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Transplantation and Transfusion

EMERGING INFECTIOUS DISEASES®

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On the Cover

Jules Adler (1865–1952)
***Transfusion of a Goat's Blood* (1892) Oil on canvas (129.5 cm × 195.6 cm)**
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About the Cover p. 1394

Synopsis

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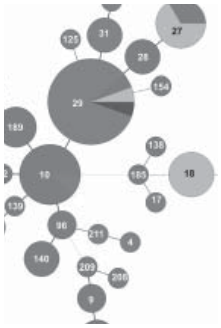
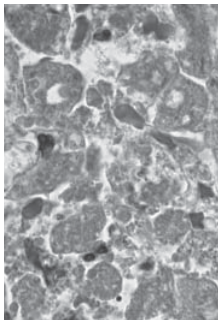


p. 1250

Research

p. 1257



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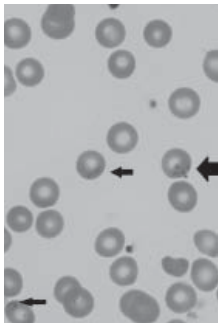
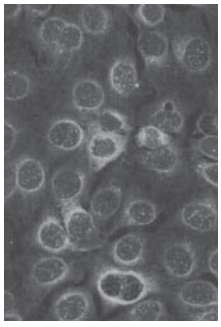


p. 1318

p. 1335



Dispatches

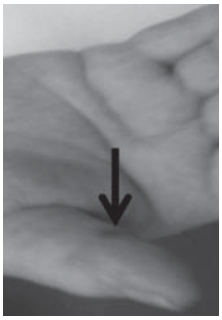
EMERGING INFECTIOUS DISEASES

August 2012

p. 1350

Letters

p. 1391



Books and Media

About the Cover

Etymologia

Online Report

Infectious Disease Transmission during Organ and Tissue Transplantation
http://wwwnc.cdc.gov/eid/article/18/8/12-0277_article.htm

Vaccination of Health Care Workers to Protect Patients at Increased Risk for Acute Respiratory Disease

Gayle P. Dolan, Rebecca C. Harris, Mandy Clarkson, Rachel Sokal, Gemma Morgan, Mitsuru Mukaigawara, Hiroshi Horiuchi, Rachel Hale, Laura Stormont, Laura Béchard-Evans, Yi-Sheng Chao, Sergey Eremin, Sara Martins, John S. Tam, Javier Peñalver, Arina Zanuzdana, and Jonathan S. Nguyen-Van-Tam

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Release date: July 9, 2012; Expiration date: July 9, 2013

Learning Objectives

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

- Assess the impact of influenza infection among health care workers
- Analyze the methodology of research into vaccination of health care workers
- Evaluate the effects of health care worker vaccination on rates of influenza infection among patients
- Distinguish other patient-related outcomes of health care worker vaccination programs

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Health care workers (HCWs) may transmit respiratory infection to patients. We assessed evidence for the effectiveness of vaccinating HCWs to provide indirect protection for patients at risk for severe or complicated disease after acute respiratory infection. We searched electronic health care databases and sources of gray literature by using a predefined strategy. Risk for bias was assessed by using validated tools, and results were synthesized by using a narrative approach. Seventeen of the 12,352 identified citations met the full inclusion criteria, and 3 additional articles were identified from reference or citation tracking. All considered influenza vaccination of HCWs, and most were conducted in long-term residential care settings. Consistency in the direction of effect was observed across several different outcome measures, suggesting a likely protective effect for patients in residential care settings. However, evidence was insufficient for us to confidently extrapolate this to other at-risk patient groups.

Respiratory disease is a leading cause of deaths worldwide, and influenza and pneumococcal infections are major contributors. Certain groups, such as persons ≥65 years of age or with chronic underlying health problems (*1*) are particularly vulnerable to severe respiratory disease and have poorer outcomes after infection than does the general population. These persons are likely to be frequent users of health care facilities, and outbreaks have been described in a range of high-risk environments, including acute care (*2*,*3*), pulmonary (*4*), and infectious diseases wards (*5*); organ transplant departments (*6*); children's wards (*7*,*8*); neonatal intensive care units (*9*); and nursing homes (*10*,*11*). Severe respiratory infections often occur despite high vaccine coverage rates among patients, suggesting that seroconversion is suboptimal (*10*). Although the origin of infection often is difficult to establish, evidence from some outbreaks (*5*,*7*,*10*–*14*) suggests that transmission from HCWs to patients is likely.

It is estimated from previous influenza seasons that ≈20% of HCWs have evidence of infection (*15*), although not necessarily acquired in the workplace. Young healthy adults often have asymptomatic infection, and ≈28%–59% might experience subclinical infection (*15*). Many persons with mild or subclinical illness continue to work while infectious, and even when illness is recognized, virus might be shed before symptom onset. In a randomized controlled trial among health care professionals, Wilde et al. demonstrated that influenza vaccine was 88% efficacious for reducing serologically confirmed influenza A infection and 89% efficacious for reducing serologically confirmed influenza B infection (*16*). Therefore, vaccination of HCWs has been widely recommended to provide direct protection for themselves and indirect protection for their patients (*1*,*17*).

Despite efforts to encourage influenza vaccination of HCWs, coverage has been historically poor. Recently, ethical arguments for mandatory influenza vaccination have been raised that focus not only on the direct and indirect benefits to staff and patient health but also on the economic consequences. Burls et al. (*18*) suggested that at a cost of £51–£405 (US$85–$675) per life-year saved, mandatory vaccination is likely to be cost-effective. However, evidence for the effectiveness of vaccinating HCWs for protecting vulnerable patients is limited.

Two recent systematic reviews considered the evidence for indirect protection of vulnerable patient groups after staff influenza vaccination (*18*,*19*). They suggest that vaccination of HCWs might be effective for reducing death and influenza-like illness (ILI) among elderly residents, but we are unaware of comparable data related to other at-risk groups. We aimed to identify and assess further evidence for the effect of vaccinating HCWs on patient groups most vulnerable to severe or complicated respiratory illness.

Methods

The full study protocol is registered with the UK National Institute for Health Research International Prospective Register of Systematic Reviews (www.crd.york.ac.uk/PROSPERO [registration no. CRD420111092]). We searched several electronic health care databases, sources of evidence-based reviews, guidelines, and gray literature in accordance with the specifications of each database (Figure). In addition, we contacted domain experts and vaccine manufacturers to identify unpublished data and undertook citation and reference tracking for all included papers. Thesaurus-indexed and free text terms were defined for the population, intervention, and outcome parameters; peer reviewed; and adapted as necessary for each search engine.

Eligibility criteria were defined a priori as follows:

- Types of study: any experiment, observational study, or systematic review reporting on the effectiveness of vaccination (including influenza or pneumococcal vaccines) of HCWs for protecting patients at higher risk for severe or complicated respiratory infection.
- Types of participants: persons at higher risk for severe or complicated illness as a result of acute respiratory infection (as defined in World Health Organization [*1*] and Advisory Committee on Immunization Practices guidance [*17*]), who have received or are receiving care from an HCW.
- Types of intervention: influenza or pneumococcal vaccination of any worker providing medical, nursing, social, or personal health care (because no uniformly accepted definition of an HCW exists, it

was defined by the peer-reviewed terms specified in the search strategy).

- Types of outcome measure: cases or consultations, death or hospitalization for acute respiratory disease, influenza, ILI, or pneumococcal disease.

Published and unpublished reports from any year that were written in Chinese, English, French, Japanese, Portuguese, Russian, or Spanish were considered. A 3-stage process was used to assess eligibility for inclusion screening first by title, then abstract, and then full text. Two reviewers undertook this in parallel for stages 1 and 2 and independently for stage 3. Consensus was reached by discussion; when reviewers disagreed, a third reviewer was consulted for a final decision. Where multiple reports were identified for the same piece of original research, the most recent peer-reviewed source was selected.

Two reviewers independently extracted data from each included, by using a predefined, piloted template. The risk for bias was assessed by using the Cochrane Collaboration tool (*20*) for experimental and prospective cohort studies, the Downs and Black tool (*21*) for other observational studies, and the US Agency for Healthcare Research and Quality (*22*) domain and element-based evaluation instrument for systematic reviews. Again, consensus was reached by discussion, with engagement of a third reviewer as necessary. No additional information was sought from corresponding authors. Data were synthesized qualitatively by using a narrative approach in accordance with the framework described by the Economic and Social Research Council and recommended by the University of York Centre for Reviews and Dissemination (*23*).

Results

Study Selection

We identified 12,352 citations (Figure): 10,713 from health care databases and the remainder from additional sources. Seventeen studies met the inclusion criteria at the full text stage; 3 others were identified from citation or reference tracking. Of these, 14 were primary research articles; 4 were cluster randomized controlled trials (RCTs), and 10 were observational studies. Four of the remaining 6 articles were different versions of a report relating to 1 systematic review, and the other 2 were different versions of a report relating to a second systematic review. One of these systematic reviews (*18*) provided a qualitative analysis of 2 of the earliest cluster RCTs (*24*,*25*), and the other (*19*) provided a quantitative meta-analysis of all 4 cluster RCTs (*24*–*27*) and 1 additional observational study (*28*). We used the most recent and detailed version of each review published in a peer-reviewed source in this study.

All of the primary studies considered influenza vaccination of HCWs (online Appendix Table 1, wwwnc.cdc.gov/EID/article/18/8/11-1355-TA1.htm); therefore, we

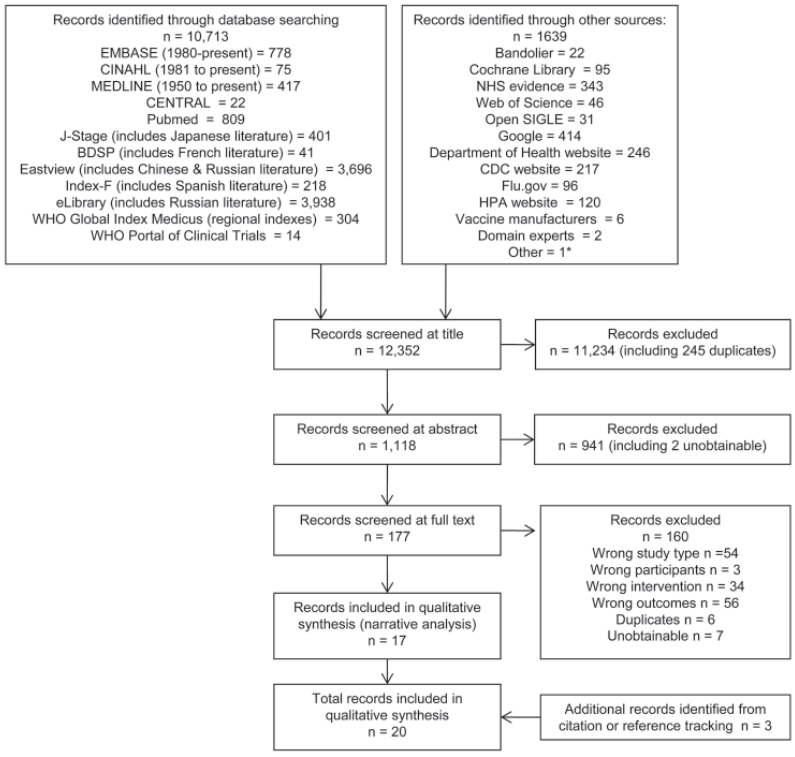

Figure. Study selection for a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease.

Table 1. Risk for bias assessed by using the Cochrane Collaboration tool in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease*

Study	Sequence generation	Allocation concealment	Blinding of participants, personnel and outcome assessors: Primary outcome	Blinding of participants, personnel and outcome assessors: Secondary outcomes	Incomplete outcome data: Primary outcome	Incomplete outcome data: Secondary outcomes	Selective outcome reporting	Other sources of bias
Lemaitre et al. (*26*)								
Hayward et al. (*27*)								
Carman et al. (*24*)								
Potter et al. (*25*)								
Saito et al. (*33*)								
Oshitani et al. (*28*)								

*Black shading, low risk of bias; light gray shading, uncertain risk of bias; dark gray shading, high risk of bias; blank cells, no secondary outcome measure reported.

discarded our planned subanalysis relating to pneumococcal vaccination. Only 4 studies (*24*–*26*,*29*) defined HCW, even though this definition is likely to affect the probability of transmission and therefore the magnitude of observed effects. Where reported, vaccination among staff ranged from ≈35% to 70% in the intervention arm and from none to 32% in the control arm of experimental studies and from 12% to 90% in observational studies. Eleven of the primary research studies were conducted in long-term care facilities; the remainder were conducted in renal dialysis facilities (*30*), a pediatric hospital (*31*), and an adult oncology hospital (*32*) (1 study each). Where reported, vaccination coverage among patient populations ranged from 0% to ≈90%, and few studies considered additional infection control practices, such as hand washing, duration of contact, or use of face masks, which vary and again influence the propensity for transmission.

Risk for Bias

Cochrane Collaboration Tool

Concerns arose largely from the lack of blinding of participants or study personnel (Table 1). Although the effect was likely to be minimal with regard to the primary outcome for all 4 RCTs (all-cause mortality), it might have resulted in underestimation or overestimation of additional, more subjective, outcome measures, such as incidence of ILI.

All studies, except for that by Lemaitre et al. (*26*), were judged to be at some further risk for bias. This included selection bias (inadequate description of selection criteria [*24*,*25*,*33*] or sequence allocation [*25*,*28*,*33*]), performance bias (lack of detail about allocation concealment [*25*,*26*]), and measurement bias (no clearly defined outcome measure [*28*]).

Downs and Black Tool

The Downs and Black tool (Table 2) considers 5 assessment domains, but because most observational studies identified were primarily descriptive, we excluded the power domain in this review. Scores ranged from 3/27 (*34*) to 10/27 (*29*,*30*,*35*), with higher scores representing lower risk for bias. None of the studies provided sufficient detail about the patient population, and only 1 (*29*) described principal confounders. Other concerns about reporting related to lack of detail of study objectives (*29*,*32*,*34*), a priori definition of outcome measures (*32*,*34*–*37*) or those lost to follow up (*35*), failure to provide sufficient detail of statistical analysis (*29*,*30*,*34*–*37*), lack of randomization or blinding, and failure to adjust outcome measures.

Agency for Healthcare Research and Quality Tool

We assessed the 2 identified systematic reviews (*18*,*19*) by using the Agency for Healthcare Research and Quality tool (*22*). Both appeared to be at a comparatively low risk for bias, providing a clearly defined research question, search strategy, inclusion and exclusion criteria, and description of outcomes. However, details were lacking about blinding of reviewers to authorship and measurement

Table 2. Risk for bias by using the Downs and Black tool in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease

Study	Type of score (maximum score)*: Reporting (11)	External validity (3)	Internal validity, bias (7)	Internal validity, confounding (6)	Total (27)
Ando et al. (*30*)	5	2	2	1	10
Shugarman et al. (*35*)	6	0	1	3	10
Kanaoka et al. (*29*)	5	1	3	1	10
Monto et al. (*36*)	5	0	2	2	9
Weinstock et al. (*32*)	4	0	4	1	9
Stevenson et al. (*37*)	4	1	2	1	8
Munford et al. (*34*)	2	0	0	1	3

*Maximum score indicates lowest risk of bias for each domain.

Table 3. Cases of and consultations for acute respiratory disease in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease*

Outcome measure (study)	Study design	Method of assessment	Measure of effect in patient population	Effect estimate (95% CI)
Clinically defined episodes of viral illness (Potter et al. [*25*])	Cluster RCT	Not defined. No. episodes recorded by study nurses.	OR, nonvaccinated and vaccinated patients	**0.64 (0.48–0.87)**
			OR, vaccinated patients	**0.40 (0.26–0.62)**
			OR, nonvaccinated patients	0.98 (0.65–1.48)
Lower respiratory tract infection				
Potter et al. (*25*)	Cluster RCT	Defined as 1) pulmonary crackles, wheeze, or tachypnea plus temperature >37.0°C or leukocyte count >10 × 10^9/L or 2) a positive sputum culture. No. episodes recorded by study nurses.	OR, nonvaccinated and vaccinated patients	0.69 (0.40–1.19)
			OR, vaccinated patients	0.59 (0.25–1.38)
			OR, nonvaccinated patients	0.77 (0.38–1.57)
Thomas et al. (*19*)	Pooled data		OR, adjusted for clustering	0.71 (0.29–1.71)†

*RCT, randomized controlled trial; OR, odds ratio. **Boldface** indicates statistical significance.
†p = 0.44. p value not reported for other categories.

of agreement in extracting data, which might have resulted in measurement bias.

Synthesis of Results

Cases or Consultations for Acute Respiratory Disease

One RCT reported data (*25*) for 2 measures of consultation for respiratory disease; episodes of lower respiratory tract infection and suspected viral illness (Table 3). In addition, the estimate for lower respiratory tract infection was adjusted for clustering by Thomas et al. (*19*). Both measures demonstrated reduced odds, and results were significant for suspected viral illness when vaccinated and nonvaccinated patients were considered together.

The study by Potter et al. (*25*) was considered to be at a higher risk for bias than the other RCTs identified; thus, the strength of evidence for these outcomes is questionable. In addition, the measures considered are nonspecific, and the observed effects cannot necessarily be attributed to reduced influenza infection. Nasopharyngeal samples were taken from a subset of patients within 48 hours after symptoms developed; no samples were positive for influenza on immunofluorescence assay.

Cases or Consultations for Inf uenza or ILI

Data were reported in 13 studies for 5 outcome measures of influenza/ILI. Eight primary studies measured clinically defined influenza/ILI (online Appendix Table 2, wwwnc.cdc.gov/EID/article/18/8/11-1355-TA2.htm; Table 4).

