreak, however, coincides with a period during which several tropical-themed festivals and activities were hosted, July–August 2014. These activities attracted a high number of local and international visitors to the park.

Before this 2014 outbreak, dengue fever was diagnosed for a German traveler who had returned from Japan in 2013 (*14*). Neutralization tests confirmed that the traveler's infection was caused by DENV-2. She was reportedly bitten by mosquitoes when in Fuefuki, but she had also traveled to Tokyo and Kyoto during her trip to Japan. No local DENV cases were reported in 2013, although cases may have been underreported because of the lack of local dengue outbreaks in Japan for the past 70 years.

Because adult *Ae. albopictus* mosquitoes cannot survive the winter in Japan, only eggs overwinter (*15*). Thus, the outbreak of autochthonous dengue fever is expected to end as mosquito activity decreases during autumn. The Japanese government is currently strengthening vector control measures and increasing awareness among residents to prevent similar outbreaks.

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Treatment of Ebola Virus Infection with Antibodies from Reconvalescent Donors

Thomas R. Kreil

Clinical evidence suggests that antibodies from reconvalescent donors (persons who have recovered from infection) may be effective in the treatment of Ebola virus infection. Administration of this treatment to Ebola virus–infected patients while preventing the transmission of other pathogenic viruses may be best accomplished by use of virus-inactivated reconvalescent plasma.

The largest outbreak of Ebola virus infection to date included 15,935 reported cases and 5,689 deaths as of November 26, 2014 (World Health Organization Ebola Response Roadmap Situation Report Update, http://apps.who.int/iris/bitstream/10665/144498/1/roadmapsitrep_26Nov2014_eng.pdf). Nearly all infections have been reported in West Africa. The outbreak has reminded public health systems of the minimal medical options available for the treatment of persons affected by this disease. Beyond the management of symptoms, no vaccine or proven causal treatment is available, and interventions that are in development remain at early stages.

Supported by scarce yet positive clinical evidence (*1*) and some recent animal model data (*2*), the use of whole blood or plasma transfusions from reconvalescent donors (persons who have recovered from Ebola infection) that contain antibodies to the Ebola virus has received substantial (also media) attention as a treatment alternative. However, several aspects associated with this approach need consideration to potentially enable treatment at a scale reasonably commensurate to the ongoing outbreak and at a level of safety with respect to the possible transmission of viruses that is consistent with currently accepted standards. The primary choice among options would be between use of whole blood or plasma only.

The use of whole blood transfusions is probably the least desirable choice. For this option, a donor would only be able to donate approximately once per quarter; thus, the number of treatment courses that could be collected from any donor would be fairly limited. In addition, the required matching of blood type (ABO) and antigen (Rh negative/positive) in a whole blood unit for transfusion would add a layer of complexity. Whole blood also cannot be treated by any of the currently approved virus-inactivation methods (reviewed in [*3*]), which would leave virus testing as the only option available to prevent the transmission of infectious agents that the donor may carry, particularly HIV. In resource-rich countries, the implementation of serologic testing for HIV, starting in the mid-1980s, greatly reduced the risk for transmission by blood transfusion (*4*), but rare cases still occur despite use of the most sensitive nucleic acid tests (*5*). This aspect is of particular importance because HIV prevalence in adults is ≈1% in 3 of the affected countries, Liberia, Sierra Leone, and Guinea (http://www.unicef.org/infobycountry).

On a larger scale, the limitations of testing have been highlighted by transmission of West Nile virus (WNV) through blood transfusions in the United States even after implementation of sophisticated nucleic acid testing schemes for the blood supply (*6*). By contrast, the demonstrated WNV inactivation capacity embedded into the manufacturing processes of plasma derivatives (*7*) has effectively prevented WNV transmission, although plasma for fractionation collected and used in the same geographic region is not tested for WNV.

Many challenges are associated with establishing and operating a virus-testing laboratory in an environment that lacks the equipment infrastructure or trained personnel. Within these circumstances, it is difficult to ensure that predonation test results for "HIV, HBV, HCV, syphilis, and other locally transmitted infections, as applicable" would be generated within 48 hours, or otherwise repeated at donation, as recommended by interim guidance from WHO (http://apps.who.int/iris/bitstream/10665/135591/1/WHO_HIS_SDS_2014.8_eng.pdf). In addition, the economic aspects of such a testing endeavor would appear challenging.

Transfusion of plasma alone would alleviate a number of the concerns inherent in the use of whole blood. Donor-to-recipient matching complexity would be reduced because only blood type compatibility needs to be established for plasma transfusion. In addition, if plasma were collected by plasmapheresis, a donor could, depending on health status, donate up to twice each week or up to 50 times each year, and up to several hundred milliliters of plasma could be collected per donation. Health care infrastructure and cold-storage capability necessary for effective inventory management are now being deployed to the areas affected by the Ebola outbreak, and addressing the logistics around the installation of an automated plasmapheresis capacity, including providing the required training and supplies, has also received

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support (*8*). Further, the volumes of antibody-containing material that could be collected by this approach are an order of magnitude higher than the volumes available through whole blood collection, which would enable multiple treatments of patients if neutralizing antibody titers, reported to be highly variable in survivors (*9*), were found to be insufficient to stop virus replication after a single transfusion.

Another possibility is that, if antibody testing could be implemented, screening the general population in affected areas might prove beneficial to identify persons who have seroconverted in response to asymptomatic infection (*10*). These persons would have uncompromised health status and thus could be even more effective plasma donors, although the level of protection afforded by their Ebola virus antibody spectra would have to be verified through collaboration with specialized laboratories.

After collection, plasma from any donor source could be virus inactivated by an approved method, such as S59 + UVA Intercept or riboflavin + UV Mirasol treatment (reviewed in [*3*]) or by a solvent/detergent (SD) treatment (*11*). These methods would enhance the virus safety margin of plasma units for transfusion by several orders of magnitude. The unique robustness of SD in inactivating all the lipid-enveloped viruses tested (*12*) would seem to make this method the preferred choice for removing concerns about transmission of HIV, HBV, HCV or even the Ebola virus itself. The Intercept and Mirasol technologies have shown a somewhat more limited virus inactivation capacity for certain lipid-enveloped viruses (*3*).

As has been argued, "Capacity building for the collection and testing of sufficient convalescent blood or plasma from recovered Ebola patients is crucial" (*13*). Whereas some testing can safely be replaced by the more broadly effective inactivation approach described, establishing the infrastructure for, for example, sterile pooling and SD treatment of plasma may still present a challenge. As an alternative, individual plasma units could be SD treated in a commercially available, integral disposable processing bag system (*14*), a system that was developed for use in resource-limited blood bank settings.

To provide for a treatment without any matching requirements that would also make higher virus antibody titers available, laboratories could perform fractionation of reconvalescent plasma into hyperimmune intravenous immunoglobulin preparations. The feasibility of this approach has recently been confirmed at commercial scale during the 2009 influenza A(H1N1) pandemic (*15*). The US-based donor population for this preparation was, however, fully qualified and consistent with the stringent standards required for current plasma fractionation, a situation entirely different from the biosafety challenges associated with bringing reconvalescent plasma from the current Ebola-endemic regions into fractionation facilities licensed according to good manufacturing practices. In addition, available manufacturing capacities for the production of plasma derivatives are used already to support the treatment of persons who have hemophilia or immune deficiencies.

For these reasons, the interim WHO guidance on use of blood and plasma for treatment of Ebola virus infection would benefit from inclusion of a chapter on virus inactivation for plasma. As an effective and readily deployable technical approach, SD-inactivated plasma transfusions might even be proposed as the standard of care.

In conclusion, although the continued development of long-term scalable solutions such as a vaccine for Ebola remains critical, existing technology and protocols could help fill the gap. Establishment of a supply of virus-inactivated reconvalescent plasma for treatment of persons infected with this virus may be the most feasible treatment option.

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Tuberculosis Microepidemics among Dispersed Migrants, Birmingham, UK, 2004–2013

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To determine if local transmission was responsible for rising tuberculosis incidence in a recently dispersed migrant community in Birmingham, UK, during 2004–2013, we conducted enhanced epidemiologic investigation of molecular clusters. This technique identified exact locations of social mixing and chains of apparent recent transmission, which can be helpful for directing resources.

For tuberculosis (TB) control programs, detection of recently acquired infection provides opportunities to stop ongoing transmission. As a proxy for recent infection, shared genotyping patterns are widely used (*1*–*3*). Universal genotyping of mycobacterial strains has been made possible by high-throughput, 24-loci mycobacterial repetitive-unit variable-number tandem-repeat (MIRU-VNTR) typing (*4*). However, inferring genuine recent transmission is complex. False molecular clusters can arise from reactivation of latent infection acquired in the distant past, limited strain variation over long periods, or lengthy stability of MIRU-VNTR markers (*5*). Among migrants from TB-endemic countries, determining whether TB infection was acquired locally or in the country of origin is difficult. We investigated TB incidence in a recently dispersed migrant community in Birmingham, UK, by using conventional and molecular epidemiologic techniques to define possible chains of recent transmission.

Birmingham has ≈1.1 million residents, of whom 20% were born abroad (*6*). In 2012, the local TB incidence was 58 cases per 100,000 population; 70% of cases were in foreign-born persons (Birmingham and Solihull TB Service, unpub. data). Birmingham has an established migrant community from the Indian subcontinent. In the past decade, a wave of new communities settled in Birmingham after implementation of a compulsory dispersal policy for asylum seekers (*7*), which aimed to ease housing and other social pressures in London and southeastern England (*8*). Changing migration patterns may have altered TB patterns; since 2005, incidence rates have stabilized in London but have been increasing in urban centers outside London (*9*).

The Study

In February 2012, the Birmingham and Solihull TB Service noted 2 distinct 24-loci MIRU-VNTR strain types of *Mycobacterium tuberculosis* (clusters A and B) isolated from 4 Eritrea-born patients with pulmonary TB. The diagnoses were made in July (cluster A) and December 2011 (cluster B). No epidemiologic links between any clustered patients were detected by routine contact investigation. Epidemiologic links were considered to be patients naming each other as contacts, sharing a contact without naming each other, or sharing a location during the infectious period of 1 patient. Case managers interviewed patients to elicit names of persons they may have had regular contact with over the infectious period; contacts in nonhousehold settings (e.g., workplace, leisure sites) were also included, but geographic locations were not always defined. The infectious period was defined as the date of onset of respiratory symptoms, if known, or 3 months before diagnosis.

Surveillance data revealed an increased number of Eritrea-born TB patients (1, 4, and 19 cases in 2004, 2006, and 2011 respectively); estimated incidence was 960 cases per 100,000 persons. To investigate whether transmission had occurred in the United Kingdom and to identify opportunities for wider community intervention, we invited all Eritrea-born TB patients (with and without strain typing available) reported during 2004–August 2012 for an extended interview with a dedicated TB nurse. Semistructured questions examined potential sources of infection and secondary cases. To systematically explore locations frequented by patients, nurses asked patients to complete a 24-hour work/rest/play diary. Since November 2011, extended interviews have been routinely conducted for all MIRU-VNTR clustered TB patients in Birmingham; no ethics approval was required. According to routine practice, clustered Eritrea-born patients continued to be interviewed through December 2013. All participants gave written consent.

During 2004–2013, a total of 88 cases occurred among Eritrea-born persons. There were no UK-born Eritrean (born to Eritrean parents) patients. Most patients were male (65%) and had pulmonary TB (67%); median

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Table 1. *Mycobacterium tuberculosis* strain type clusters involving Eritrea-born patients and associated cluster members born elsewhere, Birmingham, UK, 2004–2013*

Cluster designation (24-loci MIRU-VNTR)†	No. patients in cluster	No. patients born in Eritrea	No. patients born elsewhere (not Eritrea), country‡	No. patients epidemiologically linked to ≥1 other patient in cluster
A (32433 2242515321 233323462)	12	12	0	12
B (32433 2512511322 132443383)	5	3	2, Yemen	2
C (32435 2332517333 455443382)	3	3	0	2
D (32433 2512511322 131443373)	5	4	1, Kenya	0
E (32432 2311514322 124523342)	3	1	2, Lithuania and Pakistan	2
F (42235 2642515333 342423374)	6	4	2, Pakistan	2
Unique 24-loci strains	NA	18	NA	NA
Only 15 loci available§	NA	15	NA	NA
Culture negative	NA	28	NA	NA
Total	34	88	7	20

*MIRU-VNTR, mycobacterial interspersed repetitive units variable-number tandem-repeat; NA, not applicable.
†24-loci MIRU-VNTR was available beginning in 2010.
‡All cases were in recent migrants (arriving in the past 5 y). No epidemiologic links between those from different countries were found except in cluster E.
§15-loci MIRU-VNTR typing was available beginning in 2003.

age was 29 (interquartile range 25–35) years, and median length of time in the United Kingdom before diagnosis was 4 (interquartile range 2–5) years. Of 62 (71%) patients who could be located, 49 participated in extended interviews. Except for homelessness (1 patient), no other social risk factors (e.g., drug and/or alcohol misuse, imprisonment, or mental health problems) were noted. Socializing occurred mainly in places of worship (43/49 patients frequented 9 places of worship) and private homes (10/49 patients frequented 7 residences).

MIRU-VNTR typing was available for all 61 culture-confirmed cases; 46 isolates had 24 loci, and 15 isolates had 15 loci (Table 1). Of the isolates with 24 loci, 27 (59%) clustered in 6 strain types. To identify risk factors for strain-type clustering, we compared demographic characteristics of clustered and nonclustered patients (Table 2). According to bivariable analysis results, a noncongregate location was significantly associated with cluster A only (Table 2).

The Figure shows the overlap of social networks and MIRU-VNTR strain-type clusters for Eritrea-born TB

Table 2. Demographic and clinical characteristics of 46 Eritrea-born TB patients with 24-loci MIRU-VNTR strain typing available, Birmingham, UK, 2004–2013*

Characteristic	Clustered case, n = 27	Nonclustered case, n = 19	p value†
Median age, y (IQR)	31 (26–37)	29 (26–33)	0.26‡
Male sex, no. (%)	21 (78)	10 (53)	0.11
Regular attendance at noncongregrate location			
Cluster A			<0.01
RV 1	12/12	1/19	
Other venue	0/12	7/19	
Cluster B			0.06
RV 1	1/3	1/19	
Other venue	2/3	7/19	
Cluster C			0.21
RV 1	0/3	1/19	
Other venue	3/3	7/19	
Cluster D			>0.99
RV 1	0/4	1/19	
Other venue	2/4	7/1	
Cluster E			0.45
RV 1	0/1	1/19	
Other venue	1/1	7/1	
Cluster F			>0.99
RV 1	0/4	1/19	
Other venue	2/2	7/1	
Previous BCG vaccination	1 (4)	2 (11)	0.56
History of previous TB	0	0	NA
Pulmonary TB	20 (74)	11 (58)	0.25
Sputum smear positive	6 (30)	4 (36)	>0.99
Isoniazid-monoresistant isolate	3 (11)	1 (5)	0.632

*Data are no. (%) patients unless indicated otherwise. IQR, interquartile range; BCG, Bacillus Calmette-Guérin; MIRU-VNTR, mycobacterial interspersed repetitive units variable-number tandem-repeat; NA, not applicable; RV, religious venue; TB, tuberculosis.
†Fisher exact test, unless indicated otherwise.
‡Mann-Whitney U test.