Three RCTs (*25*–*27*) measured cases of ILI, and these data were pooled by Thomas et al. (*19*) to demonstrate a statistically significant reduction in odds. Two observational studies (*28*,*33*) also measured cases of clinically defined ILI, demonstrating statistically significant reductions in risk, although the threshold of staff vaccination coverage used to categorize facilities in these studies varied (Oshitani [*28*] considering facilities where more or fewer than 10 staff were vaccinated, and Saito [*33*] comparing facilities with ≤40%, 40%–59%, and ≥60% coverage among staff). A third observational study (*29*) reported no correlation between

Table 4. Clinically defined outbreaks and clusters of ILI in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease*

Study	Study design	Method of assessment	Measure of effect in patient population	Effect estimate (95% CI)
Oshitani et al. (*28*)	Prospective cohort	Defined as ILI >10% of total resident population. Mandatory reporting by survey.	OR, unadjusted facilities with ≥10 staff members vaccinated vs. those with <10 staff members vaccinated	**0.30 (0.09–0.69)†**
Stevenson et al. (*37*)	Cross-sectional	No definition provided. Reporting by survey.	χ^2 test for trend; logistic regression	χ^2 p = 0.03; logistic regression p = 0.08
Shugarman et al. (*35*)	Cross-sectional	Defined as ≥3 residents within a 72-h period with influenza-like symptoms, sudden onset of fever, or "feverishness" and ≥1 of the following respiratory symptoms; sore throat, runny nose, cough, or nasal congestion. Reporting by survey.	OR, facilities with staff vaccination coverage >55% and patient vaccination coverage >89%, vs. those with lower coverage	**0.39 (0.17–0.87)†**

*ILI, influenza-like illness; OR, odds ratio. **Boldface** indicates statistical significance.
†p value not reported.

staff vaccination coverage and cases of influenza in patients, although the relative change in vaccination coverage (79%–91%) was small and thus any difference in the number of cases was probably difficult to detect. The magnitude of reported effects varied, most notably by influenza season in the study of Hayward et al. (*27*), and with patient vaccination status in the study of Potter et al. (*25*).

One study measured general practitioners consultations for ILI (*27*). An inconsistent effect was demonstrated across different periods of influenza activity, but pooled data suggested an overall statistically significant reduction in the odds of consultation after vaccination of HCWs.

Three observational studies (*28*,*35*,*37*) demonstrated a statistically significant protective effect of staff vaccination against clinically defined outbreaks of ILI in patients (Table 4). The thresholds used to categorize facilities on the basis of staff vaccination coverage again varied among studies, and these data were considered to be at relatively high risk for bias.

Measures of laboratory-confirmed infection (online Appendix Table 3, wwwnc.cdc.gov/EID/article/18/8/11-1355-TA3.htm) were less frequently reported and generally based on small samples of data at high risk for bias. Five studies measured laboratory-diagnosed influenza (*24*,*25*,*31*,*32*,*36*), although 1 reported no statistical analysis (*25*). Different methods of defining laboratory confirmation were used (online Appendix Table 3). Thomas et al. (*19*) pooled data from the 2 RCTs (*24*,*25*) to demonstrate a small nonsignificant protective effect. This result is supported by evidence from 2 additional observational studies (*31*,*32*), which indicated a statistically significant reduction in the proportion of laboratory-confirmed cases of nosocomial influenza among inpatient pediatric and oncology patients after implementation of vaccination campaigns. In addition, Monto et al. (*36*) measured outbreaks of laboratory-diagnosed influenza, and this was the only study not to demonstrate a protective effect of vaccinating HCWs. The authors reported a higher, but nonsignificant, median vaccination coverage among staff in homes experiencing outbreaks.

Deaths from Respiratory Infection, ILI, or Acute or Respiratory Disease or Its Complications

Evidence for 5 measures of death was identified (Table 5). All 4 RCTs (*24*–*27*) considered all-cause death as their primary objective, providing the strongest evidence on the basis of study design. Although not defined a priori as an outcome of interest for this review, data were therefore extracted. These were pooled by Thomas et al. (*19*) to demonstrate a statistically significant protective effect.

Although at higher risk for bias, supporting data were provided for 4 more-specific measures. Thomas et al. (*19*) pooled data from 2 RCTs, 1 measuring deaths after

Table 5. Measures of death in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease*

Outcome measure and study	Study design	Method of assessment	Measure of effect in patient population	Effect estimate (95% CI), p value
All-cause mortality				
Potter et al. (*25*)	Cluster RCT	Death certificate	OR, vaccinated and nonvaccinated patients	**0.56 (0.40–0.80)†**
			OR, vaccinated patients	**0.57 (0.35–0.91)†**
			OR, nonvaccinated patients	**0.56 (0.34–0.94)†**
Carman et al. (*24*)	Cluster RCT	Not stated	OR	0.62 (0.36–1.04), p = 0.092
Hayward et al. (*27*)	Cluster RCT	Reporting by lead nurse	Rate difference, epidemic period 1	**–0.05 (–0.07 to –0.02)**, p = 0.002
			Rate difference, epidemic period 2	–0.01 (–0.04 to 0.02), p = 0.49
			Rate difference, nonepidemic period 1	0.00 (–0.03 to 0.03), p = 0.93
			Rate difference, nonepidemic period 2	0.01 (–0.03 to 0.04), p = 0.70
Lemaitre et al. (*26*)	Cluster RCT	Not stated	OR	0.86 (0.72–1.02), p = 0.08
Thomas et al. (*19*)	Pooled data		OR, adjusted for clustering	**0.68 (0.55–0.84),** p<0.001
Respiratory deaths: Lemaitre et al. (*26*)	Cluster RCT	Reporting by study nurses	OR	1.55 (0.59–4.10), p = 0.38
Pneumonia-associated deaths				
Potter et al. (*25*)	Cluster RCT	Reporting by lead nurse	OR, vaccinated and nonvaccinated patients	**0.60 (0.37–0.97)†**
			OR, vaccinated patients	0.56 (0.28–1.13)†
			OR, nonvaccinated patients	0.64 (0.33–1.23)†
Thomas et al. (*19*)	Pooled data		Risk ratio, adjusted for clustering	0.87 (0.47–1.64), p = 0.67
Death with influenza-like illness				
Hayward et al. (*27*)	Cluster RCT	Reporting by lead nurse	Rate difference, epidemic period 1	–0.01 (–0.02 to 0.01), p = 0.24
			Rate difference, epidemic period 2	–0.01 (–0.03 to 0.00), p = 0.08
			Rate difference, nonepidemic period 1	–0.01 (–0.04 to 0.02), p = 0.59
			Rate difference, nonepidemic period 2	0.01 (–0.01 to 0.02), p = 0.35
Thomas et al. (*19*)	Pooled data		OR, adjusted for clustering	0.72 (0.31–1.70), p = 0.45
Laboratory-diagnosed influenza at death: Carman et al. (*24*)	Cluster RCT	Nasal swab within 12 h before death	Difference in proportions, influenza positive at death	20%, p = 0.055

*RCT, randomized controlled trial; OR, odds ratio. **Boldface** indicates statistical significance. Shaded fields represent pooled data.
†p value not reported.

Table 6. Measures of hospitalization in a review of the vaccination of health care workers to protect patients at risk for acute respiratory disease*

Outcome measure and study	Study design	Method of assessment	Measure of effect in patient population	Effect estimate (95% CI)
Hospitalization				
Hayward et al. (*27*)	Cluster RCT	Reporting by lead nurse	Rate difference, epidemic period 1	–0.02 (–0.05 to 0.02), p = 0.35
			Rate difference, epidemic period 2	0.00 (–0.03 to 0.04), p = 0.84
			Rate difference, nonepidemic period 1	0.00 (–0.04 to 0.03), p = 0.80
			Rate difference, nonepidemic period 2	0.00 (–0.03 to 0.03), p = 0.86
Lemaitre et al. (*26*)	Cluster RCT	Not stated	OR	1.03 (0.76–1.40), p = 0.85
Thomas et al. (*19*)	Pooled data		OR, adjusted for clustering	0.90 (0.66 to 1.21), p = 0.47
Hospitalization for respiratory causes: Lemaitre et al. (*26*)	Cluster RCT	Not stated	OR	1.01 (0.43–2.34), p = 0.98
Admissions to hospital with influenza-like illness: Hayward et al. (*27*)	Cluster RCT	Reporting by lead nurse	Rate difference, epidemic period 1	–0.02 (–0.03 to 0.00), p = 0.009
			Rate difference, epidemic period 2	0.00 (–0.02 to 0.02), p = 0.99
			Rate difference, nonepidemic period 1	–0.01 (–0.02 to 0.01), p = 0.32
			Rate difference, nonepidemic period 2	0.01 (0.00–0.02), p = 0.31

*RCT, randomized controlled trial; OR, odds ratio. **Boldface** indicates statistical significance. Shading indicates pooled data.

pneumonia (*25*), the other measuring respiratory deaths (*26*), and demonstrated a small nonsignificant protective effect. However, the validity of this pooled analysis was questionable because how these outcomes were defined was not clear. Nonsignificant reductions in risk also were observed for laboratory-diagnosed influenza at death (*24*) and death after ILI (*27*). Again, the direction of the observed effects was largely consistent with other measures, providing further support for a hypothesis of indirect protection.

Admission to a Health Care Facility or Any Other Suggestion of Impact

Hospitalization was measured in 2 RCTs (*26*,*27*), pooled data suggesting a small, nonsignificant effect (Table 6). One RCT also measured hospitalization for respiratory causes (*26*) and 1 admission to hospital with ILI (*27*), although neither demonstrated any apparent effect. This result is particularly noteworthy given the observed decrease in deaths and might reflect health-seeking behaviors.

Discussion

Evidence is limited for the effectiveness of vaccination of HCWs for protecting patients at higher risk for severe or complicated respiratory illness. Despite the broad question posed, extensive searching, and large number of resultant hits, our search resulted in a low yield of studies, all of which focused on influenza with no consideration for pneumococcal infection. This finding is perhaps not surprising because pneumococcal vaccination is not routinely recommended for HCWs and little, if any, evidence exists of nosocomial spread. A consistent direction of effect was observed across multiple outcome measures, with virtually all studies noting a trend toward a protective effect of vaccinating HCWs. This consistency adds to the degree of confidence in interpreting our overall findings. Given that most studies were carried out in long-term care facilities, we conclude that vaccination of HCWs against influenza is likely to offer protection for this patient group. However, future reviews that specifically examine the effect of vaccinating other outpatient providers, such as home HCWs and hospital staff in acute care, short-stay settings, would clearly be of value. These findings are more difficult to extrapolate to other at-risk groups, although some, albeit limited, evidence was identified from other settings to suggest a similar effect.

The results of all 4 RCTs (*24*–*27*) and 1 of the observational studies identified (*28*) previously had been pooled in a quantitative meta-analysis (*19*). The authors of this analysis concluded that evidence is lacking that vaccinating HCWs prevents influenza infection in elderly patients because the apparent benefits were confined to nonspecific outcome measures. We considered additional observational data that demonstrate consistency in the direction of the observed effects across specific and nonspecific outcome measures. Although the strength of evidence for more-specific measures is generally much weaker, these findings add greater weight to the hypothesis of a potential protective effect.

The recent position statement by the Society for Healthcare Epidemiology of America (*38*) suggests that further studies are not needed because the biological rationale for vaccination does not vary by practice setting. However, effect size might vary considerably because of patient characteristics and care patterns (staff deployment and duration of inpatient stay), and further evidence is needed among the most at-risk groups where benefits are probably greatest, to enable prioritization of resources, particularly where vaccine shortages or resource limitations might exist.

Previous authors have suggested that vaccination of HCWs might enable development of herd immunity.

Realistically, herd immunity is difficult to achieve in health care settings, especially acute care short-stay settings, because of patient admissions and discharges, visitors, and staff turnover. That said, herd immunity might not be necessary to benefit patients; modeling studies (*39*) suggest a direct association between coverage and attack rates. Such studies (*39*) also suggest variation in the potential for transmission of infection by different staff groups, which should be explored in further detail.

This field of research has some inherent problems. These difficulties result in part from the difficulty of isolating the effect of HCW vaccination, disentangling it from other factors that might influence patient outcomes, such as patient vaccination (as demonstrated by Potter et al. [*25*]) and background influenza activity (as demonstrated by Hayward et al. [*27*]). Staff vaccination itself might be linked to additional confounding variables, such as organizational culture and professional beliefs. In fact, such confounding might explain the difference in findings between the work of Monto (*36*) and the other authors. Prospective collection of information relating to relevant transmission factors and infection control measures that were largely overlooked by the studies in this review should be used to enable appropriate adjustment in future studies. Furthermore, the most appropriate outcome measures are difficult to define because not all persons with laboratory-confirmed infection have symptoms of illness and vice versa. Future studies thus need to demonstrate consistent effects for a range of clearly defined outcomes by using valid measures across several different influenza seasons, with sufficient power to detect true underlying effects.

The findings of our review are subject to several limitations. Because 11 of the 14 primary research articles considered outcomes in long-term care facilities, generalizability to other at-risk groups is limited. In addition, we did not attempt to contact authors of original studies, and the conclusions drawn are limited by the reported detail. Although the number of reviewers was limited as far as possible, some inconsistency might have occurred in the selection, extraction, and assessment of data introducing potential bias, particularly where the opportunity for subjective judgment existed. We attempted to minimize inconsistency by using several standard assessment tools, but their use was limited by lack of information where components were not conducted because of the nature of the study design. Meta-analysis of the 4 RCTs identified had already been conducted, and although we identified additional observational data, the observed heterogeneity limited any further quantitative analysis.

Some wider possible effects of HCW vaccination, such as reduction in absenteeism because of illness, are beyond the scope of this review. Ethically, autonomy needs to be balanced with nonmaleficence, and this need must be addressed when policy decisions about vaccination are considered. Anikeeva et al. (*40*) reported that in a review of 15 studies focusing on the reasons staff accept influenza vaccine, self-protection was the most important. However, patient protection also was perceived as important, particularly among HCWs in settings with higher risk patients (*40*). Nevertheless, HCWs would be justified in claiming that the current evidence base is not especially strong and heavily weighted toward the benefits to patients receiving care in long-term care facilities, although limited evidence would not necessarily legitimize nonacceptance.

The existing evidence base is sufficient to sustain current recommendations for vaccinating HCWs on the grounds that some protection of high-risk patients against influenza seems likely. However, vaccination should be considered 1 element of a broad package of infection prevention and control measures, such as good hand and respiratory hygiene, environmental cleaning, protection against respiratory droplets, and cohorted care during outbreaks. Well-designed studies that strengthen the evidence base might increase compliance with guidelines, resulting in improved coverage.

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G.D. and J.S.N.-V.-T., the primary and senior authors, respectively, take responsibility for the work and act as guarantors of the data. G.D., R.C.H., R.H., and J.S.N.-V.-T. designed the study protocol. G.D., R.C.H., M.M., H.H., L.B., Y.C., S.E., S.M., J.T., J.P., A.Z., and R.H. executed the search strategy and screening. G.D, R.C.H., M.C., R.S., G.M., M.M., H.H., and L.S. analyzed the risk for bias and acquired the data. G.D., and J.S.N.-V.-T. analyzed and interpreted the data. G.D., R.C.H., and J.S.N.-V.-T. prepared the manuscript.

The University of Nottingham Health Protection Research Group receives research funds from GSK. The group has recently accepted an unrestricted educational grant for influenza research from F. Hoffmann-La Roche. Research on influenza funded by an unrestricted educational grant from Astra-Zeneca is also under way. J.S.N.-V.-T. has given a talk on a related topic for which expenses were paid by the European Society for Clinical Microbiology and Infectious Diseases; he has received speaker honoraria from Sanofi-Pasteur MSD, F. Hoffmann-La Roche, and GSK; and he has received remuneration for consultancy work from Baxter AG, GSK, F. Hoffmann-La Roche, Novartis,

and Solvay. All such paid consultancy and speaker engagements ceased in September 2010. J.S.N.-V.-T. is a former employee of SmithKline Beecham, F. Hoffmann-La Roche, and Sanofi-Pasteur MSD, all before 2005. R.H. currently works on a project funded by Astra-Zeneca, which considers attitudes to the use of intranasal influenza vaccine.

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VIM-2–producing Multidrug-Resistant *Pseudomonas aeruginosa* ST175 Clone, Spain

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A total of 183 patients were colonized or infected with multidrug-resistant *Pseudomonas aeruginosa* isolates at a hospital in Spain during 2007–2010; prevalence increased over this period from 2.8% to 15.3%. To characterize these isolates, we performed molecular epidemiologic and drug resistance analysis. Genotyping showed that 104 (56.8%) isolates belonged to a single major clone (clone B), which was identified by multilocus sequence typing as sequence type (ST) 175. This clone was initially isolated from 5 patients in 2008, and then isolated from 23 patients in 2009 and 76 patients in 2010. PCR analysis of clone B isolates identified the bla_{VIM-2} gene in all but 1 isolate, which harbored bla_{IMP-22}. ST175 isolates were susceptible to only amikacin (75%) and colistin (100%). Emergence of the ST175 clone represents a major health problem because it compromises therapy for treatment of *P. aeruginosa* nosocomial infections.

Members of the bacterial genus *Pseudomonas*, especially *P. aeruginosa*, are among the major nosocomial pathogens because of their ubiquitous nature and ability to colonize and survive in hospital reservoirs and because of their role in causing infections in immunocompromised and critically ill patients (*1*). *P. aeruginosa* shows a high level of intrinsic resistance to antimicrobial drugs and an ability to become even more drug resistant. These characteristics are caused by selective pressure of mutations in chromosomal genes that lead to *ampC* hyperexpression, repression or inactivation of *oprD*, and overexpression of efflux pumps (*2*). In addition, *P. aeruginosa* is able to acquire other drug-resistance determinants by horizontal transfer of mobile genetic elements coding for class B carbapenemases (also called metallo-β-lactamases [MBLs]), which hydrolyze all β-lactams except aztreonam (ATM) (*3*).

Because they can be disseminated horizontally through transfer of resistance determinants, MBLs have become a serious concern in hospitals worldwide over the past decade. Such acquired MBLs include the IMP and VIM types SPM-1, GIM-1, SIM-1, AIM-1, KHM-1, NDM-1, and SID-1 (*4*,*5*). MBL genes are normally encoded in class 1 integrons along with other resistance determinants, such as the aminoglycoside-modifying enzymes. The integrons are frequently located in plasmids or transposons, the dissemination of which contributes to the global spread of this resistance mechanism (*6*,*7*). The versatility and ability of *P. aeruginosa* to combine different resistance mechanisms has led to emergence of strains that are resistant to multiple antimicrobial drugs, which severely limits therapeutic options for treating infections (*8*,*9*). Interim definitions defining multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant bacteria, including *P. aeruginosa*, have been recently reported (*10*).

Although the prevalence of *P. aeruginosa* strains producing carbapenemases in Spain was considered low (0.4% of carbapenem-resistant isolates) (*11*) compared with prevalence in other countries in Europe, such as Italy (12.6% of carbapenem-resistant isolates) (*12*), detection of these isolates is no longer sporadic (*13*,*14*). Recent evidence from multicenter studies indicates an ≈10-fold increase in prevalence of these isolates in the past 5 years (*15*). During 2007 and 2008, a polyclonal outbreak of VIM-2–producing *P. aeruginosa* was detected in a hospital in Spain. At the same time, another outbreak in the hematology department of a hospital in Spain was caused by a *P. aeruginosa* ST235 clone, which produced GES-1 and GES-5, 2 extended-

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spectrum β-lactamases (*16*). We have observed a sharp increase in infections with drug-resistant *P. aeruginosa* that produces carbapenemase. Thus, we conducted a study to determine the clinical and molecular epidemiologic characteristics of drug-resistant *P. aeruginosa* isolates detected at a major hospital during 2007–2010.

Materials and Methods

Study Population

We conducted a retrospective study of all non–cystic fibrosis adult patients who were colonized or infected with *P. aeruginosa* isolates during January 2007–December 2010 at the Hospital Universitario 12 de Octubre in Madrid. This hospital is a 1,300-bed tertiary-care facility serving a population of 600,000 persons (≈42,000 admissions/year). We classified resistance patterns in *P. aeruginosa* according to recently published proposed interim definitions (*10*). An isolate was defined as MDR if it was resistant to ≥1 drug in ≥3 categories of drugs and XDR if it was resistant to ≥1 drug in ≤2 drug categories. The drugs on which our categorization was based included antipseudomonal cephalosporins (ceftazidime [CAZ], cefepime [FEP]), carbapenems (imipenem [IMP], meropenem [MER]), piperacillin/tazobactam (PIP-TZ), ATM, fluoroquinolones (ciprofloxacin [CIP]), and aminoglycosides (gentamicin [GEN], tobramycin [TOB], amikacin [AMK]).

Unique (nonduplicate) clinical isolates from colonized or infected patients were collected during the study. A case was defined as nosocomial if infection or colonization was detected in a person ≥48 hours after admission or if a person had documented evidence of hospitalization within the previous 12 months. Colonization was defined as isolation of *P. aeruginosa* from ≥1 clinical specimens in the absence of clinical signs consistent with infection. Medical charts were reviewed and demographic, clinical, and microbiological data were collected.

Antimicrobial Drug Susceptibility Testing

Identification and antimicrobial drug susceptibility testing of *P. aeruginosa* isolates included in this study were performed by using semi-automated microdilution panels (Soria, Melguizo, Spain) (*17*). The antimicrobial drugs tested were PIP-TZ, CAZ, FEP, ATM, IMP, MER, CIP, GEN, TOB, AMK and colistin (COL). Break points were applied according to Clinical and Laboratory Standards Institute (CLSI) guidelines (*18*).

Genotyping Analysis

Epidemiologic relatedness of isolates was studied by using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE was conducted by macrorestriction of chromosomal DNA with *Spe*I and separation of restriction fragments by using a CHEF DRIII PFGE system (Bio-Rad Laboratories, Hercules, CA, USA). Migration of DNA fragments was normalized by using an appropriate mass marker, and computer-assisted analysis of PFGE patterns was conducted by using Bionumerics software (Applied Maths, St-Martens-Latem, Belgium). PFGE types were defined on the basis of DNA banding patterns in accordance with criteria defined by Tenover et al. (*19*).

MLST was performed on selected isolates according to published protocols (*20*). Standard DNA amplification and sequencing of 7 housekeeping genes (*acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A, and *trp*E) were performed. Isolates were assigned a sequence type (ST) number according to the allelic profiles available in the MLST database (http://pubmlst.org/paeruginosa).

Characterization of Acquired MBLs and Integron Analysis

The presence of horizontally acquired β-lactamases was determined by using phenotypic and genetic approaches. Phenotypic tests included analysis with Etest MBL strips (AB Biodisk, Solna, Sweden) for detection of class B carbapenemases. On the basis of positive results from preliminary phenotypic tests, the potential presence of genes encoding acquired metallo-β-lactamases was explored by using PCR amplification and DNA sequence analysis.

Described primers and conditions were used to amplify genes encoding VIM and IMP type β-lactamases (*11,13*). After PCR amplification, sequencing reactions were performed by using the BigDye Terminator Kit (PE Applied Biosystems, Foster City, CA, USA), and sequences were analyzed by using an ABI prism 3100 DNA Sequencer (PE Applied Biosystems). Resulting sequences were compared with those available in GenBank (www.ncbi.nih.gov/BLAST). Integrons harboring MBL-encoding genes were characterized by PCR and DNA sequencing by using specific primers to amplify the IntI1 and qacEΔ1 markers, the DNA region located between intI1 and qacEΔ1, and the corresponding MBL-encoding gene (*11,13*).

Statistical Analysis

Univariate analysis was performed by using the *t* test for continuous variables and the χ^2 or Fisher exact tests for categorical variables. A p value ≤0.05 was considered significant. Data were stored and analyzed by using SPSS version 17.0 for Windows software (Analytical Software, St. Paul, MN USA).

Results

General Characteristics

During the 4-year study, of 2,145 patients who were infected or colonized with *Pseudomonas* spp., 183 harbored MDR or XDR isolates: 13 (2.8%) of 460 in 2007, 32 (6.2%) of 517 in 2008, 38 (7.4%) of 514 in 2009, and 100 of (15.3%) 654 in 2010. The estimated annual incidence of MDR *Pseudomonas*–infected persons increased from 0.04/1,000 bed-days in 2007 to 0.34/1,000 bed-days in 2010 (Figure 1). Of 183 isolates, 177 were identified as *P. aeruginosa* and 6 as *P. putida*.

The mean ± SD age of patients was 65.1 ± 15.7 years, and 70.5% were men. Most (95%) cases were nosocomially acquired. The main clinical wards in which drug-resistant bacteria were isolated were internal medicine (57 cases, 31.1%), surgery (32, 17.5%), intensive care (24, 13.1%), pulmonology (21, 11.5%), and hematology (15, (8.2%). Of 183 patients, 143 (78.1%) were considered infected, including 36 (19.7%) with lower respiratory tract infection, 30 (16.4%) with urinary tract infection, 28 (15.3%) with bacteremia, and 22 (12%) with intraabdominal infection. A total of 42 (23%) patients died during hospitalization (Table).

Molecular Typing of an MDR *P. aeruginosa* Clone

Genotyping analysis of clinical isolates by PFGE showed that 104 isolates belonged to a single major clone (clone B), 29 belonged to a second clone (clone A), and 2 belonged to 2 clones (clones C and D). The remaining isolates, including the 6 *P. putida* isolates, showed unique PFGE patterns.

MLST analysis was performed on 9 isolates in clone B to determine their relationship to other strains that had been described. All isolates that we examined exhibited the same allelic profile (*acsA* [28], *aroE* [22], *guaA* [5], *mutL* [3], *nuoD* [3], *ppsA* [14], and *trpE* [19]), and were identified as ST175 according to the MLST database. MLST analysis of isolates in clone A had already been performed, and these isolates were classified as ST235 (*16*).

Clone B was initially isolated in February 2008 from a patient admitted to the pulmonology ward with a diagnosis of lower tract respiratory infection. Subsequently, 4 additional clone B isolates were detected in 2008, 23 in 2009, and 76 in 2010 (Figure 1). Twelve patients infected or colonized with this clone were referred to Hospital Universitario 12 de Octubre from 4 other hospitals in Madrid and from a fifth hospital in the Canary Islands. From 5 of these patients, the MDR *P. aeruginosa* isolate was recovered at the time of admission to Hospital Universitario 12 de Octubre.

Comparison of clinical characteristics for patients infected or colonized with clone B and other MDR *P. aeruginosa* clones did not show major differences, but patients infected with clone B were slightly older (mean age 67.5 vs. 61.9 years; p = 0.016) and they were more frequently admitted to the internal medicine (37.5% vs. 22.8%; p = 0.033) and pulmonology wards (16.3% vs. 5.1%; p = 0.018). Patients infected with clone B had more

Table. Characteristics of 183 patients colonized or infected with clone B or nonclone B multidrug-resistant *Pseudomonas* isolates, Spain, January 2007–December 2010*

Characteristic	Patients with clone B, n = 104	Patients with non–clone B, n = 79	p value
Mean ± SD age, y	67.5 ± 15.4	61.9 ± 15.7	0.016
Male sex	74 (71.1)	55 (69.6)	0.822
Hospital location			
Surgical ward	15 (14.4)	17 (21.5)	0.211
Intensive care unit	11 (10.6)	13 (16.4)	0.243
Internal medicine ward	39 (37.5)	18 (22.8)	0.033
Hematology ward	4 (3.8)	11 (13.9)	0.014
Pulmonology ward	17 (16.3)	4 (5.1)	0.018
Hospitalization within previous 3 mo	48 (46.1)	37 (46.8)	0.927
Surgical procedure	29 (27.9)	32 (40.5)	0.073
Transplant	4 (3.8)	8 (10.1)	0.089
Hematologic malignancy	8 (7.7)	11(13.9)	0.171
Solid tumor	21 (20.2)	13 (16.4)	0.520
Diabetes mellitus	29 (27.9)	21 (26.9)	0.845
Chronic obstructive pulmonary disease	38 (36.5)	18 (22.8)	0.046
Central catheter	83 (79.8)	59 (76.7)	0.410
Urinary catheter	60(57.7)	44(55.7)	0.441
Infections	82 (78.8)	61 (77.2)	0.791
Respiratory tract	25 (24)	11 (13.9)	0.088
Intraabdominal	7 (6.7)	15 (19)	0.012
Wound	4 (3.8)	8 (10.1)	0.089
Urinary tract	19 (18.3)	11 (13.9)	0.432
Bacteremia	12 (11.5)	16 (20.2)	0.105
Died during hospitalization	20 (19.2)	22 (27.8)	0.170

*Values are no. (%) unless otherwise indicated.

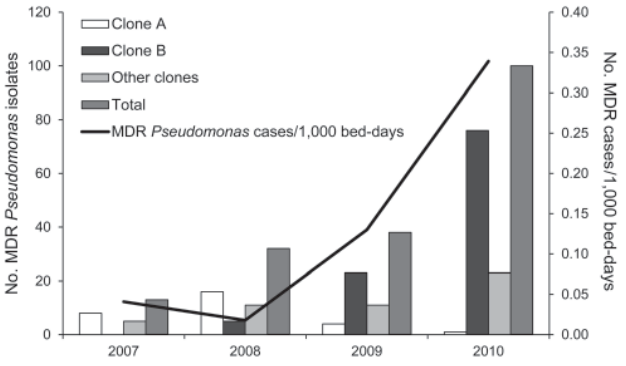

Figure 1. Annual incidence of infections/colonizations by multidrug-resistant (MDR) *Pseudomonas* spp. and temporal distribution of human cases according to clonal type, Spain. A color version of this figure is available online (www.cdcnc.gov/EID/article/18/8/11-1234-F1.htm).

frequent underlying respiratory disease such as chronic obstructive pulmonary disease (36.5% vs. 22.8%; p = 0.046) (Table) than patients infected with other clones. We also found similar differences between patients infected with clone A and those infected with other MDR *Pseudomonas* spp. clones as reported (*16*).

Antimicrobial Drug Susceptibility

Antimicrobial drug–resistance patterns of 183 *Pseudomonas* spp. isolates were CAZ (100% resistant), FEP (100%), IMP (100%), MER (100%), GEN (97.3%), TOB (96.7%), and CIP (94%), and resistance to PIP-TZ (50.3%), AMK (42.1%), and ATM (41%) was more variable (online Appendix Table, wwwnc.cdc.gov/EID/article/18/8/11-1234-TA1.htm). When break points recommended by the European Committee on Susceptibility Testing (EUCAST; www.eucast.org) were applied, the percentage of PIP-TZ–resistant and ATM-resistant isolates increased to 98.4% and 97.8%, respectively. There were no differences in MIC ranges for the other drugs tested when either CLSI or EUCAST break points were applied. All isolates were susceptible to COL.

Resistance profiles of MDR isolates in clone B were CAZ (100% resistant), FEP (100%), IMP (100%), MER (100%), CIP (100%), GEN (100%), and TOB (100%), and some isolates were also resistant to ATM (13.5%) and AMK (25%). The percentage of isolates resistant to PIP/TZ according to break points recommended by CLSI and EUCAST were 20.2% and 97.1%, respectively (online Appendix Table).

Resistance profiles of isolates in clone A were CAZ (100% resistant), FEP (100%), IMP (100%), MER (100%), CIP (96.5%), GEN (100%), TOB (89.6%), AMK (86.2%) and ATM (86.2%). As a result, this clone was classified as XDR, as were clones C and D (online Appendix Table).

Detection of MBL Genes

All isolates in clone B were positive by phenotypic methods for MBL. PCR analysis and sequencing identified MBL VIM-2 in 103 isolates and IMP-22 in 1 isolate (online Appendix Table). VIM-2 was also detected in isolates in clone C, in 4 unique clones of *P. aeruginosa*, and in 5 of 6 isolates of *P. putida*. In 1 *P. putida* isolate, VIM-1 and VIM-2 MBLs were detected. A VIM-1-type MBL was also found in 1 clone of *P. aeruginosa* (online Appendix Table). No MBL genes were detected among isolates in clone A, which demonstrated that these isolates were GES-1/GES-5 extended-spectrum β-lactamase/class A carbapenemase producers (*16*). Overall, the percentage of MBL-producing MDR or XDR *Pseudomonas* spp. isolates during the study was 63.9%. Moreover, if one considers the clone producing GES-1-GES-5, the overall prevalence of isolates producing acquired carbapenemases reaches 79.8%.

To investigate the genetic content of integrons from clone B isolates, we conducted PCR mapping of blaVIM-2 and blaIMP-22 genes for 4 strains. The structure of integrons from isolates producing VIM-2 was IntlI-VIM2-aac6'Ib-qacEΔ1 (Figure 2) in all isolates. IMP-22 was also found to be encoded in a class 1 integron, but we were unable to amplify the fragment between blaIMP-22 and qacEΔ1 by PCR, perhaps because of the large size of this DNA fragment.

Discussion

Outbreaks by MBL-producing *P. aeruginosa* have been documented in hospitals in several countries, and VIM-is the most dominant MBL variant in Spain and worldwide (*14*,*21*–*27*). We report a large outbreak of VIM-2–producing MDR *P. aeruginosa*. The predominant clone belonged to ST175, a strain that was first detected on February 2008, and it has become increasingly common in Hospital Universitario 12 de Octubre, affecting 104 patients. This MDR *P. aeruginosa* clone was found in in several wards of the hospital, although it was more frequently associated with patients in the internal medicine and pulmonology wards.

The spread of the ST175 clone should be considered an emerging pandemic. It was first identified in 2005 in the United Kingdom and Canada (http://pubmlst.org/paeruginosa) and has been reported in Hungary, the Czech Republic, Poland, Spain, the Unites States, and China (*15*,*28*–*31*). Our study also identified this clone in patients referred to Hospital Universitario 12 de Octubre from 5 other hospitals in Spain, supporting the notion that this clone has disseminated nationwide (*28*). ST175 has been associated with multidrug-resistant isolates and acquisition of different β-lactamases, mostly located on mobile elements such as integrons (*15*,*31*,*32*).

In our study, all MDR *P. aeruginosa* isolates belonging to ST175 producedVIM-2 type MBLs, except for 1 that produced IMP-22. PCR mapping showed that VIM-2 was inserted in a class 1 integron with an aminoglycoside-modifying enzyme (*aac*6′-1b). The IMP-type MBLs are most common among *Pseudomonas* spp. isolates in Asia, although they have been reported less frequently in some countries in Europe such as Italy and Austria (*33*,*34*). We report a lineage of ST175 MDR *P. aeruginosa* that produces IMP-22, which adds this MBL to the list of acquired β-lactamases associated with this epidemic clone. A thorough understanding of the genetic mechanism involved and horizontal and longitudinal dissemination is necessary, particularly for those carrying integron- and plasmid-borne MBLs, given their additional capacity for intraspecies and interspecies spread of multidrug resistance.

Although the origin of the ST175-VIM-2 MDR *P. aeruginosa* strain is unknown, this clone emerged in Hospital Universitario 12 de Octubre in February 2008, seven months after the first detection of VIM-2 type MBLs in a strain of *P. putida*. This species and other *Pseudomonas* species might play a major role as potential reservoirs for MDR determinants by enhancing their transfer to *P. aeruginosa* clones (*3*).

The ST175 clone was able to persist in Hospital Universitario 12 de Octubre for ≥34 months and has disseminated widely in spite of control measures that have been implemented, such as strict isolation of patients, active surveillance of patients at the time of entry into intensive care units, and environment investigation of possible sources of colonization. The spread of this clone among patients admitted to different sections of the hospital and high selective pressure for antimicrobial drug resistance may encourage its persistence. The design and implementation of infection control strategies in these hyperendemic situations is challenging. We recently faced a similar situation in Hospital Universitario 12 de Octubre with a large outbreak of MDR *Acinetobacter baumannii* that persisted for >30 months but that was finally controlled (*35*).

This study also detected emergence of multiple strains of *Pseudomonas* spp. that produced VIM-2- and VIM-1-type MBLs, including ≥6 *P. aeruginosa* and 6 *P. putida* clones. The polyclonal nature of MBL-based resistance might have major epidemiologic implications because sporadically isolated strains may eventually spread in the hospital environment or act as a reservoir for horizontal transfer of resistance determinants.

The emergence of MBL-producing MDR *P. aeruginosa* is a major health problem because it leaves the clinician with almost no therapeutic options for treating nosocomial infections caused by *P. aeruginosa*. Our results showed that isolates belonging to ST175 had susceptibility only to PIP/TZ (79.8%), ATM (86.5%), AK (75%), and COL (100%). The difference in PIP/TZ susceptibility depending on the break point criteria applied is remarkable. When the EUCAST susceptibility testing criteria were applied, only 2.9% isolates were susceptible to PIP/TZ compared with ≈80% when criteria recommended by CLSI were applied. A recent report concluded that in *P. aeruginosa* bacteremia caused by isolates with reduced PIP/TZ susceptibility (32/4 μg/mL or 64/4 μg/mL), empirically prescribed PIP/TZ therapy was associated with increased patient deaths (*36*). In our study, most isolates had PIP/TZ susceptibility in this range. Fortunately, the 2012 CLSI PIP/TZ break point (*37*), which was implemented during the review of this report, has been reported as 16/4 μg/mL, thus agreeing with the break point established by EUCAST. Confluence of susceptibility testing criteria among agency standards are useful for optimizing strategies to treat severe MDR *P. aeruginosa* infections.

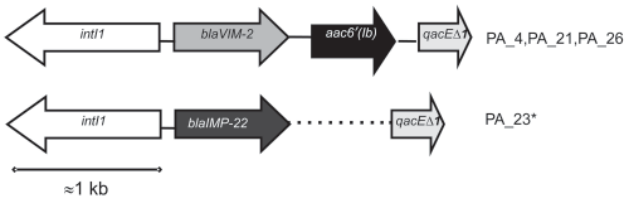

Figure 2. Structure of the class I integron detected in 4 representative isolates of the *Pseudomonas* spp. epidemic multidrug-resistant sequence type 175 clone, Spain. PA, *P. aeruginosa*. *Dotted line indicates undetermined part not amplified by PCR.