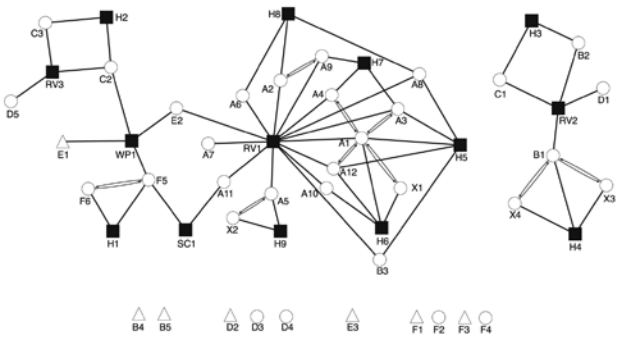

Figure. Social network of Eritrea-born patients with TB in relation to 6 distinct 24-loci MIRU-VNTR strain-type clusters and associated cluster members born elsewhere, United Kingdom, January 2009–December 2013. Nonclustered Eritrean patients are included if ≥1 epidemiologic link to clustered patients is known. Circles denote Eritrea-born patients; triangles denote patients born elsewhere; solid squares denote places of social mixing. For patients, labels denote strain type cluster (A–F; X, no strain typing available) and chronological order of case notification within each strain type cluster. For places, labels denote type (H, private home; RV, religious venue; SC, school; WP, workplace). Patients associated with private homes may or may not usually reside at the address. Double lines with arrows denote connections between TB patients who named each other as contacts during routine contact tracing investigations. Detached symbols at the bottom indicate persons for whom no epidemiologic links to any other case were detected. MIRU-VNTR, mycobacterial interspersed repetitive units variable-number tandem-repeat; TB, tuberculosis.

patients. Cluster A grew from 2 to 12 cases within 8 months. Few cases were linked by routine contact investigation alone because patients named the place of congregation (designated as religious venue [RV] 1 in the Figure) in different ways. The extended interviews defined geographic locations to street level, and it was determined that RV1 was used for multidenominational worship and therefore was recognized by different names. Routine contact investigation also failed to elicit other households where patients socialized (H5–8 in the Figure). As a result of the study, location-based contact tracing at RV1 was undertaken. An additional 68 persons were assessed, of which 19 (28%) had latent TB (16 started treatment and 14 completed treatment) and 1 had active TB. Latent TB was confirmed by a single interferon-g release assay; therefore, infection resulting from distant exposure could not be ruled out.

Combined strain typing and in-depth interviewing also uncovered apparent casual transmission involving cluster E at a workplace (WP1 in the Figure). Six months before diagnosis, patient E2 had worked at WP1 for 2 weeks, during the infectious period of patient E1. As a result, 5 new workplace contacts were investigated; 3 cases were diagnosed (no patients were born in the United Kingdom, and interferon-g release assay conversion was not documented), and these patients completed treatment for latent TB. A third case (in patient F5) occurred at WP1, 6 months after illness of patient E2, but the isolate from patient F5 was of a different strain type. Given the low probability of transmission, no further location-based contact tracing was undertaken at WP1. Discrepant strain types between patients known to have socialized at RV2, RV3, and school (SC) 1 also suggested that these cases did not involve the same chain of transmission. Thus, no additional contact investigation was undertaken. At RV2, the infectious period for patient B1 preceded the arrival of patient B2 in Birmingham, and local transmission was thought to be unlikely; both patients originated from the same town in Eritrea.

Genetically homogenous strain lineages prevalent in the countries of origin may falsely cluster persons from the migrant population (*10*); in our study, no patients within cluster D could be linked despite extensive epidemiologic investigation. However, microepidemics within such clusters do occur.

Conclusions

We have demonstrated the value of identifying places of social mixing during contact investigation for recognizing such microepidemics early. This approach has been found useful in other settings (*11*,*12*). A recent evaluation of the TB strain typing service in the United Kingdom found that strain typing did not significantly affect time to diagnosis or the median number of secondarily infected persons found per index case (*13*). However, strain typing can highlight gaps in current contact investigation procedures in specific patient populations and can help focus resources on scenarios in which recent transmission is more likely. Whole-genome sequencing may offer the ability to identify more recent strain evolution and transmission (*14*).

TB services should be vigilant for emergence of TB microepidemics in new communities. Conventional epidemiologic methods should be improved to complement molecular epidemiologic methods and increase their effect on TB control.

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Echinococcus vogeli in Immigrant from Suriname to the Netherlands

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To the Editor: Neotropical echinococcosis, caused by polycystic larvae of the tapeworm *Echinococcus vogeli* and unicystic larvae of *E. oligarthrus*, is an emerging infection in rural South America (*1*,*2*). The parasites are propagated in a predator–prey cycle; the final and intermediate hosts for *E. vogeli* are bush dogs (*Speothos venaticus*) and pacas (*Cuniculus paca*), respectively (*1,2)*. Human infections occur in rural areas and have been reported from several South American countries, mostly Brazil (*1–3*). Prompted by the recent diagnosis of an *E. vogeli* infection in a Surinamese patient in the Netherlands (*4*), we performed a retrospective analysis of all recent echinococcosis cases seen at the Amsterdam Medical Center. We describe molecular and immunohistochemical analyses from another case of *E. vogeli* infection.

In 2009, a 48-year-old female schoolteacher from Suriname sought care at the Amsterdam Medical Center for recently increasing retrosternal pain. Born in rural Suriname, she moved to the capital city of Paramaribo at 2 years of age. She had worked in the Brokopondo District for 1 year, then worked in urban Morocco, and immigrated to the Netherlands in 1990. Physical and laboratory examination findings were unremarkable. Esophago-gastro-duodenoscopy showed no abnormality. Abdominal ultrasonography and subsequent computed tomography revealed a lesion with solid and liquid components in liver segment 4, considered consistent with a biliary cystadenoma or an echinococcal cyst. Result of an echinococcosis indirect hemagglutination test with *E. granulosus* hydatid fluid antigen (Fumouze, Levallois-Perret, France) was strongly positive (titer 1:2,560; cutoff 1:160). An uncomplicated central liver resection of an 8-cm polycystic tumor was performed. Microscopic examination of resected tissue found vesicles containing protoscolices surrounded by periodic acid-Schiff–positive membranes.

Based on these findings, the initial diagnosis was cystic echinococcosis caused by *E. granulosus*, most likely contracted in Morocco. Postoperative treatment was albendazole, 400 mg twice daily for 8 weeks. Findings from a 5-year follow-up examination were unremarkable.

Current histologic reanalysis from archived formalin-fixed paraffin-embedded surgical specimens revealed laminated layers of the parasites, characteristic of *E. multilocularis* and *E. granulosus* larvae (i.e., thin convoluted and very thick areas, respectively). All *Echinococcus* species can be distinguished by the size and form of their rostellar hooks from protoscolices (*1*); for protoscolices from the patient reported here, the mean lengths of the small and large hooks were 34 and 43 μm, respectively.

We performed PCRs of the cestode-specific 12S rRNA gene (*4*) and cytochrome oxidase subunit 1 (*cox1*) (*5*). BLAST (http://blast.ncbi.nlm.nih.gov) analysis of the 282-bp and 375-bp amplicons, respectively, showed 100% and 99% homology with *E. vogeli* (GenBank accession nos. KM588225, KM588226). Phylogenetic modeling based on the *cox1* sequence showed that this *E. vogeli* isolate clustered with isolates from Colombia and Brazil (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/3/14-1205-Techapp1.pdf). Immunohistochemistry with monoclonal antibody Em2G11 raised against an *E. multilocularis* laminated layer antigen (Em2) (*6*) showed a faint and patchy pattern of the laminated layer in the *E. vogeli* lesion (Figure). Neither the previously described typical complete staining of the laminated layer as found in *E. multilocularis* larvae nor the entire absence of staining as described for *E. granulosus* metacestodes (*7*) was seen in the *E. vogeli* lesion. The typical staining of small particles of *E. multilocularis* (spems), characteristically seen adjacent to *E. multilocularis* vesicles (*7*), was completely absent in this specimen. Immunohistochemical examination with a monoclonal antibody against echinococcal cytoskeleton protein EM10 (*8*) showed staining of the germinal layer and protoscolices of *E. multilocularis* and *E. granulosus* larvae but only partial staining of the protoscolices of *E. vogeli* larvae. According to the proposed staging scheme for polycystic echinococcosis (*1*), this case was assigned to stage 1.

Approximately 220 *E. vogeli* infections have been reported, including 10 from Suriname (*1*,*4*,*9*) and the case reported here. Only 1 case outside echinococcosis-endemic areas has been described in 2013; namely, in a patient from rural Suriname who immigrated to Amsterdam (*4*). The striking similarities between both cases extended to their clinical presentations.

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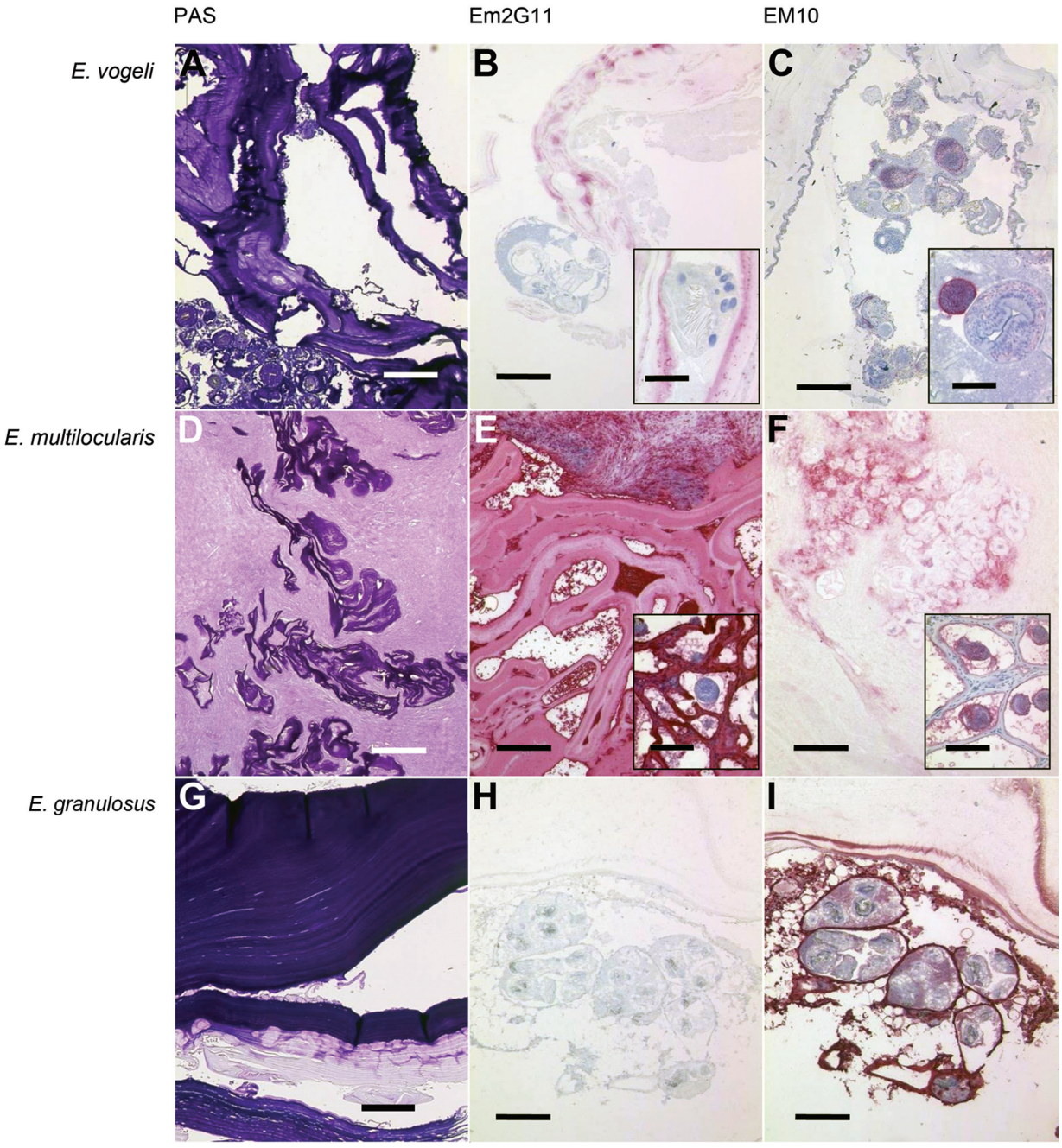

Figure. Periodic acid–Schiff (PAS) staining and immunohistochemical analysis of samples from *Echinococcus vogeli* lesions, with monoclonal antibodies against Em2 and EM10 of *E. vogeli* (A, B, C), *E. multilocularis* (D, E, F), and *E. granulosus* (G, H, I) lesions. Staining was performed on archived tissue from human patients with alveolar and cystic echinococcosis for comparison, and from the patient with *E. vogeli* infection who immigrated to the Netherlands from Suriname (*E. vogeli* infection in 2009). B and C insets) Protoscolex, with rostellar hooks clearly visible in inset B. E and F insets) Tissue from infected rodents (laboratory-infected *Meriones unguiculatus* gerbils) because *E. multilocularis* lesions in humans only rarely contain protoscolices. A, D, G) PAS-stained sections of the respective echinococcal lesions. Scale bars indicate 500 μm. B, E, H) Lesions with *E. vogeli*, *E. multilocularis*, and *E. granulosus* infection, respectively, stained with the monoclonal Em2G11 antibody against Em2 (for staining details see [7]). *E. multilocularis* lesions show intense staining, *E. granulosus* lesions show no staining, and *E. vogeli* lesions show patchy stains. Scale bars indicate 200 μm; scale bars of the insets indicate 50 μm. C, F, I) Respective lesions stained with antibodies against EM10 (dilution of the primary antibody 1:50; further steps as in Barth et al. [7]). Germinal layer and protoscolices of *E. multilocularis* and *E. granulosus* larvae are stained, but the protoscolices of the *E. vogeli* metacestode are only partly stained. Scale bars indicate 200 μm; scale bars of the insets indicate 50 μm.