In summary, we report a large outbreak of infections caused by a VIM-2–producing ST175 MDR *P. aeruginosa* strain that was responsible for 76% of infections or colonizations by MDR *P. aeruginosa* in 2010 at Hospital Universitario 12 de Octubre, and >50% of infections or colonizations during the study period. This epidemic clone is also circulating in other hospitals in Spain and other countries. The underlying reasons for the widespread success of this clone still need to be elucidated fully, including the potential for an enhanced ability to acquire MDR determinants that facilitate persistence under conditions of antimicrobial drug selective pressure encountered in the hospital environment (*21*,*22*). Deciphering the epidemiologic and molecular aspects driving the emergence and spread of such strains is crucial to the implementation of efficient measures to control their dissemination.

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etymologia

Pseudomonas

[soo″do-mo′nəs]

From the Greek *pseudo* (“false”) + *monas* (“unit”). In 1894, German botanist Walter Migula coined the term *Pseudomonas* for a genus he described as, “Cells with polar organs of motility. Formation of spores occurs in some species, but it is rare.” Migula never clarified the etymology of the term. However, the description of *Pseudomonas* as “false unit” does not make much sense, and an alternative explanation posits that Migula “had not traced directly the Greek ancestry of the name, but had simply created the name *Pseudomonas* for the resemblance of the cells to those of the nanoflagellate *Monas* in both size and active motility.” *Monas* was coined by Danish naturist Otto Friedrich Müller in 1773 to describe a genus of “infusoria” characterized as “*vermis inconspicuous*, *simplicissimus*, *pellucidus*, *punctiformis*” (“inconspicuous worm, simple, transparent, tiny”).

Pseudomonas aeruginosa [adj. fem. of *aerūginōsus*] from Latin *aerūgō* (“copper rust or verdigris,” hence green) + *-ōsus* (added to a noun to form an adjective indicating an abundance of that noun) is named for the greenish-blue color of bacterial colonies. The organism has emerged as one of the most serious causes of nosocomial infections.

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Outbreak of Extended-Spectrum β-Lactamase–producing *Klebsiella oxytoca* Infections Associated with Contaminated Handwashing Sinks[1]

Christopher Lowe, Barbara Willey, Anna O'Shaughnessy, Wayne Lee, Ming Lum, Karen Pike, Cindy Larocque, Helen Dedier, Lorraine Dales, Christine Moore, Allison McGeer, and the Mount Sinai Hospital Infection Control Team

Klebsiella oxytoca is primarily a health care–associated pathogen acquired from environmental sources. During October 2006–March 2011, a total of 66 patients in a hospital in Toronto, Ontario, Canada, acquired class A extended-spectrum β-lactamase–producing *K. oxytoca* with 1 of 2 related pulsed-field gel electrophoresis patterns. New cases continued to occur despite reinforcement of infection control practices, prevalence screening, and contact precautions for colonized/infected patients. Cultures from handwashing sinks in the intensive care unit yielded *K. oxytoca* with identical pulsed-field gel electrophoresis patterns to cultures from the clinical cases. No infections occurred after implementation of sink cleaning 3×/day, sink drain modifications, and an antimicrobial stewardship program. In contrast, a cluster of 4 patients infected with *K. oxytoca* in a geographically distant medical ward without contaminated sinks was contained with implementation of active screening and contact precautions. Sinks should be considered potential reservoirs for clusters of infection caused by *K. oxytoca*.

Klebsiella oxytoca is an opportunistic pathogen that causes primarily hospital-acquired infections, most often involving immunocompromised patients or those requiring intensive care. Reported outbreaks have most frequently involved environmental sources (*1*–*4*). *K. oxytoca*, like other *Enterobacteriaceae*, may acquire extended-spectrum β-lactamases (ESBL) and carbapenemases (*1*,*5*); outbreaks of multidrug-resistant *K. oxytoca* infection pose an increasing risk to hospitalized patients.

We report an outbreak of infections caused by ESBL-producing *K. oxytoca* in the intensive care unit (ICU), step-down unit, and medical care unit at a hospital in Toronto, Ontario, Canada, during a 4-year period. Contributing to the ongoing difficulties in the containment of this outbreak has been the contamination of handwashing sinks in the ICU. We describe a retrospective review of all *K. oxytoca* isolates intermediate or resistant to third-generation cephalosporins identified from inpatients from April 1997 through December 2011, the investigation of the source of the *K. oxytoca* outbreak, and the interventions implemented to contain the outbreak.

Methods

The outbreak occurred at an acute tertiary-care facility in Toronto with 472 beds, including a 16 single-bed medical-surgical ICU, a 6-bed cardiac care unit , and two 4-bed step-down units. Outbreak cases of *K. oxytoca* were defined as hospital-acquired isolates with pulsed-field gel electrophoresis (PFGE) patterns belonging to 2 related clonal groups; all such isolates produced an Ambler class A ESBL. Isolates were considered hospital acquired if the first specimen (clinical culture or rectal swab) yielding resistant *K. oxytoca* was obtained ≥3 days after the admission date or if the specimen was obtained <3 days after admis-

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[1]Preliminary results of this research were presented at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, September 17, 2011, Chicago, IL, USA.

sion in a patient who had been hospitalized at the outbreak hospital within the previous 3 months. Patients were characterized as infected or colonized on the basis of National Healthcare Safety Network definitions (*6*).

Hospital infection-control guidelines provide that each patient colonized or infected with class A ESBL-producing *Enterobacteriaceae* be moved to a separate, single room with contact precautions in place. Risk factor–based admission rectal swab screening for ESBL-producing *Enterobacteriaceae* was initiated in 2004. High-risk populations are all patients admitted to an ICU and general medicine or surgical patients being transferred from an acute or long-term care facility or having a history of recent hospitalization or colonization/infection with a multidrug-resistant organism. Periodic prevalence screening is undertaken on medical and surgical wards, and potential clusters of clinical ESBL-producing isolates are investigated. ESBL colonized or infected patients are flagged in an electronic clinical system. Additional precautions are continued until weekly rectal swab specimens are negative over 4 weeks, at which point patients are placed in private rooms with ongoing periodic screening for 6 months.

Clinical specimens were processed by using conventional microbiological techniques. The VITEK 2 system (bioMérieux, Marcy l'Etoile, France) was used for identification and antimicrobial drug susceptibility testing of *K. oxytoca* isolates. Rectal screening swabs were plated on MacConkey agar with cefpodoxime (2 μg/mL). Tap water was cultured by vigorously swabbing the inside of each faucet with a cotton swab, turning the tap on and collecting 50 mL of water, vortexing the tube containing the water and the swab, centrifuging the sample twice at 3,500 × *g* for 15 minutes, and resuspending the resulting pellet of precipitated material in 3 mL of brain–heart infusion broth. Other environmental samples were obtained by inoculating premoistened cotton swabs (for dry surfaces) or by adding ≈0.25 mL of gel/liquid to 3 mL of brain–heart infusion broth. For sink cultures, cotton swabs were used to sample ≈10 cm^2 areas of the surface of the sink rim or basin. Drains were sampled by rotating swabs inserted 5–7 cm through the sink drain. Inoculated brain–heart infusion broth was incubated overnight at 37°C and then plated onto MacConkey agar with cefpodoxime. Clinical isolates intermediate or resistant to cefpodoxime (MIC ≥4 μg/mL) and colonies growing on the MacConkey agar with cefpodoxime underwent disk diffusion phenotypic confirmation (ceftriaxone, ceftazidime and aztreonam plus/minus clavulanic acid and cefoxitin) on Mueller-Hinton agar (*7*). PFGE was performed by using the restriction enzyme *Xba*I, with a run time of 20 h and switch times of 5 to 35 s at 12°C and 6 V/cm (CHEF-DR II System; Bio-Rad, Hercules, CA, USA); profiles were analyzed by using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Outbreak Description

Isolation of ESBL-producing *K. oxytoca* was uncommon in the 9 years before the outbreak; from January 1, 1997, through September 30, 2006, 10 clinical isolates (no bacteremias) and 6 colonized patients were identified. All but 1 colonized patient acquired the organism in the hospital, and 16/19 (84.2%) patients were previously or currently admitted to the ICU at the time of culture. PFGE of these isolates revealed that 5 (26.3%) isolates belonged to pattern A, 3 (15.8%) isolates belonged to pattern B, 3 isolates were closely related to each other but unrelated to isolates of pattern A or B, and 3 isolates had unique patterns. Two isolates were unavailable for typing. Only 1 case (April 2004) was identified between April 2003 and September 2006.

From October 2006 through March 2011, ESBL-producing *K. oxytoca* was isolated from 87 patients (Figure 1); 21 were not part of the outbreak. Eight of these nonoutbreak patients had isolates from clinical cultures or screening specimens obtained within 72 hours of first admission to the hospital, and each isolate had a unique pattern by PFGE. The remaining 13 had isolates first identified ≥3 days after admission (n = 11) or had been previously admitted to this hospital (n = 2), but each isolate had a unique PFGE pattern, with no temporal or geographic clustering. The remaining 66 patients were classified as outbreak case-patients.

All 66 outbreak case-patient isolates carried Ambler class A β-lactamases. Clinical *K. oxytoca* isolates were identified from 27 patients; among these, 24 patients had 25 hospital-acquired infections (9 urinary tract infections, 4 of them bacteremic; 8 asymptomatic bacteriurias; 4 soft tissue infections, 1 of them bacteremic; 3 primary bacteremias; and 1 pneumonia with bacteremia). Of the 9 bacteremias,

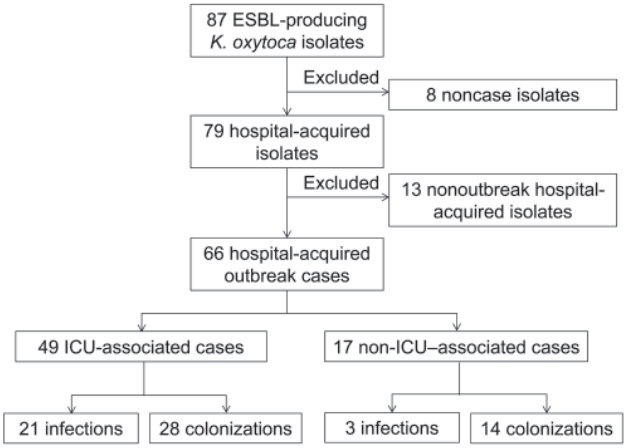

Figure 1. Flow of extended-spectrum β-lactamase (ESBL)–producing *Klebsiella oxytoca* infection and colonization in patients at a hospital in Toronto, Ontario, Canada, October 2006–March 2011. ICU, intensive care unit.

8 were PFGE pattern A. On the basis of study definitions, 3 patients had clinical specimens (2 sputum samples and 1 bronchoalveolar lavage culture) that were not associated with infection. In 11 cases, clinical cultures were preceded by identified rectal colonization; median time to first identification of a clinical isolate after recognition of colonization was 10 days (mean 12.5 days, range 1–31 days). Of the remaining 16 cases with clinical isolates of *K. oxytoca*, 13 patients had a prior negative result from an ESBL rectal screening. Thirty-nine patients were identified as colonized by rectal swab screening but no subsequent clinical isolate was identified. Patients remained colonized for variable periods of time, with the proportion colonized still positive on repeat screening as follows: 7 days (30/49, 61.2%), 14 days (20/41, 48.7%), 21 days (15/38, 39.4%), 28 days (14/33, 42.4%), 2 months (6/22, 27.3%), and 3 months (4/17, 23.5%).

Figure 2 summarizes the occurrence of outbreak-related ESBL-producing *K. oxytoca* clinical isolates over time in the ICU, where most cases occurred (49/66, 74%). Cases with clinical isolates were identified regularly from October 2006 through December 2009; however, no clinical isolates have been recovered from patients in or exposed to the ICU since that time. The 6 outbreak cases in 2010 and 4 in 2011 were identified only by rectal swab screening. The number of newly identified cases on point-prevalence screens was 13/1,049 in 2008 (1.2%), 7/1,744 (0.4%) in 2009, 6/921 (0.7%) in 2010, and 1/754 (0.1%) in 2011 ($p = 0.01$). Because prevalence screens were performed more frequently when new cases were occurring, the total number of prevalence screens decreased in 2010 and 2011 compared with 2008 and 2009.

Seventeen case-patients who had not been admitted to the ICU were identified as colonized or infected with outbreak strains. Eleven of these were part of 3 clusters. In August 2010, a total of 4 patients (3 colonized, 1 infected) from whom isolates were identified that were indistinguishable by PFGE (pattern A) acquired the strain on a single medical ward. In the medical step-down unit, 3 patients were identified as becoming colonized during June–September 2010; an additional 4 patients became colonized in February and March of 2011 (all pattern A). The remaining cases were sporadically identified on the surgical step-down unit (n = 4) and in general surgery/gastroenterology units (n = 2).

Outbreak-specif c Infection-control Interventions

At the onset of the outbreak (October 2006), the investigation encompassed a search for potential environmental sources. Samples for culture were obtained from potential reservoirs (e.g., shared equipment such as electrocardiogram and ultrasound machines, bronchoscopes and solutions used in endoscopy areas, glucometers, hand creams, lubricating gels, disinfectant swabs, blood gas machines, water baths, ice machines, mouthwashes, oral medications, and soaps), and prevalence screening was conducted to detect patient colonization in the ICU. No environmental sources were identified, no multidose vials or bags of parenteral fluids or oral medications were in use in the unit, and no procedures or exposures were identified that linked affected patients but not other patients. Two of 16 sinks had aerators, neither of which yielded *K. oxytoca* on culture. Tap water and sinks were not cultured at this time. All patients newly identified as colonized or infected had contact

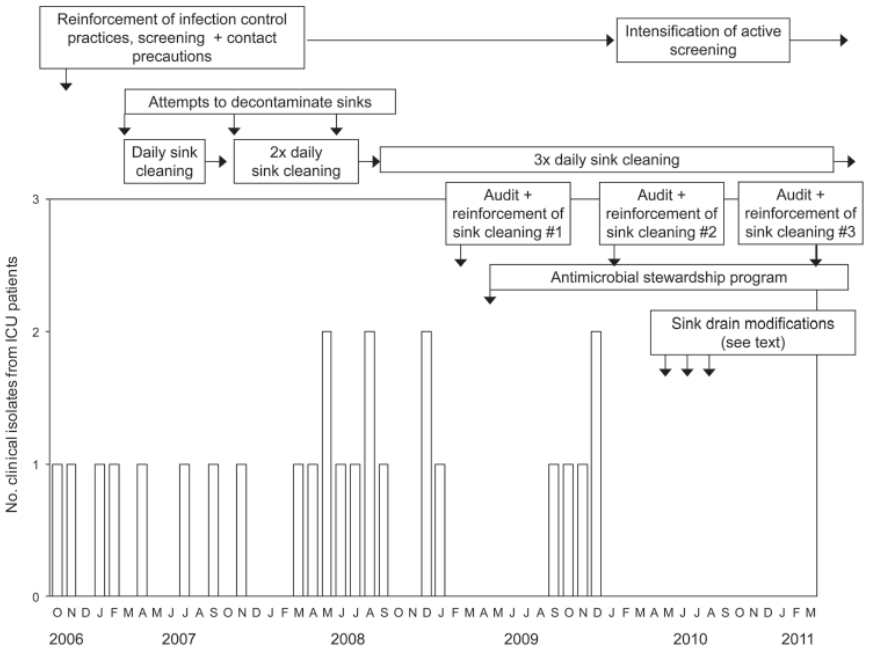

Figure 2. Nosocomial extended-spectrum β-lactamase–producing *Klebsiella oxytoca* clinical isolates from patients in the intensive care unit of a hospital in Toronto, Ontario, Canada, and the associated interventions implemented to contain the spread of the outbreak, October 2006–March 2011. ICU, intensive care unit.

precautions applied. Hand hygiene practices, adherence to contact precautions, and appropriate management of water/liquid/gels were reinforced. These interventions had no apparent effect on the rate of new clinical infections; new patients acquired the organism after weeks without identified colonized patients in the ICU.

In April 2007, results of repeat screening of potential environmental sources were negative, as were samples of tap water; however, multiple handwashing sinks were found to be contaminated with the outbreak strains. Sinks in the ICU are foot pedal–operated, free-standing, porcelain hand hygiene sinks located just inside the door to each ICU room. Although intended only for hand hygiene, they were also used for disposal of fluids, including body fluids. When sinks were identified as a potential reservoir, use of the sinks for hand hygiene only was reinforced. Attempts were made to reduce or eradicate *K. oxytoca* contamination by cleaning sinks and leaving them unused for 48 hours with disinfectant standing in traps. When this process failed, routine daily sink disinfection was initiated; sink surfaces, including taps, rims of sinks, and basins, were cleaned with a 1:16 dilution of Virox (Virox Technologies Inc., Oakville, CA, USA), and ≈250 mL of the diluted solution was poured down the drain. Neither this daily cleaning, nor month-long trials of cleaning with bleach and with a foaming hydrogen peroxide product, resulted in reduced sink colonization rates. Sink cleaning was increased to 2×/day in late 2007 and 3×/day in August 2008. Adherence to cleaning standards, particularly frequency of cleaning, was variable. Regular reminders to cleaning staff were required, and the identification of new hospital-acquired cases usually resulted in recognition that adherence had decreased.

Figure 3 shows the overall rates of recovery for patients with outbreak-related ESBL-producing *K. oxytoca* infection associated with handwashing sinks in the ICU. ICU sink culture screens were performed on 29 separate occasions, yielding a total of 910 cultures. The average rate of sink contamination during the outbreak period was 16.4% (149/910). After implementation of 3×/day cleaning/disinfection of sinks (October–December 2008), the sink colonization rate decreased to 3.9% (3/77) during the quarter; the rate increased to 16.7% (71/424) the following quarter (January–March, 2009), when adherence to routine sink cleaning was noted to have decreased.

Many of the ICU sinks had old patented opening drains (a pipe connecting the sink basin to the sink trap), a design that allowed drainage from the overflow hole to mix with the regular drainage water, potentially impairing adequate drainage. During February–June 2010, all drains were changed, eliminating the connection with the overflow drain; the overflow holes were decommissioned; the strainers in the sink basin were replaced by strainers containing a larger number of smaller holes to reduce backsplash; and sink traps were replaced.

Investigation of the medical unit on which 4 patients acquired outbreak strains during summer 2010 failed to identify an environmental source; all sink cultures were negative. After initiation of standard contact precautions for the colonized patients, no additional colonized or infected patients were identified in that unit. In contrast, the outbreak strain of ESBL-producing *K. oxytoca* was recovered from sinks, but not other environmental sources, in the medical step-down unit during August 2010. The implementation of regular sink cleaning and contact precautions for colonized patients resulted in no cases being identified during September 2010–February 2011. When new cases were identified in 2011, the previously described sink modifications were implemented in the step-down unit.

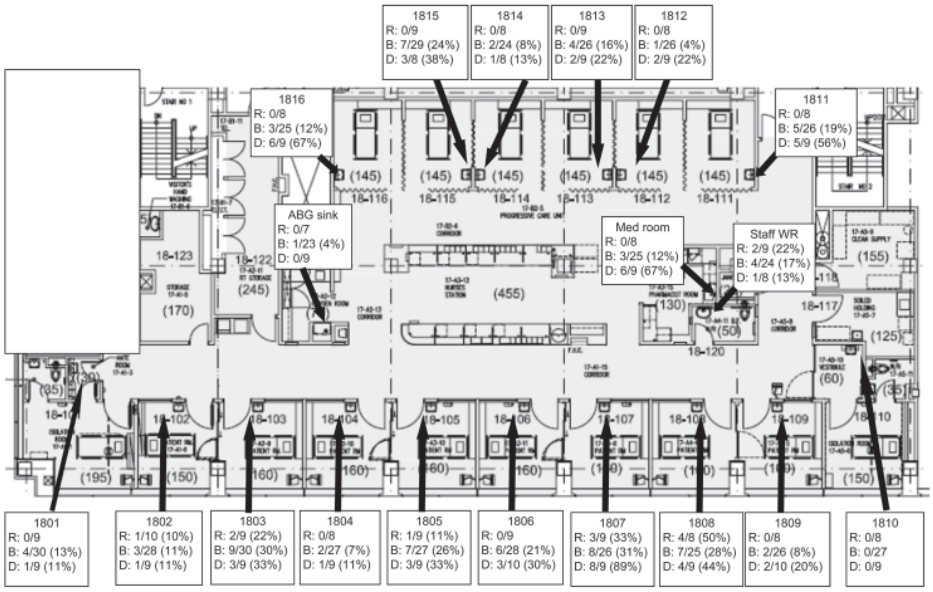

Figure 3. Locations of environmental screening for extended-spectrum β-lactamase–producing *Klebsiella oxytoca* in the intensive care unit sinks. Numbers indicate room numbers. R, sink rim; B, sink basin; D, sink drain; WR, washroom; med room, medication room (pharmacy); ABG, arterial blood gas room.

Concomitant Infection-control Interventions

As part of an ongoing program to improve adherence to hand hygiene, routine observational hand hygiene audits started throughout the hospital in fall 2008. Compliance rates improved gradually over the course of the outbreak, from 59.4% in 2008 to 69.8% in 2011.

An antimicrobial stewardship program was initiated in only the ICU in February 2009. An audit and feedback program was instituted and run by an infectious diseases physician and a pharmacist. During the first year of the program, the mean antibacterial defined daily dose per 100 patient-days decreased 9.2% compared with the same time period in the previous year (*8*).

Discussion

Outbreaks of health care–associated infection caused by *K. oxytoca* have most often been associated with contamination of environmental reservoirs such as disinfectants (*9*), multidose vials or parenteral fluid bags (*10*,*11*), humidifiers (*2*), and ventilators (*3*). This outbreak suggests that handwashing sinks in high-intensity hospital care areas may be a reservoir for *K. oxytoca* and that person-to-person transmission may also occur.

Patients in this medical-surgical ICU continued to acquire the outbreak organism despite review of routine practices, hand hygiene education and auditing, screening to identify colonized patients, and implementation of contact precautions for colonized and infected patients. In contrast, this approach seemed to control transmission in the medical ward, where contamination in sinks was not found, and has been reported to be successful in the control of other outbreaks of ESBL-producing *Enterobacteriaceae* (*12*). In this hospital, transmission of the outbreak strains of *K. oxytoca* seemed to occur both from sinks and from colonized/infected patients. As the emergence of carbapenemase-producing organisms focuses attention on health care–associated infections due to *Enterobacteriaceae,* other reservoirs may also be recognized. Recently, a clone of *K. pneumoniae* possessing SHV-1 and CTX-M-15 ESBLs was implicated in a hospital-wide foodborne outbreak in Spain, in which the hospital kitchen and colonized food handlers were the presumed reservoirs (*13*).

During the outbreak reported here, patients who acquired ESBL-producing *K. oxytoca* colonization were followed up with routine rectal swab screening; 23.5% remained colonized after 3 months. Colonization with ESBL-producing *Enterobacteriaceae* can persist for months to years (*14*,*15*); data are insufficient to determine whether duration of carriage is different for different species or clones of *Enterobacteriaceae*. In 1 study, only 6.8% of colonized patients cleared carriage over 3 years of follow-up (*14*). In addition, colonized patients may have intermittently positive rectal screening results, which suggests carriage at concentrations below the limit of detection for rectal swab specimens (*16*).

At the hospital in this study, patients to whom contact precautions are applied remain under these guidelines for 1 month and in private rooms for 6 months. However, for resistant gram-negative bacteria of epidemiologic importance (e.g., carbapenemase-producing organisms), extending the duration of contact precautions until discharge may be warranted. Because of the long duration of colonization, hospitalization is also likely to be a risk factor for community-onset infection with multidrug-resistant *K. oxytoca*, as has recently been described in Athens, Greece (*17*). The existence of asymptomatic colonized patients compounds the difficulty of containing the spread of these organisms; containing outbreaks without active surveillance may not be possible (*18*).

The outbreak-associated clones of *K. oxytoca* found in this study were ubiquitous in sinks in the ICU, cultured from 15/16 patient rooms as well as from other sinks (e.g., staff washrooms). Increased sink cleaning and auditing was associated with a decline in clinical isolates, but these measures proved difficult to sustain. Achieving persistent reductions in the degree of contamination in ICU sinks is difficult but has been a necessary intervention in outbreaks of *Pseudomonas aeruginosa* (*19*,*20*). In these outbreaks, structural changes, including renovation to sinks and plumbing or alteration of water temperature, reduced but did not eliminate the outbreak organism from sink drains. As in our experience, although the organisms could still be recovered after alterations to improve drainage and reduce splashing, these modifications were temporally associated with persistent declines in the rate of clinical infections. Persistence of *Pseudomonas* spp. in ICU sinks has been attributed to biofilm formation, which allows stable attachment to environmental surfaces and protection from disinfection (*20*,*21*). Biofilm formation has also been described for *K. oxytoca* on filtration membranes (*22*) and is probably a factor in the persistence of *K. oxytoca* in sinks in this outbreak.

This outbreak also emphasizes the challenges associated with limited space and sinks in older hospitals. Presumptively, these handwashing sinks became contaminated because they were used for the disposal of body fluids from colonized patients. While this is clearly unacceptable, nurses in the ICU are required to walk past several rooms (and out of isolation rooms) to reach the dirty utility room for disposal of body fluids, an activity that is also associated with risk. As we increasingly recognize the risks associated with hospital water and sinks, the design of ICUs becomes critical for protecting patients from these risks.

In conclusion, we describe an outbreak in which colonized sinks were a contributing reservoir for ESBL-producing class A *K. oxytoca.* A multifaceted approach including reinforcement of infection control policies (hand hygiene,

contact precautions, isolation and admission/routine rectal screening, clear delineation between handwashing sinks and sinks for other purposes), intensified cleaning of sinks, structural changes to the sinks, and antimicrobial stewardship has reduced but not eliminated transmission of the outbreak strain. Although *K. oxytoca* is in the family *Enterobacteriaceae*, its epidemiology is not clearly defined, and it may be more likely than other *Enterobacteriaceae* to be associated with environmental reservoirs in hospitals. Sinks should be considered potential reservoirs when clusters of infection caused by *K. oxytoca* are investigated.

Dr Lowe is a resident in medical microbiology at the University of Toronto. His current research interests are focused on identifying optimal methods for infection control of multidrug-resistant gram-negative organisms.

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Population Diversity among *Bordetella pertussis* Isolates, United States, 1935–2009

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Since the 1980s, pertussis notifications in the United States have been increasing. To determine the types of *Bordetella pertussis* responsible for these increases, we divided 661 *B. pertussis* isolates collected in the United States during 1935–2009 into 8 periods related to the introduction of novel vaccines or changes in vaccination schedule. *B. pertussis* diversity was highest from 1970–1990 (94%) but declined to ≈70% after 1991 and has remained constant. During 2006–2009, 81.6% of the strains encoded multilocus sequence type *prn2-ptxP3-ptxS1A-fim3B*, and 64% were multilocus variable number tandem repeat analysis type 27. US trends were consistent with those seen internationally; emergence and predominance of the *fim3B* allele was the only molecular characteristic associated with the increase in pertussis notifications. Changes in the vaccine composition and schedule were not the direct selection pressures that resulted in the allele changes present in the current *B. pertussis* population.

Pertussis, or whooping cough, is caused by the bacterium *Bordetella pertussis* and is the most frequently reported bacterial vaccine-preventable disease in the United States (*1*). Vaccination against pertussis began in the 1940s in the United States, using a whole-cell formulation (wP) that resulted in a dramatic decrease in infections and deaths (*2*). Acellular pertussis vaccines (aP) were licensed for the fourth and fifth doses of the childhood booster series in 1991 and were recommended for all 5 doses of the childhood series by 1997; in 2005, a single-dose adolescent and adult booster (tetanus-diphtheria-aP, or Tdap) was recommended (Figure 1). Despite a successful US childhood vaccination program with high coverage, the number of reported pertussis cases has increased since the early 1980s, with 27,550 cases reported in 2010 (*3*).

Before the current study, US *B. pertussis* isolates from 1935–1999 were characterized by pulsed-field gel electrophoresis (*4*), and a subset of isolates was analyzed for 2 genes, *prn* and *ptxS1* (*5*). Genetically, the *B. pertussis* population was largely homogeneous during this period, and only a few strain types caused most disease in the United States (*4*). Recently, the molecular typing methods multilocus variable number tandem repeat analysis (MLVA) and multilocus sequence typing (MLST) have been used to assess *B. pertussis* population trends in other countries (*6–9*); used together, these methods offer discriminatory power similar to that of pulsed-field gel electrophoresis (*9,10*). We used MLVA and MLST to type a large selection of *B. pertussis* isolates from the United States and examined a selection of molecular changes that occurred over time and how these changes related to increases in pertussis notifications or changes in vaccine policy.

Methods

Strain Selection

We selected 661 *B. pertussis* isolates of US origin from the Centers for Disease Control and Prevention (CDC) collection by using random sampling stratified by geography (US states and territories) and period. The strains were divided in advance as follows: period 1 (prevaccine era), 1935–1945, n = 3; period 2 (early wP era), 1946–1969, n = 16; period 3 (late wP era), 1970–1990, n = 76; period 4 (aP transition for 4th and 5th dose of childhood series), 1991–1996, n = 86; period 5 (early aP), 1997–1999, n = 159; period 6 (middle aP), 2000–2002, n = 98; period 7 (late aP), 2003–2005, n = 98; and period 8 (early Tdap booster), 2006–2009, n = 125 (Figure 1). Stratification was

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used to ensure that all states and territories with isolates in the strain bank were represented in the random sample. The geographic distribution of strains and information regarding location and year of isolation are provided in the online Technical Appendix (wwwnc.cdc.gov/EID/eid-static/spreadsheets/12-0082-Techapp.xlsx). We could not correct for the lack of representativeness of the isolates in the collection because CDC does not receive an isolate for every report of illness in the United States. After 3 days of incubation at 37°C, DNA was extracted by heat-lysis preparation from each isolate and stored at -20°C until ready for use in PCR.

MLVA

Analysis was performed by using a 6-target multiplex similar to that described (*8*) with some modifications. Using the HotStarTaq kit (QIAGEN, Valencia, CA, USA) yielded a final reaction volume of 20 µL. Master mix 1 consisted of fluorescently labeled oligonucleotides for variable number tandem repeats (VNTRs) 1 (0.13 µmol/L each primer), 5 (0.09 µmol/L each primer), and 6 (0.09 µmol/L each primer) and was supplemented with 1 mol/L betaine (Sigma-Aldrich, St. Louis, MO, USA) to facilitate primer–template interaction. Master mix 2 consisted of fluorescently labeled oligonucleotides for VNTRs 2 (0.08 µmol/L each primer), 3 (0.23 µmol/L each primer), and 4 (0.08 µmol/L each primer) and was supplemented with 10% dimethyl sulfoxide (Sigma-Aldrich). PCR was performed in single-target reactions (*6*) for some targets that were not efficiently amplified by using the multiplex assay format. Amplified products were diluted 1:50 and 1:100 and mixed with 0.5 µL MapMarker X-Rhodamine labeled 400-bp ladder (BioVentures, Murfreesboro, TN, USA). Sizes were determined by using the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA); VNTR sizes were determined by using GeneMapper version 4.0 software (Applied Biosystems). Sizing data for all strains were compared with those found for the *B. pertussis* prototype strain, Tohama I, to determine the repeat count for each locus. The assignment of an MLVA type was based on the combination of repeat counts for VNTRs 1, 3a, 3b, 4, 5, and 6 and was consistent with international nomenclature. Novel MLVA combinations were submitted to the laboratory of Frits Mooi (National Institute for Public Health and the Environment, Bilthoven, the Netherlands) for MLVA type designation.

MLST

Our algorithm consisted of 4 DNA targets: the pertactin (*prn*) gene, the first gene in the pertussis toxin operon and its respective promoter (*ptxP-ptxS1*), and the fimbrial protein-encoding gene (*fim3*). The *prn* and *fim3* genes were amplified by using oligonucleotides and conditions as described (*6,11*). The *ptxP-ptxS1* region was amplified by using oligonucleotides Ptox1Fpert (5′-CCCTC-GATTCTTCCGTACATCC-3′) and Ptox2R (5′-CGC-GATGCTTTCGTAGTACA-3′), resulting in an amplified product of 964 nt. Products were sequenced and analyzed as described (*10*).

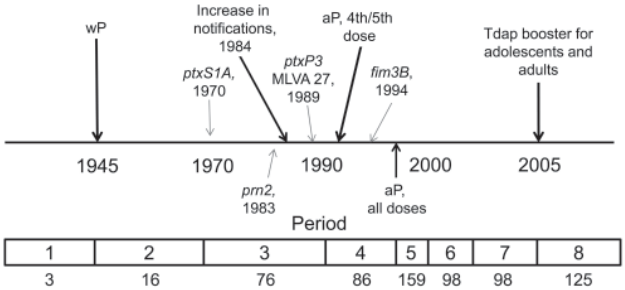

Figure 1. Timeline of pertussis vaccine introduction in the United States and appearance of alleles within the *Bordetella pertussis* population, 1935–2009. The 8 periods used in this study are indicated at bottom; numbers below indicate number of selected strains during that period (N = 661). wP, whole-cell pertussis vaccine; MLVA 27, multilocus variable number tandem repeat analysis type 27; aP, acellular pertussis vaccine; Tdap, tetanus-diphtheria-aP.

Population Analysis

Typing data among strains were compiled by using BioNumerics software version 5.01 (Applied Maths, Sint-Martins-Latem, Belgium). Minimum spanning trees (MSTs) were generated by using default settings and the Manhattan coefficient. The Simpson index of diversity (DI) and 95% CIs were calculated as described by Hunter and Gaston (*12*) and Grundmann et al. (*13*), respectively. DI was calculated by using a combination of MLVA + MLST to define types. For example, MLVA 27-*prn2-ptxP3-ptxS1A-fim3A* was considered a unique type from MLVA 27-*prn2-ptxP3-ptxS1A-fim3B*. DI is represented as $1 - D \times 100$ so that the level of diversity is proportional to the percentage. The Pearson correlation coefficient (r) was used to detect linear dependence between pertussis notifications and predominant molecular changes.

Results

Identif cation of Strains using MLVA + MLST

The prevaccine era (period 1, 1935–1945) is depicted in Figure 2, panel A, left side; all 3 strains encoded the same MLST profile, *prn1-ptxP1-ptxS1D-fim3A*. The strain identified in Figure 2, panel A, as MLVA 167 is the 10536 strain used in the manufacture of the Sanofi-Pasteur (Swiftwater, PA, USA) aP in the United States (*7*). Neither the MLVA types found (167 or 205) nor the MLST profile for

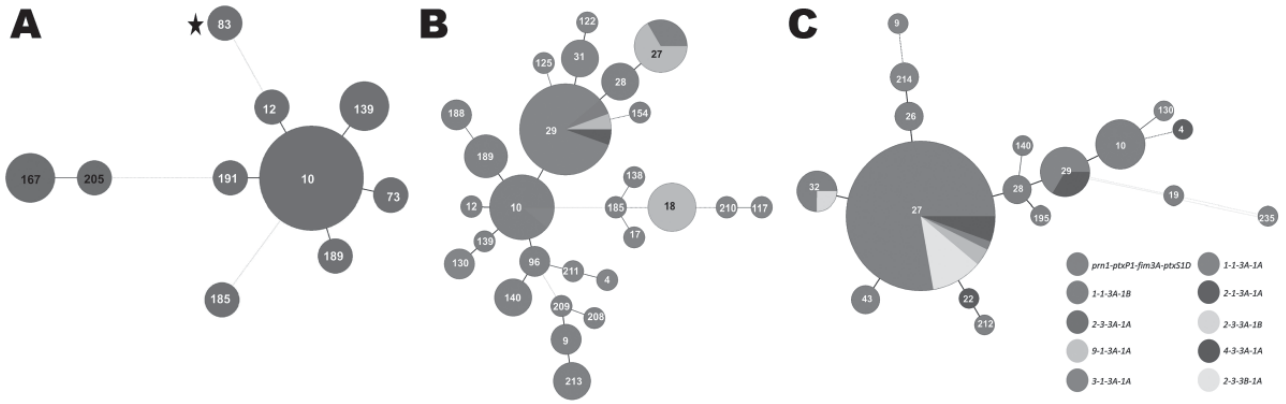

Figure 2. Minimum spanning trees depicting changes within the *Bordetella pertussis* population, United States, 1935–1996. Multilocus variable number tandem repeat analysis (MLVA) types are represented by circles and are scaled within each panel to member count; multilocus sequence typing (MLST) types are represented by shading. A) Periods 1 and 2, 1935–1945 (n = 3) and 1946–1969 (n = 16), respectively. These 2 periods were combined for the generation of the tree. Period 1 (prevaccine era) strains are shown on the left, distantly related to period 2 strains (right side) from the early whole-cell pertussis vaccine (wP) era. B) Period 3, 1970–1990, n = 76. During this period, when wP was in use, a high degree of diversity was identified; 2 predominant MLST types differed by the *ptxS1* allele. C) Period 4, 1991–1996, n = 86. During the transition from wP to acellular pertussis vaccine for the 4th and 5th dose of the childhood series, MLVA 27, *ptxP3*, and *prn2* were dominant, and the *fim3B* allele emerged. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0082-F2.htm).

period 1 are seen again in later periods. The dotted line between MLVA circles 191 and 167 in Figure 2, panel A, indicates a distant genetic relationship between the respective clusters for periods 1 and 2. The predominant MLVA type during period 2 (1946–1969), the early wP era, was 10 (Figure 2, panel A, right side), and the MLST profile shifted to *prn1-ptxP1-ptxS1B-fim3A*, identical to Tohama I (identified as MLVA circle 83 with a star), representing a single-locus change to *ptxS1B* compared with period 1. The strain used for manufacture of the GlaxoSmithKline (Research Triangle Park, NC, USA) pertussis vaccine in the United States is Tohama I.