In a recent immunohistochemistry study (*7*), antibodies against Em2G11 have shown excellent properties for distinguishing between cystic and alveolar echinococcosis. Although reported to not cross-react with purified laminated layer fractions from in vitro–kept *E. vogeli* (*10*), antibodies against Em2G11 exhibited an unusual and possibly discriminatory staining pattern when applied to the *E. vogeli* lesion from the patient reported here. Antibodies against EM10, which has not before been used for species discrimination on tissue sections, have also shown different staining properties.

Our findings suggest that there may be more undiagnosed cases of polycystic neotropical echinococcoses in immigrants from South America. In retrospect, the treatment (although aimed at *E. granulosus*) was successful despite the polycystic and proliferative nature of *E. vogeli* lesions, as indicated by an uneventful prolonged follow-up period for this patient with a well-circumscribed liver lesion. If neotropical echinococcosis had been considered before surgery (on the basis of radiologic features and the patient's origin), the management would also have included a preoperative and prolonged course of albendazole therapy.

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Post-Chikungunya Rheumatoid Arthritis, Saint Martin

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To the Editor: In October 2013, autochthonous transmission of chikungunya was detected in the Caribbean area, which resulted in the current epidemic of chikungunya in the Western Hemisphere (*1*). The chikungunya virus strain that caused this epidemic belongs to the Asian lineage, not to the strain descending from the East/Central/South African (ECSA) lineage that spread in the Indian Ocean region after 2004. This ECSA lineage was reported mainly to cause long-lasting musculoskeletal and rheumatic disorders in chikungunya virus–infected patients (*2–8*). In 1984 in South Africa, Brighton and Simson reported post-chikungunya destructive polyarthritis (*6*). Twenty years later, the arthritogenic pathogenesis of viruses in the ECSA chikungunya virus lineage was confirmed after outbreaks in the Indian Ocean region (*2–5,7,8*).

Because >870,000 suspected cases of chikungunya have occurred during the past 12 months in the Western Hemisphere (http://www.paho.org/hq/index.php?option=com_content&view=article&id=9436), it is crucial to know whether infection with the epidemic Asian strain will cause chronic inflammatory and potentially destructive rheumatism. We report post-chikungunya rheumatoid arthritis from Saint Martin, the epicenter of the current epidemic.

A 70-year-old woman (artist–painter) in Saint Martin sought treatment in June 2014 for joint pains and disabilities persisting after chikungunya. Her medical history included high blood pressure, hypothyroidism, and 3 dengue infections. During October 2013, the patient had high-grade fever, intense fatigue, and a maculopapular troncular exanthema without lymphadenopathy. Five days later, she had distal polyarthritis (joint pain and swelling) in interphalangeal joints, wrists, and ankles without plantar involvement. Recent infection with chikungunya virus was confirmed (IgM and IgG against chikungunya virus was detected in 2 blood samples), and recent dengue was excluded according to the criteria of the National Reference Center on Arboviral Diseases (http://www.niaid.nih.gov/labsandresources/resources/dmid/wrceva/Pages/default.aspx).

Despite initial brief improvement, the patient never totally recovered and subsequently chronic polyarthritis developed, which involved >10 joints, including interphalangeal joints, wrists, and knees. Nonsteroidal antiinflammatory drugs did not relieve the diffuse pain, stiffness, and swelling. She was given oral corticotherapy (20 mg/day) beginning in January 2014. She was referred to another hospital in France 5 months later because of treatment failure. She reported continuous pain in the left knee and wrists and multiple tenosynovitis on flexors and extensors of the fingers (Figure). She did not report any fever or axial, shoulder, or hip pain. Radiographs of the involved joints showed no abnormalities.

The patient had mild inflammation (C-reactive protein level 13 mg/L, fibrinogen level 3.4 g/L) but no specific autoimmunity (results were negative for anticitrullinated peptide antibodies, rheumatoid factor, antineutrophil cytoplasmic antibodies, and antinuclear antibodies). Serologic results for viruses other than chikungunya virus were negative or indicated past vaccination. The patient's condition met the 2010 American College of Rheumatology/European League against Rheumatism criteria for rheumatoid arthritis (https://www.rheumatology.org/practice/clinical/classification/ra/ra_2010.asp), and the only cause observed for this disease was acute chikungunya. For this corticosteroid-resistant, seronegative, and nondestructive post-chikungunya rheumatoid arthritis, methotrexate was prescribed at a weekly low dose after exclusion of contraindications, but the patient was not followed-up after she returned to Saint Martin.

Figure. Swollen and stiff hands of a 70-year-old woman with post-chikungunya rheumatoid arthritis 10 months after acute infection with chikungunya virus, Saint Martin.

The reported case was caused by chikungunya virus infection during an epidemic in Saint Martin in October 2013. This unfavorable post-chikungunya outcome of chronic inflammatory rheumatism 8 months later indicates a probable course of post-chikungunya disorders in the Western Hemisphere, as has already been observed in Africa and Asia. Previous outbreaks in Réunion and India offer insights regarding patients' post-chikungunya chronic status with long-lasting pain and disability, impaired quality of life, and extensive treatment (*2,3,9*).

The spectrum of post-chikungunya rheumatic and musculoskeletal disorders includes multiple tendinitis and tenosynovitis, plantar fasciitis, mechanical disbalance in susceptible joints, tunnel syndromes, edematous polyarthralgia, rheumatoid arthritis, and psoriatic arthritis (*2,4,5*). Although the proportion of patients with chronic disease has decreased, post-chikungunya chronic inflammatory rheumatism, mostly rheumatoid arthritis, develops in ≈5% of these patients (*8*). These patients had a poor prognosis and were given disease-modifying anti-rheumatic drugs (DMARDs), despite the postinfectious origin of rheumatism (*4,5*). Patients with post-chikungunya rheumatoid arthritis should benefit from methotrexate, which is recommended for treatment of classic rheumatoid arthritis (*10*).

In our experience, resistance to or dependence on corticosteroids beyond the third month after disease onset is highly evocative of post-chikungunya chronic inflammatory rheumatism. This finding requires early treatment with DMARDs to control the inflammatory process, prevent bone erosions, and prevent inevitable side effects of prolonged corticotherapy. To date, the efficacy of different DMARDs for treatment of post-chikungunya chronic inflammatory rheumatism has not been evaluated. Therefore, physicians should follow the international guidelines for treatment of classic rheumatoid arthritis and psoriatic polyarthritis, which recommend methotrexate as first-line treatment for patients fulfilling chronic inflammatory rheumatism criteria after 3 months of evolution.

We found that the Asian strain of chikungunya virus has induced arthritic disorders in the Western Hemisphere. Thus, a possible increase in post-chikungunya rheumatoid arthritis should not be overlooked. Physicians and public health authorities should prepare a response to the patients'

post-chikungunya stage in the epidemic areas. Clinical vigilance is recommended to identify patients with unfavorable outcomes 3 months after disease onset and for those in whom post-chikungunya chronic inflammatory rheumatism develops and who require specific treatment. Detailed guidelines for diagnosis and treatment of these patients with chronic rheumatoid arthritis are needed.

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Molecular Detection of *Ehrlichia chaffeensis* in Humans, Costa Rica

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To the Editor: Human monocytic ehrlichiosis (HME), a tickborne zoonosis caused by the rickettsial pathogen *Ehrlichia chaffeensis* (Rickettsiales: Anaplasmataceae), is considered an emerging pathogen in the United States and, increasingly, in many countries around the world (*1*). In Costa Rica, past reports of human cases of ehrlichiosis were diagnosed solely by clinical evaluation and cytomorphology (*2*,*3*); recent studies have detected *E. canis* in dogs and their ectoparasites (*4*,*5*). However, molecular detection of natural *Ehrlichia* infection detected in humans in Costa Rica has not been reported.

In a small rural area of Zarcero, province of Alajuela, north central region of Costa Rica, blood samples were drawn from 20 patients who had histories of tick bites and nonspecific symptoms of fatigue, arthralgia, and myalgia beginning ≥1 year before sampling. The samples were referred for *Ehrlichia* molecular analysis. In addition, blood samples were drawn from 2 patients of 2 health care clinics in the Alajuela province districts of San Carlos and Alajuela who had clinical signs compatible with recent ehrlichiosis; the samples were sent for confirmation by PCR. All anticoagulated samples were transported within 4 hours to the laboratory for processing. No serologic assays were performed; cytomorphologic estimation and laboratory data were provided from the local health facilities, mostly generated 1 year before this molecular analysis.

DNA was isolated the same day of sampling from whole blood (200 μL) by using the QIAamp Blood Kit (QIAGEN, Santa Clarita, CA, USA) according to the manufacturer's instructions. Purified DNA from each blood sample was quantified by spectrophotometry, yielding 20–32 ng/μL of DNA. Nested PCR assays were performed as described (*6*,*7*). To avoid DNA contamination, first PCR, second PCR, and electrophoresis were performed in separate rooms, following strict rules of pipetting and cleaning, and repeated ≥3 times. In addition, endpoint PCR for the variable-length PCR target gene was performed on samples that were positive in the nested assay, according to Paddock et al. (*8*). For DNA sequencing, PCR reactions were performed, and products were separated by agarose gel electrophoresis. A nested PCR mixture containing water and 1 containing unrelated *Brucella abortus* DNA were used as negative controls in every assay. As an internal

Table. Clinical manifestations in patients and laboratory findings of *Ehrlichia* PCR-positive and negative blood samples, Costa Rica

Characteristics	Patient group, no. (%)	
Clinical findings at first examination	PCR-positive, n = 5	PCR-negative, n = 17
History of tick bite	5 (100)	12 (70.6)
Fever	2 (40)	1 (6)
Rash	4 (80)	11 (64.7)
General arthralgia	5 (100)	11 (64.7)
Headache	4 (80)	7 (41.2)
Detection of morulae	1 (20)	12 (70.6)
Positive response to doxycycline	1 (20)	8 (47.1)
Laboratory findings	Range (reference value)*	
Leukocytes	5.3–7.9 × 10^9 cells/L (5–10 × 10^9 cells/L)	6.0–8.9 × 10^9 cells/L (5–10 × 10^9 cells/L)
Platelets	190–299 × 10^9 cells/L (150–450 × 10^9 cells/L)	217–329 × 10^9 cells/L(150–450 × 10^9 cells/L)
Hemoglobin	12.9–14.9 (12.5–17 g/dL)	12.7–16.1 (12.5–17 g/dL)
Aspartate aminotransferase	27–39 U/L (13–39 U/L)	26–36 U/L (13–39 U/L)
Alanine transaminase	21–49 U/L (7–52 U/L)	25–41 U/L (7–52 U/L)

*Laboratory values at time of sampling for this study.

control, the 22 samples were assayed for the β-globin gene (TaKaRa Bio/Clontech, Mountain View, CA, USA). All samples were positive for the β-globin gene by PCR with primers PC04 and GH20. Fragments (bands) were excised from electrophoretic gels by using sterile scalpels. These fragments were then were placed in a PCR mixture and used as a template. PCR mixtures were pooled and purified by using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. DNA was sequenced at Macrogen Inc. (Seoul, South Korea). The sequences obtained were compared to those previously deposited in GenBank.

Three of the 20 human blood samples from Zarcero that were tested, and the 2 samples from patients of different locations, were positive for 16S rRNA gene fragment of 390 bp and showed 99%–100% identity to the *E. chaffeensis* strain Arkansas gene (GenBank accession no. NR074500.1). These fragments were sequenced and deposited in GenBank, under accession numbers CR1 San Carlos (KF888343), CR2 Alajuela (KF888344), CR3 Zarcero (KF888345), CR4 Zarcero (KF888346), and CR5 Zarcero (KF888347). Amplifed bands for 2 of the 5 samples positive by nested PCR (CR1 and CR4) were identical to those specific for the 5 repeats of variable-length PCR target gene (459 bp).

A summary of clinical manifestations and results of DNA analysis is shown in the Table. The major symptoms reported by most patients were rash, predominantly macular in extremities; general arthralgia and myalgia; and headache. Comparison of clinical and laboratory findings of patients with PCR-positive and -negative results showed no clear differences, possibly related to the length of time between the acute phase of illness and the sampling for molecular analysis, which was usually 1 year. Nevertheless, it is noteworthy that the patients' samples harbored detectable *Ehrlichia* DNA 1 year after the acute phase, which was similar to findings in recent reports (*9*). Equally, related to the 2 patients evaluated in this study who had a recent history of infection (CR1 and CR2), no major physical or biochemical alterations were observed, suggesting that disease manifestation was mild or that samples were taken during the convalescent phase. The incongruent high detection of intracellular morulae and low PCR results point out the necessity of improving techniques for early diagnosis of this disease, particularly in primary health care clinics. Clinicians and public health authorities should be aware of the presence of this pathogen in the region and should include molecular tools in the diagnosis of this zoonosis.

The arthropod vector or vectors, and vertebrate reservoir or reservoirs of *E. chaffeensis* in Costa Rica are unknown, and further ecologic studies are required to determine these aspects of human monocytic ehrlichiosis in Central America. Epidemiologic and ecologic surveys are needed to trace and control the dissemination of this public health threat.

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Disseminated *Mycobacterium tuberculosis* in Imported Sooty Mangabey, Thailand

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To the Editor: Tuberculosis caused by bacteria of the *Mycobacterium tuberculosis* complex affects humans and various species of captive and free-living wildlife (*1*). In addition, *M. tuberculosis* has been used experimentally in many different species of Old World monkeys as part of the attempt to establish a suitable model for human tuberculosis (*2*). We report a case of disseminated tuberculosis caused by *M. tuberculosis* Spoligotype International Type (SIT) 52 in a recently imported sooty mangabey (*Cercocebus atys*) from South Africa to Thailand.

A juvenile male sooty mangabey was imported from South Africa to Thailand in September 2009. Within 1 week, while in quarantine, convulsion and salivation developed in the mangabey, and it died suddenly. This animal, along with another mangabey and 4 mustached guenons (*Cercopithecus cephus*), was imported from its native Africa to Thailand for the pet trade. Complete histories of the second mangabey and the mustached guenons were not available.

A complete necropsy of the dead sooty mangabey was conducted, and full histopathologic and microbiological analysis was performed. At necropsy, the mangabey was emaciated, with no subcutaneous and abdominal fat tissues. Disseminated granulomas (up to 2 cm) were observed throughout the carcass, including the lungs, liver, spleen, kidneys, multiple lymph nodes (hilar, mediastinal, mesenteric, splenic, hepatic, renal, and pancreatic), and the ileum. The lung was also multifocally adhered to the thoracic wall and pleural diaphragm.

Histologically, the granulomas in all tissues examined demonstrated similar histopathologic features, characterized by a central core of caseous necrosis and surrounded by an unorganized rim of mixed inflammatory cells, including neutrophils, lymphocytes, plasma cells, and epithelioid macrophages. Numerous acid-fast bacilli were present in the cytoplasm of the epithelioid macrophages and in the necrotic area of all tissues. Acid-fast bacilli were isolated and classified as *M. tuberculosis* on the basis of 1-tube multiplex PCR (*3*) and sequencing of 16S rRNA gene results. Spoligotyping revealed that the *M. tuberculosis* isolate belonged to SIT 52.

The international wildlife trade had been reported to be a major source of imported zoonoses, particularly tuberculosis, in nonhuman primates (*4–8*). In the mangabey reported here, fulminant tuberculosis was diagnosed within 1 week after it arrived in Thailand, during the 21-day quarantine period. The granulomas were morphologically similar to the histopathologic description of tuberculosis lesions of experimentally infected cynomolgus macaques (*Macaca fascicularis*), which demonstrated lesions as early as 3 weeks after infection, with a gradual increase in severity (*2*). Previously, East African–Indian lineage (*9*) and Beijing spoligotype (SIT 1) accounted for most *M. tuberculosis* isolates in Thailand (*10*). In nonhuman primates in Thailand, *M. tuberculosis* complex had been detected at rates of up to 50% (5 positive samples from 10 test samples) by PCR from buccal swabs in long-tailed macaque (*Macaca fascicularis*) (*1*). *M. tuberculosis* belonging to SIT 52 observed in this case has been primarily isolated from countries in Africa (*9*). Only 1 case of

M. tuberculosis belonging to SIT 52 that caused tuberculous meningitis was reported in a human in Thailand (*10*), but that case was not related to the case reported here. Our finding of a relatively novel spoligotype of *M. tuberculosis* in an animal destined for the pet trade underscores the need for intensive testing of and extended quarantine for all imported nonhuman primates to prevent the spread of newly isolated *M. tuberculosis* (*4*,*7*,*8*).

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Treatment of *Mycobacterium abscessus* subsp. *massiliense* Tricuspid Valve Endocarditis

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To the Editor: *Mycobacterium abscessus* is a ubiquitous, rapidly growing mycobacteria (RGM) found in water supplies, soil, and dust. *M. abscessus* is considered the most pathogenic and difficult to treat of the RGM and is most often associated with pulmonary, skin, and soft tissue infections; it has also been reported to cause ocular infections, otitis, lymphadenitis, arthritis, osteomyelitis, disseminated disease, and prosthetic valve endocarditis (*1*,*2*). Most prosthetic valve endocarditis cases have been fatal.

M. abscessus subsp. *massiliense* is 1 of 3 subspecies of *M. abscessus*. *M. abscessus* subsp. *massiliense* has an identical 16S rRNA gene sequence to the other 2 subspecies, *Mycobacterium abscessus* subsp. *bolletii* and *Mycobacterium abscessus* subsp. *abscessus*, but can be differentiated by *rpo*β and *erm41* gene sequencing (*3*,*4*). *M. abscessus* subsp. *massiliense* grows readily in blood culture media and on sheep's blood agar within 2–4 days. Care should be taken in interpreting Gram staining of isolates because RGM is not identifiable by this method and could be mistaken for corynebacteria or diphtheroids (*5*,*6*). Such isolates could be further tested by acid-fast staining and, if positive, sent to a reference laboratory for definitive identification and susceptibility testing.

Five cases of *M. abscessus* native valve endocarditis have been reported; 4 were fatal and 1 was lost to follow-up (*1*,*5*–*9*). One of the 4 fatal cases also involved the tricuspid valve and was associated with intravenous heroin abuse

(*9*). We report a case of *M. abscessus* subsp. *massiliense* native tricuspid valve endocarditis successfully treated with antimicrobial therapy and surgical debridement.

A 52-year-old man who used intravenous drugs was admitted to our hospital describing a 25-pound weight loss, fever, and night sweats. He reported injecting crushed opioid tablets mixed with tap water. He had tachycardia and pitting edema of the legs and feet. Laboratory data revealed elevated aminotransferase levels, thrombocytopenia, and opiates in the urine. Computerized chest tomographic scan showed cavitary right upper lobe and lingular nodules. Routine blood cultures (BacT/ALERT3D; bioMérieux, Marcy l'Etoile, France) of samples drawn at admission and on hospital day 3, before the initiation of antimicrobial drug therapy, grew acid-fast bacilli (AFB) in broth medium on days 3 and 4 of incubation. A transthoracic echocardiogram on hospital day 5 revealed a 1-cm vegetation on the tricuspid valve. An empiric regimen for RGM consisting of intravenous cefoxitin and amikacin and oral clarithromycin and moxifloxacin were administered. Based on preliminary (3-day) susceptibility test results showing susceptibility to amikacin, resistance to the quinolones, and intermediate susceptibility to cefoxitin, linezolid, and imipenem, the regimen was changed to tigecycline, linezolid, clarithromycin, and amikacin (*10*). Routine blood cultures on hospital days 11 and 17 were negative.

On hospital day 19, linezolid was stopped, and imipenem was added. A transthoracic echocardiogram on hospital day 31 showed the vegetation had enlarged to 1.5 × 0.5 cm. We concluded that antibiotics alone were unlikely to be curative; cardiac catheterization was performed on hospital day 38. On the basis of hemodynamic findings, the cardiologist inferred that valve replacement would be of no value and recommended valvectomy alone.

Surgery on hospital day 41 revealed a 2-cm nodule on each anterior and posterior leaflet and a 2–3 mm nodule on the septal leaflet of the tricuspid valve. The anterior and posterior leaflets were removed, and the septal leaflet was segmentally resected. Routine cultures of valve tissues, in which *M. abscessus* would have grown, were negative. Pathologic examination confirmed suppurative vegetations with numerous bacterial colonies consistent with AFB; AFB staining disclosed numerous mycobacteria (Figure, http://wwwnc.cdc.gov/EID/article/21/3/14-0577-F1.htm).

Identification and final susceptibilities of the RGM from the original blood culture isolate revealed *M. abscessus* subsp. *massiliense* by *hsp65* PCR and *erm* gene sequencing (*4*) and 14-day susceptibility to clarithromycin (*10*). *M. abscessus* subsp. *massiliense* has a nonfunctional (truncated) macrolide-inactivating gene (*erm41*), and untreated isolates are susceptible to the macrolides (*4*).

Repeat chest tomographic scan on hospital day 69 showed nearly complete resolution of the RUL cavitary and lingular nodules/infiltrates. Tigecycline, amikacin, imipenem, and clarithromycin were continued until hospital day 77, when amikacin was stopped because of moderate hearing loss. The patient was discharged without antibiotics after 2 months of postoperative antibiotic therapy. At follow-up visits 2 and 8 weeks later he was doing well except for peripheral edema. AFB and routine blood cultures drawn at both visits were negative. He is periodically seen in the cardiology clinic; his edema has resolved with diuretic therapy.

Cure of *M. abscessus* native valve endocarditis has not been previously reported. A case of *M. chelonae* native tricuspid valve endocarditis associated with a pacemaker lead was successfully treated with wire removal, valve debridement, and antimicrobial therapy (*11*). The patient in the current study likely acquired his infection from the tap water diluent he injected. Clinicians should consider the possibility of mycobacterial endocarditis when evaluating a septic patient with intravenous drug use history or cardiac prosthetic devices.

We successfully treated mycobacterial native tricuspid valve endocarditis with combination antimicrobial therapy and surgical debridement. The location of the infection in the tricuspid valve and favorable hemodynamics enabled debridement without implantation and the subsequent possibility of intraoperative infection of a prosthetic valve.

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Rickettsia rickettsii in *Amblyomma patinoi* Ticks, Colombia

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To the Editor: *Rickettsia rickettsii* is the etiologic agent of Rocky Mountain spotted fever (RMSF), a highly lethal tick-borne rickettsioses restricted to the Western Hemisphere (*1*,*2*). In Colombia, *R. rickettsii* was first reported during the 1930s, when 62 (95%) of 65 affected persons died of RMSF in Tobia town (Cundinamarca Department) (*3*), from where highly virulent strains of *R. rickettsii* were isolated through the inoculation of patient blood or of *Amblyomma cajennense* sensu lato (s.l.) extracts into guinea pigs (*4*). Thereafter, RMSF remained unnoticed in Colombia until the 21st century, when new outbreaks with high case-fatality rates were reported in different regions, including Villeta, a nearby locality of Tobia (*1*).

Recent studies have shown that *A. cajennense* s.l., widely distributed from the southern United States to Argentina, is actually a complex of 6 different species: *A. cajennense* sensu stricto (Amazonian region), *A. mixtum* (from Texas, USA, to western Ecuador), *A. sculptum* (northern Argentina, Bolivia, Paraguay, Brazil), *A. interandinum* (inter-Andean valley of Peru), *A. tonelliae* (dry areas of northern Argentina, Bolivia, and Paraguay), and *A. patinoi* (eastern cordillera of Colombia) (*5*). With this new classification, *A. patinoi*, originally described from Villeta, is the only species of this complex known to occur in the RMSF-endemic area of Cundinamarca, Colombia (*5*).

In August 2013, we collected 15 *A. patinoi* adult ticks from cattle in Naranjal village (5°3′31.52″N, 74°26′50.24″W), Villeta town, an area of Cundinamarca, Colombia, to which RMSF is endemic. Ticks were taken alive to the laboratory, where they were frozen at –80°C for further analysis. The 15 ticks were defrosted, surface sterilized with iodine alcohol, and processed individually by the shell vial technique for isolation of rickettsiae in Vero cells, as described (*6*). Infected cells were always incubated at 28°C. Rickettsiae were observed by Gimenez staining within cells (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/3/14-0721-Techapp1.pdf) from only 1 (inoculated from a female tick) of the 15 inoculated shell vials. This isolate was subjected to at least 7 Vero cell passages, each achieving >90% infected cells.

DNA was extracted from an aliquot of first passage–infected cells and tested by a battery of PCR protocols targeting fragments of the rickettsial genes *gltA*, *ompA*, and *ompB* and the intergenic regions RR0155-*rpmB*, RR1240-*tlc5*^{b}, and *cspA-ksgA* (Table). We sequenced 1,106 bp, 512 bp, and 799 bp of the *gltA*, *ompA*, and *ompB* genes, respectively. By BLAST analyses (http://www.ncbi.nlm.nih.gov/blast), these sequences were 100% identical to corresponding sequences of *R. rickettsii* from Colombia and Brazil (GenBank accession nos. CP003306, CP003305). Generated sequences for 2 intergenic regions, RR0155-*rpmB* (228 bp) and RR1240-*tlc5*^{b} (306 bp), were 100% identical to corresponding sequences of the same 2 *R. rickettsii* isolates from Colombia and Brazil. A 337-bp sequence of the *cspA-ksgA* intergenic region was 100% (337/337 nt) identical to *R. rickettsii* from Brazil (CP003305) and 99.7% (336/337) to *R. rickettsii* from Colombia (CP003306). Partial sequences from *R. rickettsii* generated in this study were deposited into GenBank and assigned nucleotide accession nos. KJ735644–KJ735649.

Whole-body remnants of the 15 ticks used to inoculate shell vials were also subjected to DNA extraction and processed by PCR for the rickettsial *gltA* gene (Table); only 1 tick (the one that provided the rickettsial isolate) contained rickettsial DNA, indicating a 6.6% (1/15) infection rate. We confirmed the taxonomic identification of this

Table. Primer pairs used for amplification of rickettsial genes or intergenic regions, Colombia, August 2013

Target, primer pairs, primers	Primer sequences, 5′ → 3′	Fragment size, bp	Reference
gltA			
1			
CS-78	GCAAGTATCGGTGAGGATGTAAT	401	(*6*)
CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT		(*6*)
2			
CS-239	GCTCTTCTCATCCTATGGCTATTAT	834	(*6*)
CS-1069	CAGGGTCTTCGTGCATTTCTT		(*6*)
ompA			
3			
Rr190.70p	ATGGCGAATATTTCTCCAAAA	530	(*7*)
Rr190.602n	AGTGCAGCATTCGCTCCCCCT		(*7*)
ompB			
4			
120-M59	CCGCAGGGTTGGTAACTGC	862	(*8*)
120–807	CCTTTTAGATTACCGCCTAA		(*8*)
RR0155-*rpmB*			
5			
Forward	TTTCTAGCAGCGGTTGTTTTATCC	290	(*9*)
Reverse	TTAGCCCATGTTGACAGGTTTACT		(*9*)
RR1240-*tlc5*[b]			
6			
Forward	CGGGATAACGCCGAGTAATA	357	(*10*)
Reverse	ATGCCGCTCTGAATTTGTTT		(*10*)
cspA-ksgA			
7			
Forward	CATCACTGCTTCGCTTATTTT	405	(*9*)
Reverse	ATTTCTTTTCTTCCTCTTCATCAA		(*9*)

tick as *A. patinoi* by generating mitochondrial 16S rRNA partial sequences from it and from an *A. patinoi* paratype that is deposited in the tick collection of the University of São Paulo (Brazil) (accession no. CNC-1585) (*5*). Both sequences were 100% identical to each other (deposited into GenBank under accession nos. KP036466–KP036467).

A 3 mL-aliquot of the second infected cell passage was inoculated intraperitoneally into an adult male guinea pig, in which high fever (rectal temperature >40.0°C) developed during days 5–8 days post inoculation. A total of 3 guinea pig passages were performed, always followed by high fever. A second passage animal survived; scrotal necrosis developed (online Technical Appendix Figure 2), and this animal seroconverted to *R. rickettsii* with 32,768 endpoint IgG titer through the immunofluorescence assay, as described (*2*).

A highly pathogenic strain of *R. ricketsii* was isolated from an *A. patinoi* specimen collected at Villeta, where recent human cases of RMSF have been reported (*1*). More than 70 years ago, the only previous *R. rickettsii* tick isolates in Colombia were obtained from *A. cajennense* s.l. in Tobia, only 20 km from Villeta (*4*). At that time, ticks of the *A. cajennense* complex were considered natural vectors of *R. rickettsii* in Tobia (*4*). Because the *A. cajennense* s.l. complex was recently found to be represented in the eastern cordillera of Colombia (which includes Tobia and Villeta) by the species *A. patinoi* (*5*), the tick isolates obtained >70 years ago also are highly likely to have been obtained from *A. patinoi*. Therefore, *A. patinoi* ticks should be considered the main vector of *R. rickettsii* to humans in this region of Colombia.