Many MLVA and MLST types were found among the 76 strains in period 3 (1970–1990), the late wP era (Figure 2, panel B). MLVA 10 was still present from period 2, but other types also dominated, including MLVA 29. MLVA 27, the dominant type among isolates from period 8, emerged in 2 strains from Ohio and 2 from Missouri isolated in 1989. Many of the strains characterized in period 3 differed from period 2 in *ptxS1* by encoding the A allele, which was first observed in a 1970 isolate from Colorado. In addition, the first *prn2* allele was found in a 1983 isolate from Washington, DC, whereas the *ptxP3* allele was first characterized in an Ohio isolate from 1989 (Figure 1). The 1989 isolate from Ohio was the first in our random selection of US isolates that encoded the combined typing data of MLVA 27 with *prn2-ptxP3-ptxS1A-fim3A* (Figure 2, panel B). This MLST pattern was dominant for the subsequent 2 periods and represents a single-locus intermediary to the *prn2-ptxP3-ptxS1A-fim3B* MLST pattern (dominant in period 8).

The MST of 86 strains from period 4 (1991–1996) is shown in Figure 2, panel C. The aP vaccine was recommended for the fourth and fifth doses of the childhood series in 1991. During this time, MLVA 27, *ptxP3*, and *prn2* were dominant. The *fim3B* allele was first noted in an Idaho isolate from 1994. The *prn1-ptxP1-ptxS1B-fim3A* MLST type that was widely distributed throughout periods 2 and 3 was restricted to 8% of the selected strains during period 4 and corresponded with MLVA 10 (also observed in periods 2 and 3).

Period 5 (1997–1999) is depicted in Figure 3, panel A. The aP was recommended for all 5 doses of the childhood immunization series in 1997. MLVA 27 increased to 73.6% of the strains compared with 62.1% for period 4 (Figure 4). Diversity constricted to a total of 7 MLST types (10 were seen previously). The *fim3A** allele was identified in 1999 in a New Hampshire isolate and is shown within the MLVA 28 circle in Figure 3, panel A. The proportion of the population that encoded *prn2-ptxP3-ptxS1A-fim3B* (Figures 2, 3) increased from 6.9% in period 4 to 30.8% in period 5.

Figure 3, panel B, shows the MST for the mid-aP era (period 6). MLVA 27 and the *prn2-ptxP3-ptxS1A-fim3B* profile continued to dominate. However, MLVA 27 represented 76.5% of selected strains, thus reaching a plateau in frequency. Meanwhile, the *prn2-ptxP3-ptxS1A-fim3B* MLST profile increased to represent 58% of strains. The MLST type *prn1-ptxP3-ptxS1B-fim3A* that was previously found in periods 2–4 reappeared with a new MLVA type, 238.

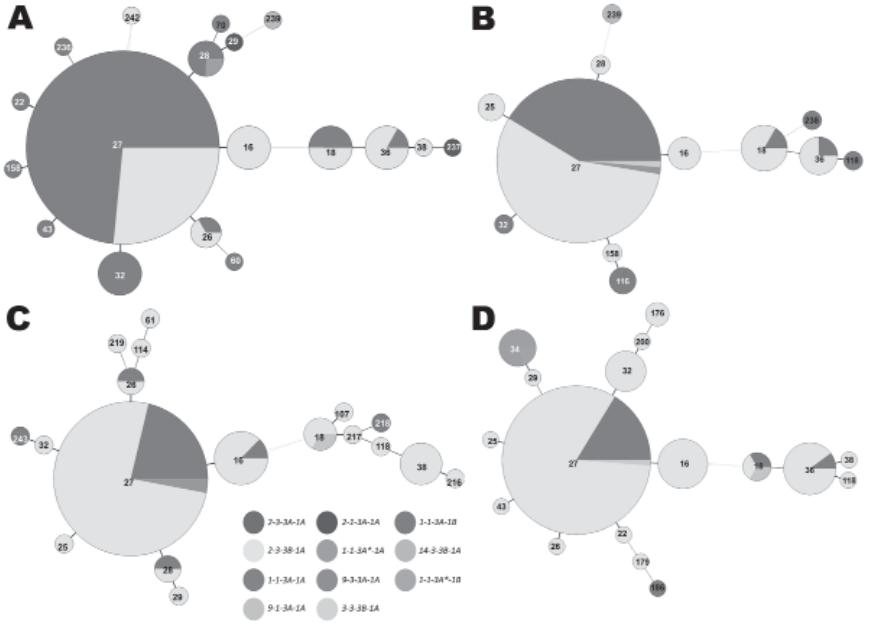

Figure 3. Minimum spanning trees depicting changes within the *Bordetella pertussis* population, United States, 1997–2009. Multilocus variable number tandem repeat analysis (MLVA) types are represented by circles and are scaled to member count within each panel; multilocus sequence typing (MLST) types are represented by shading. A) Period 5, 1997–1999, n = 159, the early years of acellular pertussis vaccine (aP) use. B) Period 6, 2000–2002, n = 98. With aP in use, MLVA 27 with the *fim3B* allele dominated. C) Period 7, 2003–2005, n = 98. In 2004, during the late aP use period, the novel pertactin allele (*prn14*) was identified in an isolate from New York. D) Period 8, 2006–2009, n = 125. After the introduction of the aP booster for adolescents and adults, MLST type *prn1-ptxP1-ptxS1B-fim3A* (previously found in periods 2–4 and 6) reappeared with a new MLVA type. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0082-F3.htm).

The late-aP era (period 7) is shown in Figure 3, panel C. During this time, a novel pertactin allele (*prn14*; GenBank accession no. HQ165753) was identified in an isolate from New York in 2004, shown in Figure 3, panel C. MLVA 27 decreased to 67.3% of the strains while the MLST profile (*prn2-ptxP3-ptxS1A-fim3B*) increased to 77.6%.

The MST for period 8 is shown in Figure 3, panel D. The Tdap booster for adolescents and adults was recommended for use in 2005. MLVA 27 frequency remained approximately the same as for period 7, 64%. The MLST profile *prn2-ptxP3-ptxS1A-fim3B* increased to 81.6% of the strains. Meanwhile, the *prn1* (n = 6), *ptxP1* (n = 6), and *ptxS1B* (n = 2) alleles reemerged; these alleles are also encoded by vaccine strain Tohama I. The last time these alleles were seen in multiple strains was in period 4, with 17 strains encoding *prn1*, 28 strains encoding *ptxP1*, and 8 strains encoding *ptxS1B*.

Trends in Typing Data during 74 Years of US History

DI values and 95% CIs are provided in the Table. During periods 1 and 2, the DI was in the mid to upper 60% range, but the CIs were large due to a low sample size. Period 3 (1970–1990) had a DI of 94.0% with a small CI that was distinct from previous time periods. To determine if the length of the time interval (20 years) was biasing the results, period 3 was subdivided into 5- and 10-year intervals; all DI values remained ≈90% with small 95% CIs. DI decreased to 75.7% in period 4 and remained relatively constant following the introduction of aP.

The frequencies of individual MLST alleles and MLVA types over time are shown in Figure 5. Changes in *ptxS1* occurred first with the transition of *ptxS1B* to *ptxS1A* beginning in the 1970s (first observed in a 1970 isolate from Colorado). Later, changes within *prn*, *ptxP*, and MLVA 27 occurred at approximately the same time, with transitions

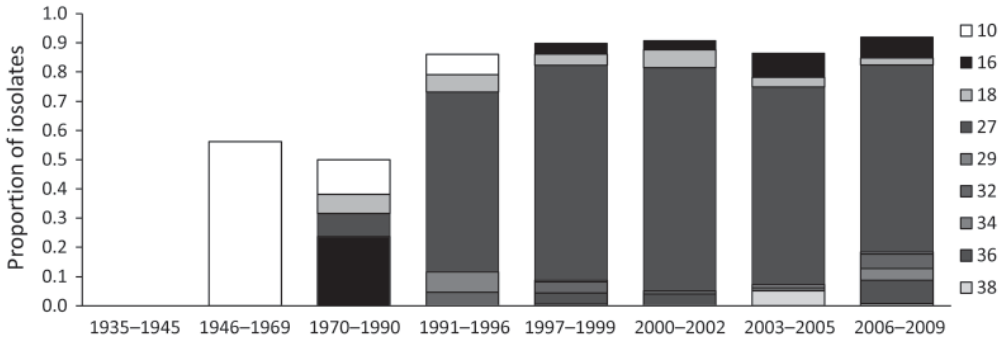

Figure 4. Frequency (by proportion of all isolates tested) of predominant multilocus variable number tandem repeat analysis (MLVA) types within the *Bordetella pertussis* population, United States, 1935–2009. MLVA 10 was dominant in period 2 (1946–1969) but decreased through periods 3 (1970–1990) and 4 (1991–1996) while MLVA 18, 27, and 29 emerged. MLVA 27 increased in proportion during period 4 and dominated the population for the rest of the study period; however, the proportion of MLVA 27 has been decreasing since period 6 (2000–2002), allowing for the emergence of other types. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0082-F4.htm).

Table. Diversity trends among selected *Bordetella pertussis* isolates, United States, 1935–2009*

Study period	Years	Vaccine use	Simpson diversity index, % (95% CI)
1	1935–1945	Pre-wP	66.7 (30.4–103.0)
2	1946–1969	Early-wP	77.8 (59.4–96.2)
3	1970–1990	Late-wP	94.0 (90.9–97.1)
4	1991–1996	wP/aP transition (4th–5th doses)	75.7 (65.9–85.5)
5	1997–1999	Early-aP	66.8 (59.5–74.1)
6	2000–2002	Mid-aP	70.2 (67.7–81.1)
7	2003–2005	Late-aP	71.6 (62.4–80.8)
8	2006–2009	Tdap	69.3 (60.7–77.9)

*wP, whole cell vaccine; aP, acellular vaccine; Tdap, tetanus-diphtheria-aP.

to MLVA 27, *prn2*, and finally *ptxP3*. In 1995, *ptxS1A* was encoded by 100% of tested strains, and in 1996, >90% of strains tested encoded *prn2* or *ptxP3*, but more recently, frequency of *prn2* and *ptxP3* has declined (period 8). The transition within *fim3* observed in the early 2000s was a more gradual increase that did not approach 100% as with the other MLST alleles.

Comparison of Typing Data and Increase in US Pertussis Notif cations

We aligned annual US pertussis notifications with vaccine coverage data, the trend lines for each of the dominant MLST alleles, MLVA 27, and DIs (Figure 6). An inverse relationship was observed between DI and notifications as well as vaccine coverage. Increases among *ptxS1A*, *prn2*, *ptxP3*, and MLVA 27 were not significantly correlated with the increase in notifications, whereas the proportion of *fim3B*-encoding strains was significantly correlated with pertussis notifications (r = 0.8608; p = 0.0277).

Discussion

Changes in the US *B. pertussis* population have followed trends that are largely consistent with other nations and yet were unique in the correlation of annual case counts with *fim3B*. Our findings regarding DI trends were similar to those found for the United Kingdom (*9*), but unlike a previous study that examined *B. pertussis* in the Netherlands (*14*), we did not find a correlation between the *ptxP3* allele and annual reporting of pertussis cases in the United States.

MLVA + MLST analysis showed that the US *B. pertussis* population changed genetically during the period covered by our study (Figures 2, 3). We found a high degree of diversity in period 3 (1970s and 1980s), with a DI of 94%, that was consistent with findings (84%) for a similar period in the United Kingdom (*7*). The results from the United Kingdom were attributed to a decline in vaccine coverage rates below 80% during 1975–1989, with a low of 31% in 1978 (*7*). Parent-supplied data from the US Immunization Survey for 1962–1985 showed US coverage among 2-year-old children for ≥3 doses of wP declined from a peak of 77.9% in 1967 to a low of 63.6% in 1985; vaccination rates then increased dramatically, to ≥94% by 1994, and have remained high ever since (Figure 6) (*15*).

Given that *B. pertussis* has no nonhuman hosts or environmental niche, vaccine-mediated immunity is the most likely selective pressure against *B. pertussis*. Therefore, vaccination coverage may have contributed to the increase in diversity during period 3. This hypothesis also supports the correlation between the decline in diversity as the rate of vaccination coverage (≥3 doses) increased to ≈95% in the mid-1990s. The emergence and subsequent dominance of MLVA 27 and the MLST alleles *prn2* and *ptxP3* (in that order, temporally) in the United States occurred during the period with the highest diversity, period 3.The timing and order of these transitions are consistent with global trends (*16*) in which the emergence of nonvaccine-type alleles for *ptxS1* and *prn* appeared 15–30 years after the introduction of pertussis vaccines. Despite increasing pertussis incidence in the United States, diversity has remained stable for *B. pertussis* during the past 20 years; a similar trend was observed in the Netherlands (*6*).

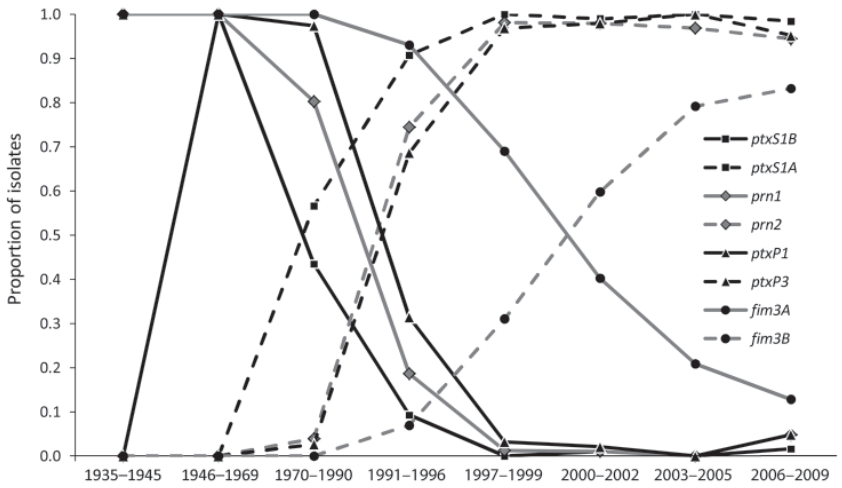

Figure 5. Transitions of frequency (by proportion of all isolates tested) of dominant alleles for each multilocus sequence typing (MLST) type target within the *Bordetella pertussis* population, United States, 1935–2009. The previous dominant type is denoted by a solid line, with the new dominant type denoted by a dashed line of the same style. The dashed lines of *prn2* and *ptxP3* overlap with each other and multilocus variable number tandem repeat analysis (MLVA) type 27 (Figure 6), which suggests they arose at approximately the same time and resulted in the new dominant MLVA + MLST profile. The transition from *fim3A* to *fim3B* occurred much later than the other transitions. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0082-F5.htm).

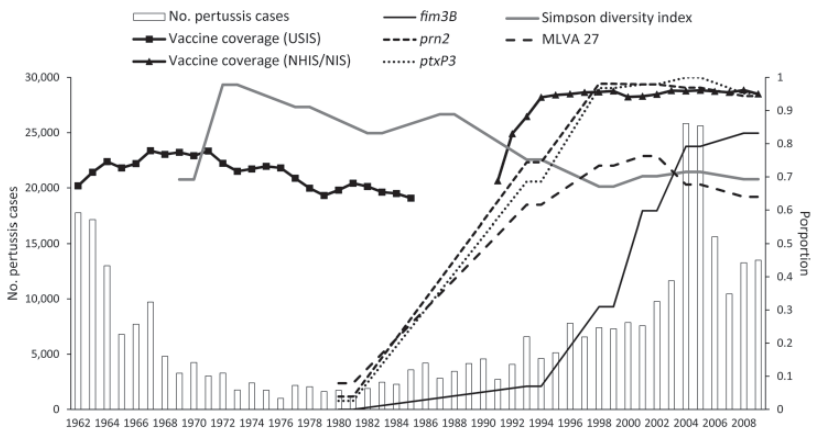

Figure 6. Comparison of number of pertussis notifications, proportion of vaccine coverage, and proportion of dominant multilocus sequence typing alleles and multilocus variable number tandem repeat analysis (MLVA) type 27 among a random selection of 661 isolates, United States, 1935–2009. Bars indicate case notifications; lines indicate 2-point moving average distributions of frequency for the time periods assigned in this study. Vaccine coverage data were collected for the United States Immunization Survey (USIS, 1962–1985), National Health Interview Survey (NHIS, 1991-1993), and National Immunization Survey (NIS, 1994-2009). No data are available for 1986-1990 because USIS was cancelled (*15*). The *fim3B* trend line was temporally and significantly associated with the rate of increase for pertussis notifications. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0082-F6.htm).

Regional clustering of *B. pertussis* MLVA types may be best exemplified by comparing the United States to Australia. Kurniawan et al. found that a single MLVA type, 70, emerged in Australia's wP era and dominated during the aP transition (reaching ≈40% of isolates), then declined once the transition to aP was complete (*8*). A similar rise and fall of MLVA 70 surrounded the transition to aP in the United Kingdom, where MLVA 70 disappeared after 2004 (*7*). However, to our knowledge, MLVA 70 has only been detected once in the United States, in a Missouri isolate from 1997. Further, MLVA 64, which represents ≈10% of the *B. pertussis* population in Australia, was not detected in the United States. Our findings, combined with the findings from the United Kingdom, do not support the conclusion that the introduction of aP was the driving factor responsible for the emergence and dominance of MLVA 70 or 64. On the contrary, the data suggest that aP helped to eliminate MLVA 70. Whole-genome sequencing has implicated regional bottlenecks as a likely contributor to the geographic restriction of particular MLVA types (*17*,*18*), which may explain the exclusive prevalence of MLVA 70 in Australia (*8*). Furthermore, the timing of the emergence and dominance of the MLVA 27 and MLST alleles *prn1*, *ptxS1A*, and *ptxP3* in the United States predate the completion of the wP to aP transition (1997) by ≈10 years (Figures 1–3, 5, 6). The emergence and dominance of the *fim3B* allele (Figure 6) probably coincides with the increase in notifications.