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Mycobacterium bovis BCG–Associated Osteomyelitis/Osteitis, Taiwan

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To the Editor: Thirty-eight patients with *Mycobacterium bovis* BCG–associated osteomyelitis/osteitis, including 8 who were previously reported (*1*), were identified during Taiwan's vaccine injury compensation program during 1989–2012; a total of 30 (79%) patients applied for compensation during 2009–2012 (Figure). In Taiwan, a laboratory program to differentiate BCG from other species of the *M. tuberculosis* complex, using a kit for the Tokyo-172 vaccine strain spoligotyping, was established in 2004 (*1*). Since 2008, the isolated extrapulmonary tuberculosis strains and pathologic specimens collected from children <5 years of age have been sent to the national reference mycobacterial laboratory for BCG detection (*2*). The detected incidence of BCG osteitis/osteomyelitis increased from 3.68 cases per million vaccinations during 2002–2006 to 30.1 per million during 2008–2012.

Parents or guardians signed written consent forms on behalf of the children when they submitted claims for the vaccine injury compensation program. After consent, children's hospital information was stored in the Taiwan Centers for Disease Control database and used for research.

Of the 38 compensated BCG osteomyelitis/osteitis patients, 18 were boys. According to chart review, no patients had immunodeficiency or other underlying conditions; however, 3 were premature babies (born at 34–36 weeks of gestation). Eighteen (47%) children had received BCG at ≤1 week of age, 12 (32%) at 1–4 weeks, 7 (18%) at 1–2 months, and 1 at >2 months. The average age at inoculation was 16.2 ± 16.6 days. Symptoms or signs began 3–32 months (average 12.4 ± 6.1 months) after BCG vaccination; for 68%, symptoms or signs developed 7–18 months after vaccination (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/3/14-0789-Techapp1.pdf). Time from vaccination to onset of symptoms or signs did not differ for the 3 premature infants.

As in previous reports (*3*,*4*), extremity bones were more commonly involved than axial bones. For 30 (79%) children, extremity bones were involved: 14 right lower limbs, 7 left lower limbs, 6 left upper limbs, and 3 right upper limbs. The tibia was the most common site (9 patients), followed by ankle bones (8 patients), femur (4 patients), radius and thumb (3 patients each), humerus and knee (2 patients each), and ulna (1 patient). Of these, 2 patients had 2 bony lesions. In 8 (21%) children, axial bones were involved: 5 sternums, 2 thoracic vertebrae, and 1 right rib. Presentation included a mass (25 [66%] children), tenderness (22 [58%]), limping (19 [50%]), redness (14 [37%]), and heat (7 [18%]). Average time from first clinical visit to final surgical management was 1.6 ± 2.1 months.

Eight (53%) of 15 patients had positive tuberculin skin test results. No specific abnormalities were found with regard to blood cell counts and inflammation markers or to chest radiographs, except for 1 child with rib

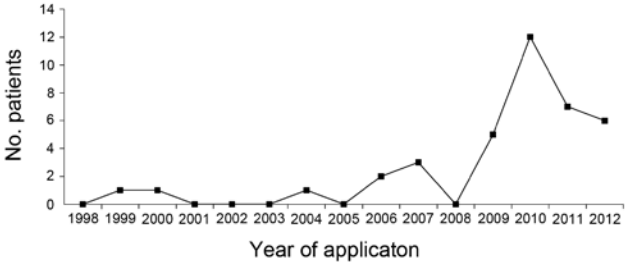

Figure. Number of patient applications for compensation as a result of *Mycobacterium bovis* BCG osteomyelitis/osteitis to vaccine injury compensation program, Taiwan, 1998–2012.

erosion. Pathologic diagnosis of *Mycobacterium* infection from bony specimens was recorded for 35 (92%) patients. For 29 (76%), diagnosis was conducted by molecular study, including 25 (66%) by the national reference mycobacterial laboratory. For 4 patients, diagnosis was confirmed by culture of *M. bovis*. Osteomyelitis/osteitis for 5 patients was considered BCG related according to pathologic diagnosis of *Mycobacterium* infection, BCG vaccination history, and lack of a history of contact with a person with tuberculosis.

Thirty-two (84%) children underwent surgery (excision, debridement, open biopsy), 4 children received arthrotomy (3 ankle and knee joint), and 2 children underwent only aspiration biopsy. All patients received isoniazid and rifampin therapy; 33 patients also received pyrazinamide, and 6 received additional ethambutol therapy. Medications were adjusted after diagnoses changed from tuberculosis to BCG infection. Two patients had major sequelae, both involving the thoracic spine and causing severe kyphosis.

Adverse reactions after BCG vaccination depend on the BCG dose, vaccine strain, vaccine administration method, injection technique, and recipient's underlying immune status (*5*). The vaccine strain and manufacturing process in Taiwan did not change during the study period. Findings were not associated with a specific batch of vaccine, inoculation age, underlying disease, or *Salmonella* spp. infection. Patients had no common birth place, hospital, or area of residence. We believe the increased number of cases resulted mainly from policy changes and laboratory facility improvements.

A surgical approach to obtain a specimen is indicated. However, because medical treatment usually yields a good outcome (*6*), extensive debridement should be avoided. Although some patients with lower extremity involvement initially limped, most were able to walk well later. Vertebral involvement is rare. Unlike previously reported cases (*7*,*8*), both patients reported here who had vertebral involvement had sequelae. For young children with suspected vertebral tuberculosis but no tuberculosis contact history, a biopsy specimen for BCG studies is preferable to spondylectomy. Although no definite immunologic deficit was found in these BCG osteomyelitis/osteitis patients, 2 other compensated infants with disseminated BCG during the same period in Taiwan had identified immunodeficiency (*9*). Studies are ongoing by the Taiwan Centers for Disease Control to evaluate medical treatment duration, long-term outcomes, and more detailed immune genetic tests.

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High Prevalence of Hepatitis Delta Virus among Persons Who Inject Drugs, Vietnam

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To the Editor: Hepatitis delta virus (HDV) is a small RNA virus that infects and persists only in persons whose samples test positive for hepatitis B surface antigen (HBsAg) (*1*). Phylogenetic analysis has revealed 8 HDV genotypes (*2*) with evidence of distinct global geographic distributions and pathogenicity (*3*,*4*). The implications of HDV infection in Vietnam have been unclear. Studies of persons who have chronic illness caused by HBV in populations of southern and northern Vietnam reported no cases or low prevalence (1.3%), respectively (*5*,*6*). In contrast, our multicenter study of chronically HBV-infected persons in 2009 showed a higher overall HDV seroprevalence rate of 10.7% (34/318) (*7*). These rates varied among regions of Vietnam and groups that had varying risk factors for infection. Higher rates were observed among persons who inject drugs (PWIDs) (20/78, 25.6%), commercial sex workers (5/57, 8.8%), and military recruits (8/45, 17.8%). A 2013 study, in which PCR-based methods were used, reported a high rate of HDV RNA detection (41/266, 15.4%) in a cohort of HBV-infected persons in the city of Ha Noi (also known as Hanoi) collected during 2000–2009 (*8*). Illnesses of these patients ranged from acute hepatitis to severe liver disease, but injection of drugs was not reported. To better clarify the prevalence of HDV, we conducted serologic and molecular testing focusing on PWIDs from different geographic regions of Vietnam.

During 2010–2011, we screened consecutive samples (n = 1,999) from PWIDs from 5 centers (Ha Noi and Hai Phong in northern, Da Nang and Khanh Hoa in central, and Can Tho in southern Vietnam) for HBsAg. In each center, we recruited PWIDs to obtain 200 participants per year following national guidelines for annual sentinel surveillance of HIV (http://www.vaac.gov.vn/Download.aspx/C64DBE4B-B9074A489283056ACF639780/1/Huong_dan_giam_sat_

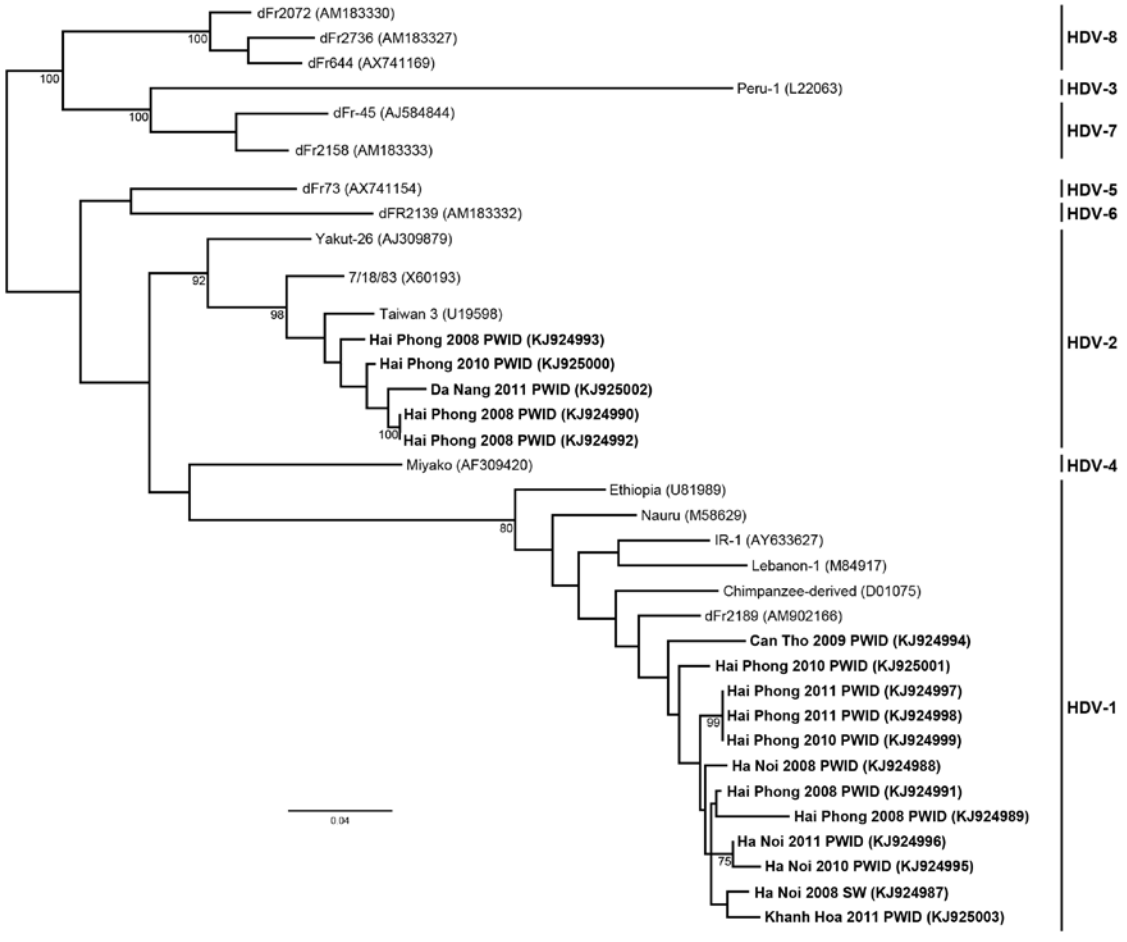

Figure. Maximum-likelihood phylogenetic tree of hepatitis delta virus (HDV) genotypes 1 and 2 from Vietnam. A 472-nt fragment (corresponding to nucleotides 802–1,273 from HDV isolate C15; Genbank accession no. KF660600) was used to construct the phylogram. HDV genotyping was done by using amplification and bidirectional sequencing of the R_0 region as described by Le Gal et al. (*2*). Bootstrap resampling was done for 1,000 replicates of the dataset using the neighbor-joining algorithm; values >70% are shown at the nodes. Bold text indicates samples from patients in Vietnam; location, year, and risk group are indicated. Genbank accession numbers are shown in parentheses. Scale indicates substitutions per position. PWID, person who injects drugs; SW, sex worker.

trong_diem_2010.doc). Ethical approval for the study was obtained from the National Institute of Hygiene and Epidemiology in Ha Noi. Samples collected from 300 (15%) persons were HBsAg positive, consistent with our previous study (*7*). Of these, 294 were subsequently screened by using ELISA for anti-HDV IgG; reactive samples were tested for HDV IgM. HDV IgG was detected in 45/294 (15.3%) samples; 20 were also HDV IgM positive (6.8% total; 44.4% of IgG-positive samples). Serologic analysis revealed considerable differences in prevalence by geographic region. HDV seroprevalence rates were high among PWIDs from northern Vietnam (30.2% and 29.4% in Ha Noi and Hai Phong, respectively), but a lower seroprevalence rate was observed in Da Nang (5.3%), and intermediate rates were found in Khanh Hoa (8.1%) and Can Tho (12.5%) in southern Vietnam (online Technical Appendix Tables 1, 2, http://wwwnc.cdc.gov/EID/article/21/3/14-1147-Techapp1.pdf).

We analyzed anti-HDV–positive samples (n = 41) for the presence of HDV RNA using a quantitative real-time PCR. HDV RNA was detected in 25/41 (61%) of IgG-seropositive samples (median 1.2×10^4 copies/mL, range 0–1.8×10^7 copies/mL) and 19/19 (100%) of IgM-seropositive samples (median 1.2×10^6 copies/mL, range 4.3×10^2–1.7×10^7 copies/mL). The viral loads of HDV IgM-positive samples were significantly higher than those of IgM-negative samples ($p<0.0001$) (online Technical Appendix Figure 1); however, when only samples with detectable HDV RNA from the IgM negative and positive groups were analyzed, there was no statistically significant difference in viral titer ($p = 0.45$; online Technical Appendix Figure 2). Comparison of HDV RNA and HDV IgM seroresponses showed evidence of superinfection with HDV persistence in 6 cases (HDV IgM negative/RNA positive; 6/22, 27.3%; online Technical Appendix Figure 1). The 6 samples that were IgM negative for detectable RNA (median 2.9×10^5 copies/mL, range 1.1×10^3–1.8×10^7 copies/mL) highlight the limitation of using IgM as a surrogate marker for HDV replication; therefore, HDV RNA investigation is more appropriate for IgG-positive samples.

To identify the genotypes of HDV involved, we completed nucleotide sequencing and phylogenetic analysis of HDV from 17 viremic patient samples from Ha Noi, Hai Phong, Da Nang, Khanh Hoa, and Can Tho collected from another study cohort during 2008–2011 (Figure) (*7*). Most (12/17, 71%) samples were HDV genotype 1 from both northern and southern Vietnam; 5 (29%) HDV genotype 2 species were identified in 4 samples from Hai Phong in northern and 1 sample from Da Nang in central Vietnam. The finding that HDV-1 was the predominant genotype is consistent with reports by Sy et al. (19/21 HDV-1; 2/21 HDV-2) (*8*), suggesting that HDV-1 is the predominant genotype in all parts of the country.

This study, the previous report from the National Institute of Hygiene and Epidemiology laboratory (*7*), and data from Sy et al. (*8*,*9*) indicate that HDV is highly prevalent in Vietnam, particularly in the northern part of the country, contrary to previous reports (*5*,*6*,*10*). In particular, our findings indicate that increased efforts are needed to improve HBV vaccination rates among PWIDs and others with risk factors for infection. Over time, these interventions may help reduce the effects of hepatitis virus–related liver disease. We also intend to study HDV in other high-risk groups, including commercial sex workers and men who have sex with men.

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Cholera in Yangon, Myanmar, 2012–2013

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To the Editor: *Vibrio cholerae* O1, a causative agent of cholera, is classified into 2 biotypes, classical and El Tor. Since 1817, cholera has spread from the Indian subcontinent to other regions of the globe 7 times (*1*). However, little information on the occurrence of cholera and *V. cholerae* in Myanmar has been published. Here, we report cholera cases and characterization of 58 clinical isolates of *V. cholerae* O1 serotype Ogawa recovered from patients with diarrhea in Yangon, Myanmar, during February 2012–June 2013.

During February and August 2012, rectal swab specimens were collected from patients suspected of having cholera in 4 hospitals in Yangon: New Yangon General Hospital, North Okkalapa General Hospital, Thingangyun Sanpya General Hospital, and Yangon Children Hospital. The specimens were cultured on thiosulfate citrate bile salts sucrose agar plates. After overnight incubation, several colonies that resembled to those of *V. cholerae* were confirmed as serogroup O1 by using slide agglutination tests with specific monoclonal antibodies (*2*).

Of the tested specimens, 34 isolates carried the *tcpA* gene, encoding the structural subunit of toxin-coregulated pilus, and the *rstR* gene, the repressor gene in the cholera toxin encoding (CTX) phage (*3*); these results may indicate that these strains belonged to the El Tor biotype. However, identification of the sequence type of the cholera toxin B subunit gene in these isolates revealed that they were of classical type ($ctxB^{Cla}$). Thus, these isolates were classified as atypical El Tor *V. cholerae* O1 (*4*) carrying $ctxB^{Cla}$ and $rstR^{El}$. Currently, the predominant clones causing cholera in Asia and Africa are atypical El Tor *V. cholerae*, CIRS101, and CIRS101-like variants (*5*,*6*). Myanmar isolates from 2012 and the CIRS101 strain contained a single nucleotide polymorphism in the *tcpA* gene at nt 266 (A→G) of the prototype seventh pandemic El Tor (N16961) strain.

Pulsed-field gel electrophoresis (PFGE) (*7*) using the 2012 isolates revealed 9 patterns (Table, http://wwwnc.cdc.gov/EID/article/21/3/14-1309-T1.htm). During the initial phase of cholera occurrences, *V. cholerae* O1 was mainly isolated from adults, and 9 pulsotypes were observed. During the later period (May–August), most isolates were from children <5 years of age, and pulsotype Y6 predominated.

We carried out multilocus variable-number tandem-repeat analysis (MLVA) (*8*) of the 2012 isolates to resolve distinct populations. MLVA yielded 13 isolate types, and all 18 isolates of pulsotype Y6 exhibited either MLVA profile 11.6.6.17.17 or a closely related profile that differed only by 1 repeat number. These data suggest that cholera was contracted mainly in adults and was caused by multiclonal *V. cholerae* O1. However, in children, *V. cholerae* has transformed from single clonal expansion since May 2012.

During March–June 2013, we extended our studies to characterize cholera organisms isolated from patients with severe diarrhea who were admitted to the original 4 hospitals as well as 2 additional hospitals, Yankin Children Hospital and Insein General Hospital. Of 24 cases, 16 patients showed symptoms of severe dehydration, including 1 patient who experienced shock. Other common symptoms in this patient population included fever (50%, 12/24), vomiting (92%, 22/24), and abdominal pain (33%, 8/24). Although fever is less common among patients with cholera-associated diarrhea (*9*), the frequency of fever was considerably high in this study. PFGE reveled 23 of the 24 isolates were identical to pulsotype Y7, which was the second-most prevalent pattern in 2012; MLVA profiles were also similar to those from 2012. Thus, the occurrences of cholera in 2013 may have been related to persistent transmission of a clone from 2012.

According to surveillance records from the Yangon Regional Health Center, the reported number of diarrhea cases in Yangon increased from 11,651 in 2010 and 11,016 in 2011 to 15,540 in 2012 and 13,919 in 2013. Although there were no reports of cholera outbreaks in Yangon, PFGE/MLVA results revealed that most of the cholera cases in this study were caused by isolates belonging to identical or closely related types. Thus, cholera outbreaks could have occurred in Yangon, and the related clone may have persisted.

In Myanmar, the illness rate for severe diarrhea is estimated to be 2.6–3.5 per 100,000 persons and the mortality

rate is 0.04–0.1 per 100,000 (*10*). In this study, the detection rates of *V. cholerae* O1 in stools from patients with severe diarrhea were 23% (49/213 cases) in 2012 and 14% (35/250 cases) in 2013, respectively. Although our investigation is merely the tip of the iceberg for studies of cholera in Myanmar, our data provide crucial initial insights into the genetic backgrounds of recent Yangon isolates of *V. cholerae* O1. Epidemiologic surveillance linked to laboratory investigations is need to minimize the risk for *V. cholerae* infection in children.

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Role of Race/Ethnicity in Pulmonary Nontuberculous Mycobacterial Disease

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To the Editor: We read with interest the study of gender and age in nontuberculous mycobacterial (NTM) lung disease case-patients in Taiwan (*1*). NTM lung disease is relatively uncommon; however, the exact prevalence of NTM lung disease and causative organisms are largely unknown in many regions of the United States because the disease is not reportable. A recent study using Medicare claims data in the United States showed that the annual prevalence of NTM lung disease increased from 20 cases/100,000 persons in 1997 to 47 cases/100,000 persons in 2007 (*2*). The study also showed that Hawaii had the highest period prevalence of cases (396 cases/100,000 persons), which was at least partially attributed to the large Asian/Pacific Islander population (*2*). During June–December 2011, we conducted a cross-sectional study to evaluate the epidemiologic and clinical significance of NTM isolated from patients in Honolulu, Hawaii; the patients had suspected pulmonary tuberculosis (TB) and were in airborne isolation at a university-affiliated, tertiary-care hospital.

NTM cases were defined according to the 2007 criteria of the American Thoracic Society/Infectious Diseases Society of America (*3*). The process required to establish a diagnosis of NTM lung disease is sometimes lengthy; thus, patients who did not initially meet the disease criteria but who had cultures positive for NTM were reviewed again 1 year after the original data were collected to see if follow-up microbiological and radiographic studies would confirm the presence of NTM lung disease. Descriptive statistics were used to describe categorical and continuous variables. During June–December 2011, a total of 113 patients with suspected pulmonary TB were placed into isolation at the tertiary-care hospital. Of these patients, 85 (75.2%) were men and 28 (24.8%) were women; the median age was 59.8 ± 17 years. Eighteen (15.9%) patients were white, 92 (81.4%) were Asian/Pacific Islander, and 1 (0.9%) was African American; for 2 (1.8%) patients, race/ethnicity was classified as not specified/other.

Of the 113 isolated patients, 21 (18.6%) were positive for mycobacteria. Of these 21 patients, 14 (66.7%) were men and 7 (33.3%) were women; the median age

was 64.3 ± 17.3 years. Three (14.3%) of these patients were white, and 18 (85.7%) were Asian/Pacific Islander. *Mycobacterium tuberculosis* and NTM were identified in samples from 3 (14.3%) and 18 (85.7%) of the 21 patients, respectively. Of the 18 patients with NTM-positive samples, 4 (22.2%) had definite NTM lung disease (all of these patients were Asian/Pacific Islander); 2 (11.1%) had probable NTM lung disease; and 12 (66.7%) had possible NTM lung disease. *M. chelonae* (identified by DNA sequencing) was the causative agent for most of the definite cases (n = 3, 75%), and the largest proportion of possible cases was caused by *M. avian* complex bacteria (n = 5, 41.7%).

Our finding that 22.2% (4/18) of the patients in Honolulu with NTM-positive clinical samples during June–December 2011 received a definite diagnosis of NTM lung disease is slightly higher than but consistent with reports from other regions, which show that 9.8%–17.0% of such patients receive a definite NTM disease diagnosis (*4*,*5*). For unclear reasons, the number of NTM disease cases appears to be highest in Asian/Pacific Islander populations. Determining the reason(s) for this discrepancy should be the subject of future research efforts.

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Rickettsial Infections in Monkeys, Malaysia

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To the Editor: The cynomolgus monkey (*Macaca fascicularis*), also known as the long-tailed macaque or crab-eating monkey, is commonly found in the Southeast Asia region (*1*). The macaque has been associated with several bacterial infections, such as those caused by hemotropic *Mycoplasma* and *Bartonella quintana* (*2*). As a result of rapid deforestation and changes in land use patterns, cynomolgus monkeys live in close proximity to human-populated areas (*1*). Human–macaque conflict may increase the risk for zoonoses.

Little is known about rickettsial and anaplasma infections in cynomolgus monkeys in Malaysia. Although *Rickettsia* spp. RF2125 and Rf31 have been identified from cat fleas in Malaysia (*3*), the presence of *Anaplasma bovis* in monkeys is not known.

Rickettsia felis, a member of the spotted fever group rickettsiae, is an emergent fleaborne human pathogen distributed worldwide (*4*). The obligate intracellular bacterium has been identified from cats, dogs, opossums, and the ectoparasites of various mammalian hosts. Several uncultured rickettsiae genetically closely related to the *R. felis*–type strain URRWXCal2 (referred to as *R. felis*–like organisms and including *Rickettsia* spp. RF2125, Rf31, *Candidatus* Rickettsia asemboensis, and others) have also been identified from various arthropods and fecal samples of primates (*5*). *A. bovis* is a gram-negative, pleomorphic, tickborne intracellular bacterium that infects a wide range of mammal species in many geographic regions (*6*).

To learn more about these infections in monkeys, we examined blood samples from 50 cynomolgus monkeys caught by the Department of Wildlife and National Parks at 12 residential areas in Peninsular Malaysia during a population management and wildlife disease surveillance program (January 2012–December 2013). Most monkeys (14 male, 36 female) were adults and were active and healthy. DNA was extracted from 200 μL of each blood sample by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). We performed PCRs selective for the rickettsial citrate synthase gene (*gltA*) by using primers CS-78 and CS-323 and for the 135-kDa outer membrane protein B gene (*ompB*) by using primers 120-M59 and 120-807 (*7*). As positive controls, we

used cloned PCR4-TOPO TA plasmids (Invitrogen, Carlsbad, CA, USA) with amplified *gltA* fragment from *R. honei* (strain TT118) and *ompB* fragment from a rickettsial endosymbiont (98% similarity to *R. raoultii*) of a tick sample. Amplification of anaplasma DNA was performed by using a group-specific primer pair (EHR 16SD/EHR 16SR) (*8*). As a positive control for the PCR, we used an *A. marginale*–infected cattle blood sample. The full-length sequences of the *Anaplasma* 16S rRNA gene were obtained by amplification with primers ATT062F and ATT062R (*9*). Sequence determination of the amplicons was performed by using forward and reverse primers of respective PCRs on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). To search for homologous sequences in the GenBank database, we performed a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis and constructed a dendrogram based on 16S rDNA sequences of *A. bovis* (*10*).

The rickettsial *gltA* gene was detected from 12 (24%) blood samples of mostly male monkeys from 8 locations. BLAST analysis of 210 nucleotides (GenBank accession no. KP126803) amplified from all samples demonstrated 100% sequence similarity with *Rickettsia* sp. RF2125 (accession no. AF516333), *Candidatus* Rickettsia asemboensis (accession no. JN315968), and *Rickettsia* spp. clone 4G/JP102 and 11TP21 (accession nos. JN982949 and JN982950), which had been identified from cat fleas in Southeast Asia, Africa, and Costa Rica, respectively. The rickettsial sequence also showed 99.0% similarity (2-nt difference) with *R. felis*–type strain (accession no. CP000053). The rickettsial *ompB* gene was amplified from 4 samples, and BLAST analysis of the sequences (556–779 bp) revealed closest match to several *R. felis*–like organisms, including *Rickettsia* sp. RF2125 (100%, accession no. JX183538) and *Candidatus* Rickettsia asemboensis (99%, accession no. JN315972). BLAST analysis of the longest *ompB* sequence (accession no. KP126804) obtained in this study showed 93% similarity with that of the *R. felis*–type strain.

Anaplasma DNA was amplified from 5 (10%) monkeys at 2 locations by using group-specific primers. Analysis of the nearly full-length sequences of the *A. bovis* 16S rRNA gene (1,457 nt) revealed 3 sequence types (GenBank accession nos. KM114611–3) with 99.1%–99.2% homology to that of the *A. bovis* strain from cattle in South Africa (accession no. U03775). The phylogenetic tree (Figure) inferred by using various *Anaplasma* species confirms the clustering of the strains from monkeys with *A. bovis* from different animals (i.e., goats, cattle, deer, ticks, wild boars, dogs, raccoons, leopard cats, eastern rock sengis, and cottontail rabbits). Co-infection of *R. felis*–like organisms and *A. bovis* was detected in only 1 sample.