Many of the allele changes we found have been identified in clinical isolates of *B. pertussis* throughout the world. However, any conclusions involving individual gene loci in clinical isolates are vulnerable to phenotypic results that arise from mutations elsewhere in the genome. The clearest way to identify the effect of allele mutations is to examine them alone and in combination in a genetically controlled bacterial background (*19*). Therefore, it is premature to associate allele changes with a phenotype, disease severity, or events of epidemiologic importance until they are functionally analyzed individually and cumulatively in a model system. Data related to the functional or clinical effects of allele changes for the MLST targets used in this study are limited.

Recently, an increase in pertussis notifications and a 1.41-fold increase in hospitalizations were correlated with the increasing presence of the *ptxP3* allele in circulating *B. pertussis* isolates (*20*). Unfortunately, the *ptxP* allele was not characterized for *B. pertussis* among hospitalized patients, so a direct correlation could not be made between *ptxP3* and disease severity. In addition, ELISA was used to demonstrate a modest increase (1.6-fold) in pertussis toxin production among *ptxP3* strains relative to *ptxP1*-encoding strains when grown in vitro. In vivo experimentation using genetically controlled *B. pertussis* mutants for *ptxP3* is needed to determine whether a 1.6-fold increase is sufficient to cause more severe disease or to overwhelm vaccine-mediated anti–pertussis toxin antibody response. Bart et al. hypothesized that *ptxP3* may be a "hitchhiker" mutation that benefited from advantageous mutations selected elsewhere in the genome (*18*); our findings lend support to this hypothesis.

More information is known about the effects of the *prn* and *ptxS1* allele changes, but studies assessing the *fim3* locus are lacking. The divergence among the pertactin alleles is proximal to the encoded RGD motif that is involved in eukaryotic cell binding and antigen presentation to B cells (*21*). In theory, such mutations could biochemically affect protein folding, host cell binding, or recognition by B cells (*14*); this hypothesis is supported by the finding that the pertactin variants 1–3 induce type-specific antibodies (*22*). However, the effects of these insertions/deletions on

the pertactin protein product have not been determined experimentally. The *ptxS1A* allele encodes 3 amino acid changes relative to the 10536 vaccine type, *ptxS1D*, and 1 amino acid change compared with *ptxS1B* for Tohama I–based vaccine (*23*). The pertussis toxin remains biologically functional despite these changes (*24*). In vivo, mouse-derived anti–pertussis toxin antibodies tolerate numerous amino acid substitutions in *ptxS1* with equal neutralization between wild-type and mutant isolates (*25*). Therefore, *ptxS1* allele changes may not be clinically or immunologically relevant.

Little is known about the functional or in vivo effects of the *fim3B* mutation on protein function, bacterial survival, and adherence. The *fim3B* allele results in an alanine-to-glutamic acid mutation at aa 87 (*11*). Biochemically, this is a potentially important residue change, and the mutation is located in a surface epitope of *fim3* (aa 79–91) that has been shown to interact with human serum (*26*). Given the significant correlation between the increases in *fim3B* and US pertussis case notifications, the effect of the *fim3* mutation needs to be functionally and clinically determined. Alternatively, this could be another example of a regional bottleneck (*17,18*); additional data regarding the prevalence of *fim3B* in other countries is needed to rule out this possibility. Moreover, the strain collection available for this study may not be fully representative of the *B. pertussis* population in the United States over time. Efforts were made to ensure that the strains selected for this study were diverse in year of isolation as well as geography within the United States. According to Mouillot (*27*), DI can be influenced by selection bias and sample size, but almost all studies evaluating a historical collection of strains encounter this limitation, including the recent study in the United Kingdom (*7*).

In summary, the US *B. pertussis* population has evolved in the time since vaccinations were introduced in the 1940s (Figures 2–5). Our findings demonstrate that the resurgence of pertussis in the United States was not correlated with the *ptxP3* allele but with the presence of the *fim3B* allele among the *B. pertussis* population. The commonly circulating strains of *B. pertussis* in the United States encode different alleles compared with the strains used for manufacture of the pertussis vaccines, but the relevance of these allele changes remains to be fully elucidated. Because *B. pertussis* has no nonhuman host, the selective pressures it encounters are limited to the human immune system and the vaccine, but the influence of this selection pressure versus natural evolution on the modern US *B. pertussis* population is unclear. For example, minor types are beginning to emerge, including the reemergence of vaccine-type alleles. In addition, the vaccine policies of other nations may have contributed to the makeup of the US *B. pertussis* population in ways that could not be measured by using this study of US-based isolates.

As vaccine coverage rates improve among adolescents and adults, changes in the *B. pertussis* population should be monitored through molecular typing. The in vivo effects of MLST allele changes in a genetically controlled model for pathogen and host should be characterized to determine what effects, if any, these allele changes have with respect to vaccine-mediated immunity to circulating *B. pertussis*. More specific studies, such as genomic sequencing of particular strains and genetic expression of the multiple alleles in animal models, should be performed to determine the virulence and pathogenesis of these variants.

Acknowledgments

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Dr Schmidtke is a microbiologist in the PulseNet Next Generation Subtyping Methods Unit at CDC. Her primary research interest is development and adaptation of molecular typing assays to better equip state and local public health laboratories for foodborne disease surveillance.

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Solid Organ Transplant–associated Lymphocytic Choriomeningitis, United States, 2011

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Three clusters of organ transplant–associated lymphocytic choriomeningitis virus (LCMV) transmissions have been identified in the United States; 9 of 10 recipients died. In February 2011, we identified a fourth cluster of organ transplant–associated LCMV infections. Diabetic ketoacidosis developed in the organ donor in December 2010; she died with generalized brain edema after a short hospitalization. Both kidneys, liver, and lung were transplanted to 4 recipients; in all 4, severe posttransplant illness developed; 2 recipients died. Through multiple diagnostic methods, we identified LCMV infection in all persons, including in at least 1 sample from the donor and 4 recipients by reverse transcription PCR, and sequences of a 396-bp fragment of the large segment of the virus from all 5 persons were identical. In this cluster, all recipients developed severe illness, but 2 survived. LCMV infection should be considered as a possible cause of severe posttransplant illness.

Lymphocytic choriomeningitis virus (LCMV), an Old World arenavirus, family *Arenaviridae*, is a zoonotic virus maintained in the house mouse (*Mus musculus*) and can be carried by pet and laboratory rodents (*1*–*7*); human exposure occurs through aerosolized excreta or by direct rodent contact. Infection in immunocompetent humans most commonly results in nonspecific febrile illness, although aseptic meningitis develops in a subset of persons (*8*). Person-to-person transmission of LCMV is unusual and has been reported only through vertical transmission from a pregnant woman to her fetus and through solid organ transplantation. In both instances, infections are associated severe disease. For instance, congenital infection can result in birth defects, including hydrocephalus and chorioretinitis (*9*–*12*), and transplant recipient infection can result in multisystem organ failure. Three previous clusters of organ transplant–transmitted LCMV infections have been identified in the United States, affecting 10 organ recipients, 9 of whom died (*13*,*14*).

In February 2011, the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) was notified of a cluster of severe illnesses (2 fatal, and 2 in persons who were recovering) among 4 organ recipients linked to 1 donor, who died in late December 2010. Postmortem evaluation of the donor showed only evidence of previous Epstein-Barr virus infection. CDC acquired multiple specimens from the donor and recipients for testing. Histopathologic findings showed multifocal hepatocellular necrosis (Figure 1) in the lung transplant recipient, and Old World arenavirus antigens subsequently were identified by immunohistochemical testing (IHC). Reverse transcription PCR (RT-PCR) and sequencing indicated LCMV infection. Subsequent testing of specimens from the donor and recipients confirmed LCMV infection in all 5 persons, marking the fourth detected cluster of transplant-associated LCMV transmissions in the United States. We describe the laboratory investigation and clinical outcomes of this recent cluster of transplant-transmitted LCMV infections (Table 1).

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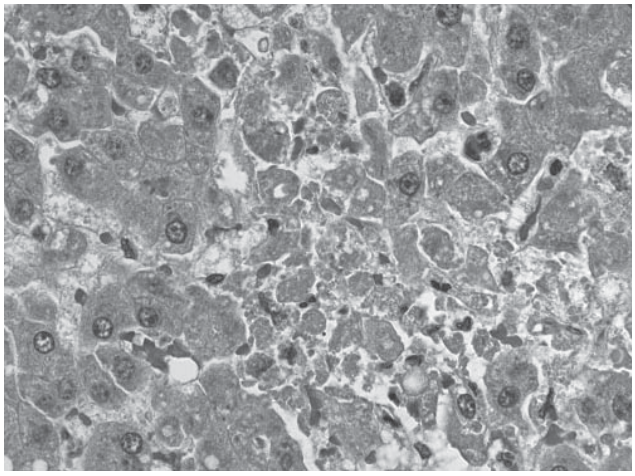

Figure 1. Liver from a 62-year-old woman (lung transplant patient) showing acute necrosis of hepatocytes and minimal inflammation. Randomly distributed single-cell necrosis, as observed in this patient, is a histopathologic feature observed in lymphocytic choriomeningitis virus infection. Original magnification ×400.

Methods

Medical teams involved in clinical care consulted with CDC. All available samples from the donor and the 4 recipients were then sent to CDC for diagnostic investigation.

IHC

Tissue specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm sections. IHCs that used an immunoalkaline phosphatase technique (Fisc13) were performed on tissue sections. The primary monoclonal antibody (81001–52-BG12, Viral Special Pathogens Branch, CDC) reacts with the GP2 epitope of Lassa virus but also will react with other Old World arenaviruses, including LCMV. Appropriate positive and negative controls were run in parallel.

RT-PCR

Total RNA was extracted from clinical specimens by using Tripure (Roche, Indianapolis, IN, USA) or from fixed tissue, as described (*15*). Because of the high genetic variability of LCMV (*16*), we used generic Old World arenavirus primers to amplify a 396-nt fragment from the large (L) segment (*17*). Negative samples underwent a second round of amplification with the same primers. The complete small (S) segment was amplified by previously described 19C primers (*18*). Resulting amplicons were purified and sequenced (GenBank accession nos. JN687949 [S segment] and JN687950 [L fragment]; because amplicon sequences from clinical samples were identical, a single sequence is provided for each amplicon). BLAST nucleotide analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to verify the presence of LCMV.

Serologic Testing

Postmortem serum from the donor, pretransplantation and/or posttransplantation serum from the recipients, and cerebrospinal fluid (CSF) collected from the right kidney recipient were sent to CDC. LCMV-specific IgM capture and IgG ELISA were performed as described (*19*).

Results

Clinical and Epidemiologic Investigations

Organ Donor

The organ donor, a 13-year-old girl with type 1 diabetes mellitus, was seen at an emergency department in December 2010 with a 2-day history of nausea and vomiting. At admission, her leukocyte count was elevated (19.1×10^3 cells/μL, with 28% band forms and 56% segmented neutrophils (reference $3.54–9.07 \times 10^3$ cells/μL, 0–5% bands, 40%–70% neutrophils]). Diabetic ketoacidosis was diagnosed and was managed with an insulin drip and aggressive fluid resuscitation. She reported a severe headache, which was not relieved by morphine. She vomited, aspirated, and required intubation and was noted to have muscle spasms and jerking before intubation. No lumbar puncture was performed. She became hypothermic and hypotensive requiring vasopressors; cerebral edema and coma developed, and the patient underwent emergency craniectomy, which was unsuccessful in preventing herniation. She was declared brain dead on day 2 of hospitalization. The family consented to organ donation, and her organs were procured the following day. Findings

Table 1. Case information, major clinical findings, and outcome of an organ donor and 4 transplant recipients, United States, 2011*

Patient	Age, y	Organ received	Major clinical findings	Outcome
Donor	13	NA	Diabetic ketoacidosis, hypothermia, hypotension, nausea, vomiting, cerebral edema, possible meningitis	Died
Left kidney recipient	53	Left kidney	Urinary leak, pelvic abscess, acute respiratory distress syndrome, respiratory failure, mild hepatitis, possible acute myocardial infarction, possible encephalitis	Died
Right kidney recipient	46	Right kidney	Encephalitis, pancytopenia	Survived
Liver recipient	62	Liver	Hepatitis, encephalopathy, urinary tract infection, atrial fibrillation	Survived
Lung recipient	60	Lung	Pneumonia, respiratory failure, pulmonary infarction, atrial fibrillation, hepatitis	Died

*All were female. NA, not applicable.

from autopsy indicated generalized brain edema; however, no evidence of an infectious or inflammatory process in the central nervous system (CNS) was noted. The left and right kidneys, liver, right lung, and corneas were procured and subsequently transplanted to 4 organ recipients and 1 cornea recipient (only 1 cornea was transplanted).

During the retrospective public health investigation, the girl's residence was visited in April 2011. Family members reported that she had been sleeping in a recently built extension to the house and recalled rodent infestation in the extension when she became ill in December 2010. The family did not report having pet rodents or any other possible rodent exposures for the organ donor.

Left Kidney Recipient

This patient was a 52-year-old woman who underwent transplantation for end-stage renal disease caused by hypertension and diabetes mellitus. Her immunosuppressive regimen consisted of mycophenolate mofetil and tacrolimus. One week after transplantation, fever, nausea, vomiting, and diarrhea developed, and a urinary tract infection (UTI) was diagnosed. The fever was attributed to a pelvic abscess, which was drained, and a urinary leak was repaired. Three weeks after transplant, acute onset of severe headache and fever developed; acute respiratory distress syndrome also had developed, and the woman was intubated. Computed tomographic scan of the brain was unremarkable. Elevated cardiac enzymes and electrocardiographic changes also developed, as did anemia, thrombocytopenia (platelets 72 × 10^9/L [reference 175–415 × 10^9/L]), and mildly elevated transaminases (alanine aminotransferase [ALT] 74 U/L [reference 7–41 U/L]; aspartate aminotransferase [AST] 173 U/L [reference 12–38 U/L]). Her neurologic function was poor when sedation was reduced, but no lumbar puncture was performed because she had intermittent positional ventricular tachycardia and was too unstable for magnetic resonance imaging. Electroencephalography demonstrated background slowing. Supportive care was withdrawn, and the patient died 30 days after transplantation. No autopsy was performed.

Right Kidney Recipient

This patient was a 46-year-old woman who underwent transplantation for end-stage renal disease resulting from polycystic kidney disease. She received induction therapy with antithymocyte globulin and received maintenance immunosuppression with mycophenolate mofetil, tacrolimus, and prednisone; mycophenolate mofetil was later discontinued. Two days after transplantation, fever, which persisted during the following week; myalgia; severe headache; nausea; and vomiting developed. Lumbar puncture was performed 25 days after transplant; CSF contained elevated protein level (95 mg/dL [reference 15–50 mg/dL]), low glucose level (45 mg/dL [reference 10–70 mg/dL]), and increased leukocytes (188 cells/μL [reference 0 cells/μL]; 65% lymphocytes, 30% monocytes, and 5% segmented neutrophils). CSF cultures and PCR for herpes simplex virus and varicella zoster virus were negative. Aseptic meningitis was diagnosed, and the patient was treated with intravenous acyclovir, but no definitive cause of illness was identified. She was discharged 30 days after transplant but was readmitted 2 days later because of altered mental status; she had a 2-day history of nausea, vomiting, anorexia, and severe headache. CSF demonstrated elevated protein level (95 mg/dL), low glucose level (45 mg/dL), increased leukocytes (188 cells/μL; 65% lymphocytes, 30% monocytes, and 5% segmented neutrophils). Magnetic resonance imaging showed scattered multifocal signal abnormalities throughout the brain parenchyma. Pancytopenia developed, but extensive investigation for an infectious agent was unrevealing. She was treated again for possible herpes simplex encephalitis with intravenous acyclovir followed by oral valacyclovir, transferred to a rehabilitation facility 11 days later, and was discharged home the following week.

Liver Recipient

This patient was a 60-year-old woman who underwent transplantation for end-stage liver disease caused by alcoholic cirrhosis. She initially received an immunosuppressive regimen of mycophenolate mofetil, prograf, and prednisone; mycophenolate mofetil was later discontinued. Her postoperative course was complicated by UTI, postoperative encephalopathy, and elevated transaminases; liver biopsy showed no evidence of rejection. She was transferred to a rehabilitation facility but was readmitted 20 days after transplant with rapid atrial fibrillation and altered mental status. Hepatic transaminases were elevated (maximum ALT 366 U/L, maximum AST 564 U/L); liver biopsy 24 days after transplant demonstrated marked macrovesicular and microvesicular steatosis (>90%) and no evidence of rejection. Rapid atrial fibrillation was managed medically; pleural effusion developed, requiring thoracentesis. She had recurrent UTI, for which she received broad-spectrum antimicrobial drugs, and had *Clostridium difficile* colitis, which also was treated. She gradually recovered in a rehabilitation facility; liver enzymes normalized; and she was discharged home 66 days after transplant.

Lung Recipient

This patient was a 62-year-old woman who underwent lung transplantation for end-stage chronic obstructive pulmonary disease. She received 2 doses of basilixumab 0 and 4 days after transplant and a maintenance

immunosuppressive regimen of mycophenolate mofetil, tacrolimus, and prednisone taper. She was extubated <1 day after transplant; however, rapid atrial fibrillation developed and was managed medically. She was discharged home after 1 week. Three days later, chills, dyspnea, fatigue, abdominal pain, nausea, and vomiting developed, and the patient's oxygen requirement increased. She was readmitted 14 days after transplant; leukocyte count was 16,600 cells/μL; computed tomographic scan of the chest demonstrated pneumonia; and she was treated with broad-spectrum antimicrobial and antifungal drugs. Bronchoalveolar lavage culture grew *Candida albicans*. The patient was intubated 3 days after admission, after which fever, hypotension requiring vasopressors, and hepatitis (maximum ALT 744 U/L; maximum AST 1,133 U/L) developed. Asystole developed, and she died 6 days after being readmitted. Autopsy revealed ischemic cardiovascular disease, acute pulmonary infarction and necrosis of the right lower lobe, bilateral pulmonary edema and effusion, and extensive congestion of the liver.