Infections caused by *R. felis*–like organisms and *A. bovis* in the cynomolgus monkeys were subclinical (i.e., monkeys showed no evident signs of infection at the time of blood sampling). The diverse range of the organisms'

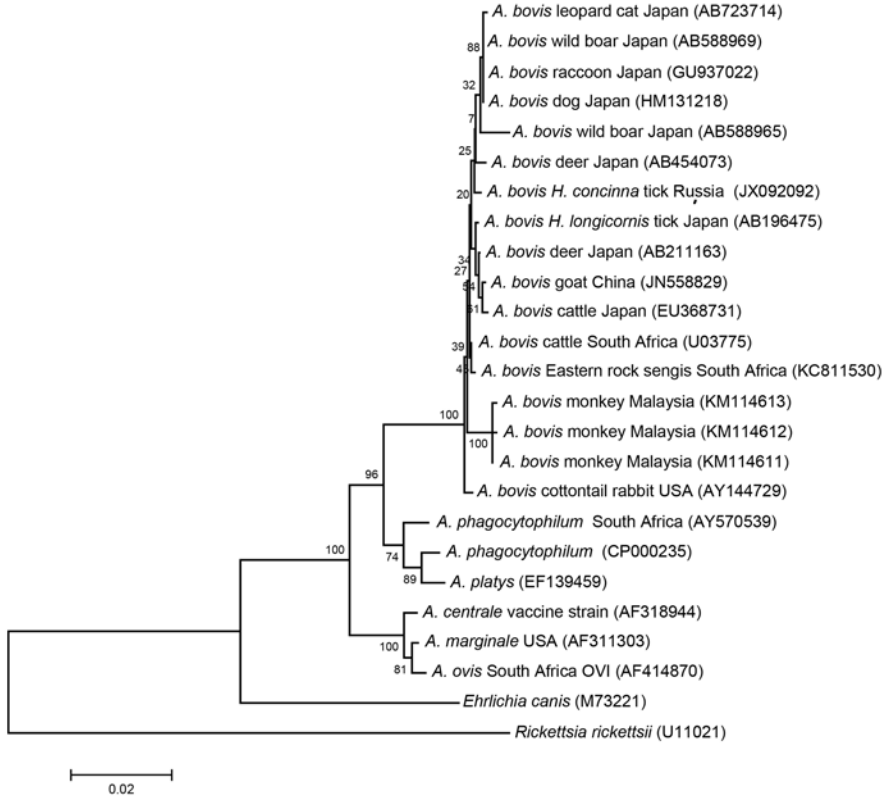

Figure. Phylogenetic relationships among various *Anaplasma* species, based on partial sequences of the 16S rRNA gene (1,263 bp). The dendrogram was constructed by using the neighbor-joining method in MEGA6 software (*10*) with the maximum composite likelihood substitution model and bootstrapping with 1,000 replicates. *Rickettsia rickettsii* (U11021) was used as an outgroup. Numbers in brackets are GenBank accession numbers. Representative Malaysian *A. bovis* sequences were deposited into the GenBank database under accession nos. KM114611–3. Scale bar indicates nucleotide substitutions per site.

ectoparasite and animal hosts raises concern about their potential risk to human and animal health. Further study on the interactions between the microbes, vectors, and reservoir hosts is needed to assess their effects on public health.

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Effect of Ciliates in Transfer of Plasmid-Mediated Quinolone-Resistance Genes in Bacteria

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To the Editor: Previous studies have suggested that protozoa may promote horizontal gene transfer among bacterial species (*1*,*2*). This process is largely, although not exclusively, responsible for increasing the incidence of antibiotic-resistant bacteria through various mechanisms, such as transformation by acquisition of naked DNA, transduction by acquisition of DNA through bacteriophages, and conjugation by acquisition of DNA through plasmids or conjugative transposons (*3*,*4*). Because antibiotic resistance may be mediated by horizontal gene transfer, it is necessary to understand whether protozoa, which are widely distributed in nature, facilitate the acquisition and spread of antibiotic resistance genes. The aim of this study was to explore whether the ciliated protozoan *Tetrahymena thermophila* promotes the transfer of plasmid-mediated quinolone-resistance (PMQR) genes in bacteria.

Two *qnr* gene–positive bacterial strains (*Klebsiella oxytoca* and *Escherichia coli*) were chosen as donors, and azide-resistant *E. coli* strain J53 was used as a recipient for the assessment of gene transfer frequency. The *K. oxytoca* and *E. coli* strains were previously isolated and identified from the Ter River (Ripoll, Spain) in the framework of a multidisciplinary study on antibiotic-resistant bacteria (*5*). Donor and recipient bacteria, previously grown in Luria-Bertani broth for 5 h at 37°C, were mixed in equal numbers (10^9 CFU/mL) with or without *T. thermophila* strain SB1969 (10^5 cells/mL) in Page's amoeba saline for 24 h, as previously described (*1*). Heat-treated ciliates, exposed for 10 min at 90°C, were also tested to determine whether viable organisms are required for gene transfer. Conjugation experiments were performed at 37°C, and, after the incubation period, the cultures were treated as previously

described (*1*). Transconjugants were then selected on Luria-Bertani agar plates containing sodium azide (100 mg/L) and nalidixic acid (6 mg/L). The gene transfer frequency was estimated as the number of transconjugants for each recipient. Antibiotic susceptibility tests were also determined by using the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (*6*). All data were derived from ≥3 independent experiments, and statistical analyses were performed by using analysis of variance modeling, in which $p<0.05$ was considered significant (SPSS 17.0 software; IBM, Chicago, IL, USA).

The results revealed that the frequency of gene transfer between bacteria exposed to ciliates increased significantly ($p<0.05$), from 1.5×10^{-7} to 2.8×10^{-6} and from 1.2×10^{-7} to 1.6×10^{-6} in *E. coli* transconjugants of *K. oxytoca* (*qnrB*-positive strain) and *E. coli* (*qnrA*-positive strain), respectively (Figure). However, there were no differences in MIC values of ciprofloxacin and ofloxacin between transconjugants obtained from cultures exposed to ciliates and those from untreated cultures. These results suggest that, even though ciliates promote the transfer of PMQR genes, they did not induce increased expression of these genes. Moreover, no statistically significant differences were found in gene transfer efficiency between cultures exposed to heat-treated ciliates and those not exposed to ciliates. This finding suggests that the cell components of ciliates do not promote gene transfer and, therefore, other mechanisms may be responsible for this phenomenon. In fact, the presence of ciliates may increase the frequency of gene transfer by facilitating contact between donor and recipient bacteria through co-accumulation in their vesicles (*1*). Because protozoa are widely distributed in diverse environments, they may constitute a key environmental reservoir for acquisition and spread of antibiotic-resistance genes among bacteria, including human pathogens.

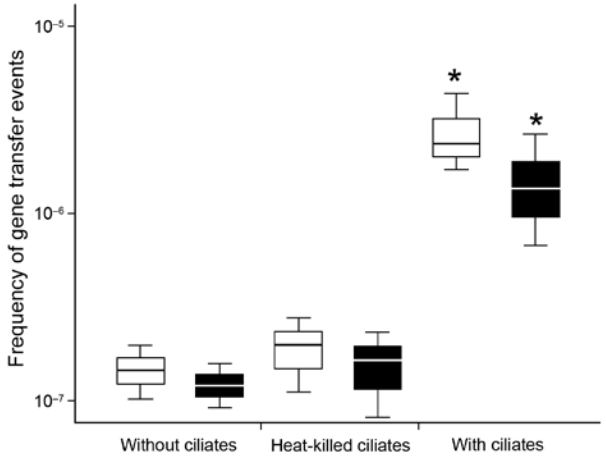

Figure. Box plot chart showing effects of ciliates on the transfer frequency of plasmid-mediated quinolone-resistance genes between *Escherichia coli* strain J53 and *qnrB*-positive *Klebsiella oxytoca* strain (white boxes) or *qnrA*-positive *E. coli* strain (black boxes). Box plots are divided by medians (black or white bars) into upper quartile and lower quartile ranges. Error bars indicate minimum and maximum values. Asterisks indicate a statistically significant difference ($p<0.05$) between treated and untreated cultures.

The study findings demonstrate that ciliates increase the transfer of PMQR genes in bacteria. These findings may therefore have important public health implications because the presence of ciliates would promote the spread of antibiotic resistance genes among bacterial species. According to recent data from the European Centre for Disease Prevention and Control (http://www.ecdc.europa.eu/), each year, ≈25,000 persons in the European Union die as a direct result of antibiotic-resistant infections. Thus, further studies are needed to determine the role of protozoa, such as ciliates, in the emergence and spread of antibiotic-resistant bacteria and to inform the implementation of appropriate public health strategies, policies, and mitigation programs. Elucidation of the mechanisms involved could lead to a better understanding of why some protozoa can promote gene transfer between bacteria.

Acknowledgments

I thank Juan C. Gutiérrez for providing the ciliated protozoan *T. thermophila* strain SB1969 and Seok Hoon Jeong for supplying the azide-resistant *E. coli* strain J53. I also extend special thanks to Miray Etyemez for her technical assistance.

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Porcine Epidemic Diarrhea Virus Replication in Duck Intestinal Cell Line

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To the Editor: Porcine epidemic diarrhea virus (PEDV) was first detected in pigs in the United States in May 2013 (*1*). Since then, according to the American Association of Swine Veterinarians (https://www.aasv.org, see link to number of new cases reported), PEDV has spread to 41 states, and as of October 15, 2014, 8,622 confirmed cases of PEDV infection have been reported in swine. PEDV (family *Coronaviridae,* genus *Alphacoronavirus*) is an enveloped, positive-sense, single-stranded RNA virus (*2*). The virus replicates in epithelial cells of small and large intestines and causes highly contagious infection in pigs. The disease is characterized by watery diarrhea, vomiting (leading to subsequent dehydration), and high rates of death, especially in young piglets; thus, outbreaks cause substantial economic losses to the swine industry (*1*). Variants of the original virulent PEDV have recently been isolated in the United States, making development of a vaccine to protect against this devastating disease even more challenging (*3*). Vero cells are used for the isolation of virus from clinical samples and for virus propagation and titration and virus neutralization studies. The addition of exogenous trypsin in culture medium is a prerequisite for efficient replication of PEDV in Vero cells (*4*): trypsin cleaves the spike protein of PEDV into 2 subunits that mediate cell-to-cell fusion and virus entry into the cells (*5*).

We examined PEDV replication in a newly established immortalized duck intestinal epithelial cell (MK-DIEC) line, which was generated from the intestinal tissues of a 19- day-old white Pekin duck embryo. MK-DIECs are cuboidal (characteristic of epithelial cells), express epithelial marker (pan-cytokeratin), and show extensive proliferation in culture. Several coronaviruses, including PEDV, use aminopeptidase N (APN) as the cellular receptor for attachment to cells (*6*). As a first step, we used a rabbit polyclonal anti-human APN antibody (Abcam, Cambridge, MA, USA) in an indirect immunofluorescence assay (IFA) to examine whether MK-DIECs express APN. We found that nearly 100% of the cells expressed APN on their surface (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/3/14-1658-Techapp1.pdf).

Next, we examined PEDV replication in MK-DIECs. The cells were cultured in medium containing equal amounts of Dulbecco modified Eagle medium; Mammary Epithelial Growth Medium (Lonza, Walkersville, MD, USA) supplemented with bovine pituitary extract (70 μg/mL), human epidermal growth factor (5 ng/mL), insulin (5 μg/mL), and hydrocortisone (0.5 μg/mL); and 2% fetal bovine serum. Near confluent cells were infected with PEDV at a multiplicity of infection of 0.1. The Colorado strain of PEDV (obtained from the National Veterinary

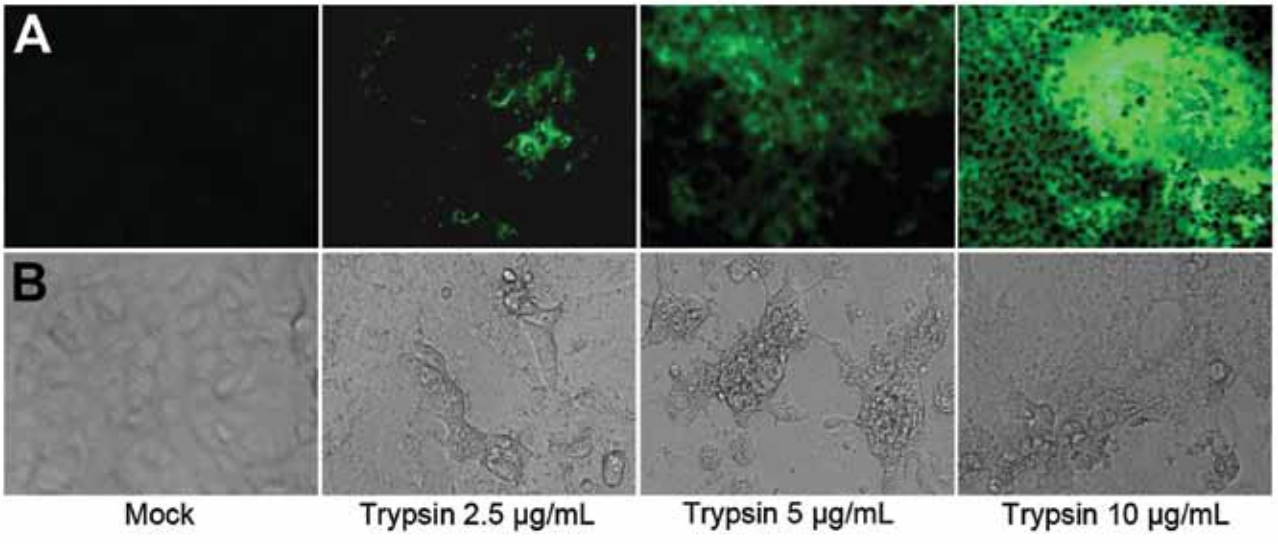

Figure. Replication of porcine epidemic diarrhea virus (PEDV) in a newly established immortalized duck intestinal epithelial cell line (MK-DIEC) infected with PEDV at a multiplicity of infection of 0.1 in the presence of different concentrations of trypsin. A) Twenty-four hours after infection, PEDV nucleoprotein in infected cells was detected by immunofluorescence assay using fluorescein isothiocyanate–labeled nucleoprotein-specific monoclonal antibody. B) PEDV-induced cytopathic effect in MK-DIEC cells 36 h after infection.

Services Laboratories, Ames, IA, USA), which was initially passaged 5 times in Vero cells, was used to infect the MK-DIECs. After adsorption for 1 h, the cells were cultured in serum-free Dulbecco modified Eagle medium supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 1% penicillin/streptomycin (infection medium). To examine the requirement of trypsin (Sigma, St. Louis, MO, USA) for PEDV replication in MK-DIECs, we added trypsin (0, 2.5, 5, or 10 μg/mL) to the infection medium.

We also cultured MK-DIECs in 96-well plates and similarly infected them with PEDV for the detection of PEDV nucleoprotein (NP) by IFA using fluorescein isothiocyanate–labeled mouse PEDV NP monoclonal antibody (SD-1F; Medgene Labs, Brookings, SD, USA). At 12, 24, and 36 h after infection, released virus in infected cells was quantified by virus titration in Vero cells by inoculating 10-fold serial dilutions. After 24 h, viral NP was detected by IFA staining. The virus titer was calculated according to the Reed–Muench method and expressed as the 50% tissue culture infectious dose/mL.

We detected PEDV NP in MK-DIECs 24 hours after infection in medium with and without trypsin (data not shown). However, the numbers of cells positive for PEDV NP was larger in cells cultured with trypsin (2.5 μg/mL and 5 μg/mL) than without trypsin (Figure, panel A). PEDV also induced cytopathic effects (CPEs) in these cells, which were characterized by rounding of cells, syncytium formation, and cell detachment. The CPEs were more pronounced in cells infected with added trypsin; as little as 2.5 μg/mL of trypsin in infection medium was sufficient to induce substantial CPEs in infected cells (Figure, panel B). No signs of CPEs were observed in uninfected control cells, and cells did not display any trypsin-mediated toxicity. The virus titers were detectable in PEDV-infected cells 12 h after infection. The titers further increased at 24 h, reaching a peak at 36 h after infection. Infected cells in infection medium with 10 μg /mL added trypsin had the highest titers (online Technical Appendix Figure 2).

Coronaviruses are RNA viruses that are prone to high levels of mutation resulting in novel reassortants. Birds and bats are considered reservoirs of coronaviruses. However, reserviors of PEDV are not yet known. In conclusion, we have demonstrated that PEDV replicates in MK-DIECs. Availability of a cell line of intestinal origin that supports PEDV replication may be of value for studying mechanisms of virus–cell interactions and for developing live attenuated and killed vaccines.

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Lack of Effect of Lamivudine on Ebola Virus Replication

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To the Editor: The unprecedented number of Ebola virus disease (EVD) cases in western Africa has compelled the world to consider experimental and off-label therapeutics to mitigate the current outbreak. For clinicians, approved drugs are an attractive solution because of known safety profiles and availability.

Oral lamivudine (GlaxoSmithKline, Brentford, UK), a US Food and Drug Administration–approved anti-HIV drug, has been suggested as a possible antiviral agent against Ebola virus (EBOV). In September 2014, a Liberian physician, Dr. Gorbee Logan, reported positive results while treating EVD with lamivudine (*1*). Thirteen of 15 patients treated with lamivudine survived presumed EVD and were declared virus free. Clinical confirmation of EVD in these cases remains to be verified.

Our laboratory had previously assessed this antiretroviral compound in drug screens against EBOV and observed no discernable antiviral activity. However, given

Table. Inhibitory effects of test compounds on Ebola virus replication*

Virus variant or subtype	Cell type	Lamivudine EC_{50}, μmol/L†	Toremifene $EC_{50} \pm$ SD, μmol/L†
EBOV/Kik	Vero E6	>80	5.7 ± 0.7
EBOV/Kik	Vero E6	>320	12.0 ± 1.0
EBOV/Gue	Vero E6	>320	≈8
EBOV/Kik	HepG2	>80	1.6 ± 0.1
EBOV/Kik	HepG2	>320	5.5 ± 0.1
EBOV/Kik	Macrophages	>320	≈25
EBOV/Kik	Macrophages	>320	18.3 ± 0.8
HIV-1	Macrophages	0.002‡	ND
HIV-1	Monocytes	0.69‡	ND
HIV-1 (multiple subtypes)	PBMC	0.002–2.5‡	ND
HBV	HepG2 (2.2.15)	0.002§	ND

*EBOV, Ebola virus; EC_{50}, 50% effective concentration; BMC, peripheral blood mononuclear cells; ND, not done; HBV, hepatitis B virus.
†EC_{50}s were determined by using an EBOV ELISA with antibodies against glycoprotein or viral matrix protein as described (*3*).
‡Data from Schinazi (*4*).
§Data from Kruining et al. (*5*).

the recent testimonials regarding lamivudine effectiveness in treating EBOV-infected patients in Africa, we conducted additional studies to determine whether our previous assertion that lamivudine lacked any direct antiviral activity was correct.

Lamivudine is a nucleoside analog reverse transcription inhibitor of HIV and hepatitis B virus that acts as a synthetic cytidine analog. Incorporation of the active triphosphate form into viral DNA results in chain termination. Studies have demonstrated that lamivudine is a weak inhibitor of mammalian α, β, and γ DNA polymerases (*2*). Lamivudine would not be expected to inhibit the replication of a negative-strand RNA virus.

The activity of lamivudine against EBOV infection was evaluated in a cell-based ELISA with 1995 isolate EBOV *H. sapiens*-tc/COD/1995/Kikwit (EBOV/Kik) (*3*). Three cell lines were tested: Vero E6 (African green monkey kidney, ATCC CRL-1586), Hep G2 (human hepatoma, ATCC HB-8065), and human monocyte-derived macrophages. Macrophages were generated by treating $CD14^+$ cells for 7 days with macrophage colony–stimulating factor and conditioned medium. Cells were treated with compounds in 3-, 4-, or 8- point dose response curves with 2-fold dilutions starting at 80 μmol/L or 320 μmol/L oral lamivudine. Toremifene (T7204–5MG; Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control for activity against EBOV and tested at 2-fold dilutions starting at 25 μmol/L. One hour after drug addition, the cells were infected at a multiplicity of infection of 0.5 or 1 with EBOV/Kik. Experiments were run on duplicate plates or the entire experiment was run on 2 separate days. At 48 hours after infection, cells were formalin-fixed and stained with a primary antibody against EBOV (antibodies against viral matrix protein or glycoprotein) and a secondary antibody (Alexa-488 or horseradish peroxidase).

No direct antiviral effect for lamivudine was observed at concentrations ≤320 μmol/L in Vero E6 cells (Table). Because optimal efficacy of the drug requires phosphorylation, lack of activity may be caused by poor phosphorylation in Vero E6 cells (*6*). Therefore, we also assessed HepG2 cells and primary human monocyte–derived macrophages sensitive to EBOV infection. Toremifene was included as a positive control. Toremifene is a US Food and Drug Administration–approved drug that was reported to have direct antiviral activity in cell culture and to protect mice infected with mouse-adapted EBOV (*3*). As expected, toremifene inhibited EBOV at low micromolar concentrations (Table).

Finally, we assessed the antiviral activity of the compounds against a recent isolate prototype from the current outbreak, EBOV *H. sapiens*-tc/GIN/2014/Guéckédou-C05 (EBOV/Gue) to test whether inhibition of EBOV/Gue by lamivudine was different from that of the reference Kikwit strain. In contrast to a known active compound (toremifene), lamivudine showed no direct antiviral activity.

The current data suggest that lamivudine does not directly inhibit EBOV RNA polymerase or replication of the virus. Systemic and off-target effects, while not previously described, might be possible. To address this possibility, we plan to assess lamivudine in the mouse model of EVD and will report these findings when available. However, on the basis of these in vitro tests, there is no foundation for recommending lamivudine for treatment of EVD in human patients.

G.G.O. was named in patent #8,475,804 assigned to the US Army on approved drugs for use for filoviruses.

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Correction: Vol. 21, No. 2

The abbreviation mL was inadvertently used in the place of µL in paragraphs 2 and 4 of the print edition of the article Potential Sexual Transmission of Zika Virus (D. Musso et al.) The article is correct online (http://wwwnc.cdc.gov/eid/article/21/2/14-1363_article.htm)

A Master Medalist, a President, Tuberculosis, and a Congress: Contributions More Lasting than Bronze

Terence Chorba

The initials VDB are well known to collectors of the Lincoln penny, the obverse (front surface) of which reflects the longest-running design in the history of US coinage. In 1909, in its first coinage, VDB appears in embossed format at the bottom of the coin's reverse, in honor of its designer, Victor David Brenner. The initial San Francisco Mint coin, the 1909-S VDB, remains a sought-after rarity; most collectors never fill the open-mouthed, empty "1909-S VDB" hole in their penny albums.

Despite a tradition of artists' signing their names or initials in coinage, controversy arising from the prominence of the VDB initials resulted in their removal that same year. Tucked into the left lowermost ridge of the bust of Abraham Lincoln, Brenner's initials were restored to the obverse of Lincoln pennies coined since 1918. Discernable by magnification, the inconspicuous placement of Brenner's signature imitates that of a Sicilian engraver, Euainetos, whose work Brenner described as having an "extraordinary decorative sense woven into every line, giving to the empty spaces as much charm as to the modeled surfaces."

Brenner, born Viktoras Baranauskas in Lithuania in 1871, immigrated to the United States in 1890, bringing with him stone-cutting skills that he had learned from his father. Employed as an engraver in New York City, he took evening classes first at Cooper Union, then at the National Academy School and the Art Students League of New York. In 1898, he began to study metal sculpting in Paris under Louis-Oscar Roty and Alexandre Charpentier and became acquainted with Auguste Rodin. Roty taught Brenner the lost wax method of casting to make medals. Brenner's work subsequently won high honors at the Paris Exhibition (1900), Pan American Exposition in Buffalo (1901), and St. Louis International Exposition (1904).

After returning to New York City in 1906, Brenner produced a medal, plaquettes, and a bas-relief for the 1909 centenary of Lincoln's birth. US President Theodore Roosevelt saw examples of those works while posing for Brenner during the making of a Panama Canal service medal, and Brenner gained two more commissions as a result. The first commission was to design a penny featuring Lincoln, a radical departure from tradition—heretofore, no president had appeared on US coins intended for widespread circulation. The second commission was to design a medal for the Sixth International Congress on Tuberculosis, to be held in Washington, DC, in 1908. Roosevelt, who was invited to preside over this Congress, wrote: "The importance of the crusade against tuberculosis… cannot be overestimated when it is realized that tuberculosis costs our county two hundred thousand lives per year, and the entire world over a million lives per year, besides constituting a most serious handicap to material progress, prosperity, and happiness, and being an enormous expense to society, most often to those in walks of life where the burden is least bearable."

The National Association for the Study and Prevention of Tuberculosis (founded by Edward Trudeau in 1904 and now known as the American Lung Association) planned the Congress. Honorary presidents of the congress included Trudeau and Robert Koch, one of the fathers of modern microbiology, who had described the causative agent of tuberculosis (TB) 25 years earlier. Many legendary figures

Victor D. Brenner (1871–1924) International Congress on Tuberculosis Medal, 1908. Bronze, 1.4 × 1.2 in /35.5 × 30.9 mm. Photography by James Gathany

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in the United States and foreign medical communities participated, including William Welch (whose image was subsequently immortalized on a Brenner plaque at Johns Hopkins University), Hermann Biggs, William Osler, Arnold Klebs, and Charles Mayo. Albert Calmette also discussed the success that he, together with Camille Guérin, had achieved in immunizing cattle against TB by using an attenuated strain of *Mycobacterium bovis*. The relatively recent breakthroughs of Koch and of Calmette and Guérin offered hope that science would soon defeat TB.

The Congress was convened in what is now the National Museum of Natural History. For the time, it was a colossal meeting: the number of delegates exceeded 5,000, and total attendance at the various sessions was nearly 100,000. Each delegate received a bronze medal (this month's cover image) designed by Brenner. On the obverse is the figure of a beautiful woman holding the hourglass of time and striding toward a radiant sun, under which is written the Latin word for light, "LVMEN." The woman represents human scientific endeavors approaching enlightenment, and as she progresses, she is trampling down an evil dragon representing disease. On the reverse is the American eagle, with the stars and stripes; underneath appears "INTERNATIONAL CONGRESS ON TVBERCVLOSIS WASHINGTON 1908" and the double-barred cross, the insignia of the campaign against TB.

At the closing of the Congress, several adopted resolutions described the underpinnings of today's federal, state, and local TB control programs. The first such resolution was as follows: "That the attention of the state and central governments be called to the importance of proper laws for the obligatory notification, by medical attendants, to the proper health authorities, of all cases of tuberculosis coming to their notice, and for the registration of such cases, in order to enable the health authorities to put in operation adequate measures for the prevention of the disease."

This Congress justifiably captured the attention of those in medicine and government. In 1908, the US death rate from TB was estimated to be 164 deaths per 100,000 population, nearly 10% of deaths from all causes. For those who marvel at the vision of Roosevelt, the challenges remain: TB still claims more than a million lives annually worldwide and still disproportionately affects "those in walks of life where the burden is least bearable."

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May 14–17, 2015
SHEA
The Society for Healthcare Epidemiology of America
Orlando, FL, USA
http://shea2015.org/attendees/registration/

May 30–June 2, 2015
American Society for Microbiology General Meeting
New Orleans, LA, USA
http://gm.asm.org/

August 10–21, 2015
14th International Dengue Course
Havana, Cuba
http://instituciones.sld.cu/ipk/14thdenguecourse/

August 24–26, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

August 29–September 2, 2015
IDBR
20th Annual Infectious Disease Board Review Course
McLean, VA, USA
http://smhs.gwu.edu/cehp/activities/courses/idbr

September 17–21, 2015
ICAAC
Interscience Conference on Antimicrobial Agents and Chemotherapy
San Diego, CA, USA

March 2–5, 2016
ISID
17th International Congress on Infectious Diseases
Hyderabad, India

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Epidemiology of Human *Mycobacterium bovis* Disease, California, USA, 2003–2011

CME Questions

1. You are consulting for an indigent care clinic in California regarding *Mycobacterium bovis* infection. According to the retrospective review by Gallivan and colleagues, which of the following statements about the epidemiology of *M. bovis* infection in California is correct?

A. The percentage of tuberculosis (TB) cases attributable to *M. bovis* decreased from 2003–2011
B. The percentage of TB cases caused by *M. bovis* was 5.4% in 2011
C. Among children younger than 15 years during 2010–2011, the percentage with at least 1 parent/guardian born in Mexico did not differ significantly between case patients with *M. bovis* and those with *M. tuberculosis*
D. One third of case patients with *M. bovis* had at least 1 parent/guardian born in Mexico

2. According to the retrospective review by Gallivan and colleagues, which of the following variables is most likely a risk factor for *M. bovis* infection in California?

A. African American race
B. Infection limited to the lungs
C. Diabetes
D. HIV/AIDS

3. According to the retrospective review by Gallivan and colleagues, which of the following statements about the public health implications of the findings regarding control of *M. bovis* infection is most likely correct?

A. Prevention efforts should target only California
B. Transmission is only via the respiratory route
C. Future educational efforts to prevent the acquisition of *M. bovis* infection should focus on non-Hispanic blacks
D. Expansion of *M. bovis* genotyping surveillance is recommended

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://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.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

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 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. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

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.

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