Cornea Recipient

The cornea recipient was examined, as part of a follow-up visit, ≈4 months after transplant. She reported no illness after transplantation.

Laboratory Investigation

We attempted to test all available samples remaining from the organ donor and recipients; for the liver recipient and the cornea recipient, we also were able to acquire follow-up serum samples. Diagnostic testing for LCMV was performed by using a combination of RT-PCR, serologic testing, and IHC (Table 2).

Organ Donor

Serum, lymph node, and spleen were tested by RT-PCR for LCMV. The lymph node yielded a positive RT-PCR result when partial L segment primers were used. Sequencing of the RT-PCR–amplified fragment confirmed LCMV and identified a sequence different from any previously sequenced LCMV isolates in our laboratory. IgM capture and IgG ELISA on archived postmortem serum were negative for LCMV-specific antibodies. The nontransplanted cornea was also tested by IHC and RT-PCR; no evidence of viral antigen or RNA was detected in the cornea.

Left Kidney Recipient

Serum collected 11 days before transplant and tested for serologic evidence of previous LCMV infection was negative for LCMV-specific IgM and IgG, indicating that this patient had not been previously infected with LCMV; in addition, RT-PCR on this specimen was negative. Posttransplant specimens were limited to 1 bronchoalveolar lavage specimen collected 22 days after transplant. RT-PCR using L segment and full-length S segment primers gave positive results. Sequencing of both RT-PCR products indicated LCMV, and L segment product yielded a sequence identical to that from the organ donor. Phylogenetic analysis of the S segment indicated that the virus belonged to lineage I of LCMV (*16*).

Table 2. Summary of laboratory test results for an organ donor and 4 transplant recipients, United States, 2011*

Patient	Time of sample collection	Specimen type	RT-PCR result	Serology	IHC
Organ donor	Postmortem	Serum	Neg	IgM neg, IgG neg	NA
	Postmortem	Autopsy tissues	Pos†	NA	Neg‡
	Postmortem	Cornea	Neg	NA	Neg
Left kidney recipient	11 d before transplant	Serum	Neg	IgM neg, IgG neg	NA
	22 d after transplant	Bronchoalveolar lavage	Pos	IgM neg, IgG neg	NA
Right kidney recipient	14 d before transplant	Serum	Neg	IgM neg, IgG neg	NA
	11 d after transplant	Bone marrow	Neg§	NA	Neg§
	32 d after transplant	CSF	Pos	IgM neg, IgG neg	NA
Liver recipient	7 d before transplant	Serum	ND	IgM neg, IgG neg	NA
	Before transplant	Liver	Neg	NA	Neg
	9 d after transplant	Liver	Pos	NA	Pos
	24 d after transplant	Serum	Pos¶	IgM pos (1,600), IgG neg	NA
	25 d after transplant	Liver	Pos	NA	Pos
	37 d after transplant	Liver	Pos	NA	Pos
	51 d after transplant	Serum	Neg	IgM pos (≥6,400), IgG neg	NA
	98 d after transplant	Serum	Neg	IgM pos (≥6,400), IgG neg	NA
Lung recipient	20 d after transplant	Autopsy tissues	Pos#	NA	Pos**
Cornea recipient	4 mo after transplant	Serum	ND	IgM neg, IgG neg	NA

*RT-PCR, reverse transcription PCR; IHC, immunohistochemical testing; neg, negative; NA, not applicable; pos, positive; CSF, cerebrospinal fluid; ND, not done.
†Lymph node positive; spleen negative.
‡Central nervous system, spinal cord, trachea, lung, gastrointestinal tract, spleen, and mesenteric lymph node negative.
§Bone marrow aspirate and biopsy specimens negative.
¶Virus was isolated from this specimen.
#Lung and liver positive.
**Bladder, pancreas, right lung, left lung, stomach, spleen, gall bladder, right adrenal gland, kidney, and liver positive; left ventricle negative.

Right Kidney Recipient

Serum collected 14 days before transplant tested negative for LCMV-specific IgM and IgG, indicating that this patient was not previously infected with LCMV; in addition, RT-PCR testing on this specimen was negative. CSF collected 32 days after transplant was positive for LMCV RNA by RT-PCR by using L segment and full-length S segment primers; sequencing of both products confirmed LCMV, and the L segment sequence was identical to that from the organ donor. The CSF specimen tested negative for LCMV-specific IgM and IgG.

Liver Recipient

Liver biopsy specimens were acquired from the explanted native liver and the transplanted liver on days 9, 25, and 37 after transplant. The native liver tested negative for evidence of LCMV infection by RT-PCR, whereas all 3 posttransplant specimens yielded positive RT-PCR results (partial L segment primers); sequencing indicated a sequence identical to that from the organ donor. LCMV antigens also were detected by IHC in all 3 posttransplant liver biopsies (Figure 2) and absent in the native liver. Serum samples were acquired 7 days before transplant and 24, 51, and 98 days after transplant. The pretransplant serum sample was negative for LCMV-specific IgM and IgG, whereas posttransplant serum had LCMV-specific IgM (titers of 1,600, ≥6,400, and ≥6,400, respectively). No LCMV-specific IgG was detected in any of the serum samples. RT-PCR was performed on all serum samples; a positive result was obtained only for the serum collected 24 days after transplant, indicating that the virus was cleared from peripheral blood by day 51 after transplant. LCMV was additionally isolated from serum collected 24 days after transplant.

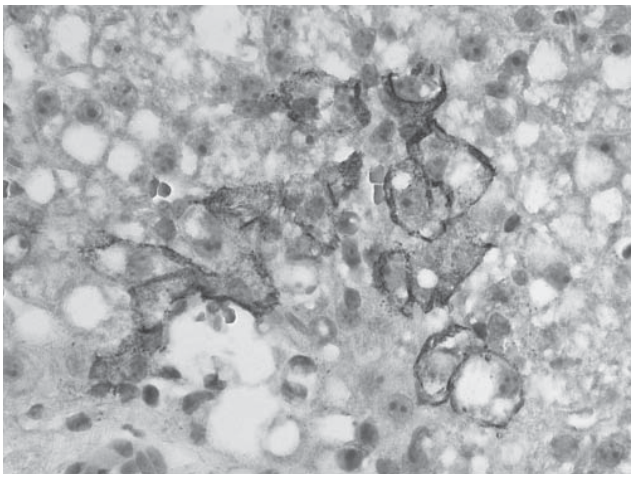

Figure 2. Immunohistochemical staining of lymphocytic choriomeningitis virus antigens in a biopsy specimen of the transplanted liver from a 60-year-old woman, which demonstrates abundant and predominantly perimembranous staining of hepatocytes. Original magnification ×200.

Lung Recipient

Lung and liver specimens collected at autopsy were RT-PCR positive by using partial L segment primers; sequencing of the amplified fragment indicated a sequence identical to that from the organ donor. Immunohistochemical evidence of LCMV infection was identified in numerous autopsy tissues, including bladder, pancreas, right lung, left lung, stomach, spleen, gall bladder, right adrenal gland, kidney, and liver.

Cornea Recipient

A serum sample collected ≈4 months after transplant from the cornea recipient tested negative for IgM and IgG. This finding indicated no evidence of previous LCMV infection.

Discussion

We investigated a cluster of LCMV infections associated with organ transplantation. We found identical viral sequences in a 396-bp fragment of the L segment in clinical samples from the organ donor and all 4 organ recipients. Evidence of LCMV infection by IHC in tissues from 2 of the organ recipients supported this finding. Additionally, serum samples collected shortly before organ transplantation from 3 of the 4 recipients indicated absence of previous LCMV infection. Serologic testing of archived postmortem serum from the donor was negative for LCMV-specific antibodies, implying a relatively acute infection.

This event is the fourth cluster of organ transplant–transmitted LCMV infections identified in the United States and the fifth such transmission event reported worldwide (*13*,*14*,*20*). The previous 4 instances involved a total of 13 infected organ transplant recipients, 12 of whom died. Ribavirin is an antiviral drug with demonstrated efficacy for improving clinical outcomes for patients with Lassa virus (a closely related Old World arenavirus) infection (*21*). Although not definitely shown to have a therapeutic role in treating LCMV in humans, the 1 person who previously survived organ transplant–associated LCMV was treated with ribavirin (*13*). In this current cluster, 2 of the 4 infected recipients survived, without receiving therapy targeted at LCMV infection.

In immunocompetent persons, LCMV infection can cause CNS disease, such as aseptic meningitis. In previously documented LCMV infections associated with organ transplantation, a variety of signs and symptoms, including multisystem organ failure, developed in transplant recipients (*13*,*14*). Similarly, a diverse set of signs and symptoms were noted among recipients in this cluster, including evidence of meningoencephalitis in 3 recipients and the donor. Both persons who died had multisystem organ involvement: the left kidney recipient had acute

respiratory distress syndrome and possible hepatic, cardiac, and CNS involvement, and the lung recipient had prominent pulmonary involvement and hepatitis. Severe pulmonary involvement was previously reported in lung recipients with transplant-transmitted LCMV (*13*). Finally, although LCMV transmission to caregivers has not been shown, the use of universal precautions for care of transplant recipients with suspected LCMV infection might be warranted, given the potential for high viral titers in body fluids of infected transplant recipients.

Multiple instances of rabies virus transmission associated with cornea transplantation have occurred (*22–24*), which provide at least some precedence for transmission of viruses through cornea transplantation. Fischer et al. (*13*) reported the absence of clinical evidence of infection in 2 recipients of corneas procured from the organ donor involved in the 2005 organ transplant–transmitted LCMV outbreak; however, no samples were available to definitively determine the absence of infection. The availability of a preserved, nontransplanted donor cornea, and follow-up serum from the recipient of the donor's other cornea, enabled us to investigate the potential for LCMV transmission through cornea transplantation. We found no evidence of LCMV in the donor cornea, and serologic evaluation indicated absence of LCMV infection in the cornea recipient. Collectively these data suggest that the potential for LCMV transmission through cornea transmission is small, although we cannot conclusively exclude the theoretical transmission of LCMV through cornea transplantation.

The absence of LCMV-specific IgG in the samples from the liver transplant recipient (including a sample 98 days after transplant) is noteworthy. These observations are consistent with those for the only previous surviving patient, who remained negative for LCMV-specific IgG 85 days after transplant (*13*). Although the availability and timing of clinical sample collection from persons with fatal outcomes from this and previous transplant clusters vary widely, no LCMV-specific IgG has been identified in any patients with fatal transplant-associated LCMV infections.

Although organ donation screening procedures reduce the risk for transmission of some bloodborne viruses, such as HIV, hepatitis B virus, and hepatitis C virus, through the donor history questionnaire and laboratory screening (*25*,*26*), screening for all possible acute viral infections from donor evaluation to organ procurement is not possible. Other notable instances of transplant-transmitted viral encephalitis from West Nile virus and rabies virus have occurred recently (*27–29*). Similarly, LCMV is not among the infectious agents routinely screened for in potential organ donors. However, for the cluster reported here, archived postmortem donor serum was negative for LCMV by RT-PCR and serologic testing, and thus testing serum before organ transplantation would not have helped recognize donor infection. Asking about exposure to rodents also might be helpful in heightening suspicion for LCMV infection in potential donors who have signs of aseptic meningitis. Although family members described clear evidence of rodent exposure for the organ donor shortly before onset of illness, this information was acquired during follow-up investigations, which occurred long after LCMV infection in the organ recipients. Current Organ Procurement and Transplantation Network guidelines require assessment of the donor's medical history and behavior, including a review of the donor's medical records (optn.transplant.hrsa.gov/policiesAndBylaws/policies.asp) before transplantation. Although recent rodent exposure by a potential organ donor would not exclude transplantation, the information might help transplant centers appropriately assess risk to the potential donor, heighten their suspicion for transplant-transmitted LCMV in the event of recipient illness, and obtain early diagnosis and treatment. The efficacy of ribavirin for treating LCMV infection in humans has not been examined; however, early detection of LCMV infection and treatment with ribavirin might improve the outcomes of transplant recipients with transplant-transmitted LCMV infection.

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Paragonimus kellicotti Fluke Infections in Missouri, USA

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Learning Objectives

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

- Analyze the epidemiology and microbiology of paragonimiasis
- Assess the clinical presentation of paragonimiasis
- Evaluate patterns of management of paragonimiasis
- Distinguish abnormal ancillary studies among patients with paragonimiasis

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Paragonimiasis is an infection caused by lung flukes of the genus *Paragonimus*. In Asia, *P. westermani* infections are relatively common because of dietary practices. However, in North America, cases of paragonimiasis, which are caused by *P. kellicotti* flukes, are rare. Only 7 autochthonous cases of paragonimiasis were reported during 1968–2008. In 2009, we reported 3 new case-patients with paragonimiasis who had been seen at our medical center over an 18-month period. Six additional case-patients were identified in St. Louis, Missouri, USA, and treated at Washington University–affiliated health centers in 2009–2010. We report detailed descriptions of these case-patients, which includes unusual clinical manifestations. We also describe public health interventions that were undertaken to inform the general public and physicians about the disease and its mode of transmission.

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Paragonimiasis is an infection caused by lung flukes of the genus *Paragonimus*. As many as 9 species of *Paragonimus* are responsible for human infections worldwide (*1*). Human paragonimiasis is common in Asia, where diets often include raw, cured, pickled, or salted crustaceans (*2,3*). In contrast, consumption of uncooked crustaceans is uncommon in North America.

In North America, paragonimiasis is caused by *Paragonimus kellicotti* flukes (*4*). *Paragonimus* spp. lung flukes have a complex life cycle, requiring snail and crustacean intermediate hosts. Definitive hosts excrete eggs in feces or sputum, which hatch in water to become ciliated miracidia. The miracidia invade soft tissue of snails where they reproduce asexually. Cercariae released from snails invade the secondary intermediate host, a crustacean. Secondary intermediate hosts for *P. kellicotti* flukes are crayfish in the genera *Cambarus* and *Orconectes*. Mammals acquire the infection when they ingest raw or undercooked crustaceans (*5*). *P. kellicotti* fluke infections have been found in cats, dogs, bobcats (*6*), raccoons (*7*), foxes (*8,9*), skunks (*9*), minks (*9,10*), and coyotes (*9*). Human infections are uncommon; only 7 cases were reported during 1968–2008 (*2,11–18*)

In 2009, we reported a cluster of 3 patients who had probable or proven paragonimiasis caused by *P. kellicotti* flukes and who were seen at a single tertiary-care center over an 18-month period (*19*). We report an additional 6 patients seen at Washington University Medical Center, St. Louis, Missouri, and at an affiliated Veterans Administration hospital over 14 months (September 2009–October 2010). The purpose of this report is to emphasize that *P. kellicotti* flukes are an emerging pathogen in Missouri, to highlight unusual clinical features observed in these patients, to educate the public in hopes of preventing new cases, and to increase awareness among the medical community to promote early diagnosis and treatment.

Patients, Materials, and Methods

Patients with proven or probable *P. kellicotti* fluke infection seen at Washington University School of Medicine and an affiliated Veterans Administration Hospital during September 2009–October 2010 were identified at time of clinical encounter. Patient characteristics, case histories, and laboratory values were obtained from medical records by infectious disease physicians. Immunoblot tests were performed at the Centers for Disease Control and Prevention (Atlanta, GA, USA), commercial laboratories, or Washington University School of Medicine as described in the Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-0335-Techapp.pdf).

Results

Clinical Features

Patient characteristics for the combined series of 9 patients are summarized in Tables 1 and 2, and detailed case descriptions for the 6 new patients are provided in the online Technical Appendix. The patients included in this series were predominantly male (88.9%), and all but 1 were adults. Patients consumed raw crayfish while on float (recreational river) trips (7/9, 77.8%), camping (1/9, 11.1%), or as a demonstration of wilderness survival skills (1/9, 11.1%). Alcohol consumption at the time of crayfish consumption was common (7/9, 77.8%). Although there were differences in timing of seeking care and signs and symptoms, patients in this series frequently had cough (100%), fever (88.9%), and eosinophilia (100%).

Table 1. Characteristics of 9 patients infected with *Paragonimus kellicotti* flukes, Missouri, USA, September 2009–October 2010*

Patient no.	Age, y/sex	Location	Incubation period, wk	Signs and symptoms	Time to diagnosis, wk	Method of diagnosis	Reference
1	31/M	Jacks Fork and Current Rivers	2	Fever, pharyngitis, cough, dyspnea, eosinophilia	3	Clinical history	(*19*)
2	26/F	Meramec River	2	Fatigue, cough, fever, eosinophilia	12	Serologic analysis	(*19*)
3	32/M	Current River	3	Fever, malaise, cough, headache, eosinophilia	12	Serologic analysis	(*19*)
4	28/M	Huzzah River	8	Fever, myalgia, malaise, cough, weight loss, eosinophilia	12	Clinical history	NA
5	10/M	Current River	16	Fever, myalgia, malaise, cough, chest pain, weight loss, eosinophilia	3	Clinical history	NA
6	20/M	Jacks Fork River	12	Fever, night sweats, malaise, cough, dyspnea, chest pain, weight loss, eosinophilia	36	Serologic analysis	NA
7	22/M	Jacks Fork River	6	Fever, night sweats, cough, dyspnea, chest pain, weight loss, eosinophilia	40	Serologic analysis, sputum ova and parasite examination	NA
8	30/M	Jacks Fork River	2	Fever, night sweats, malaise, cough, dyspnea, chest pain, weight loss, eosinophilia	16	Serologic analysis	NA
9	43/M	Missouri River	12	Cough, dyspnea, chest pain, weight loss, eosinophilia	83	Serologic analysis	NA

*Patients 4–9 were not previously reported. NA, not applicable.

Table 2. Clinical and laboratory findings for 9 patients infected with *Paragonimus kellicotti* flukes, Missouri, USA, September 2009–October 2010*

Characteristic	Value
Age, y, median (range)	28 (10–43)
Male sex	8 (88.9)
Alcohol consumption	7 (77.8)
Incubation period, wk, median (range)	4 (2–12)
Duration of symptoms before examination, wk, median (range)	2 (2–8)
Signs and symptoms	
Fever	8 (88.9)
Cough	9 (100.0)
Chest pain	6 (66.7)
Dyspnea	4 (44.4)
Night sweats	5 (55.6)
Malaise	5 (55.6)
Abdominal pain	2 (22.2)
Weight loss	7 (77.8)
Laboratory findings	
Eosinophils/mm^3 at first examination, mean (range)	1,626 (800–3,600)
% Eosinophils at first examination, mean (range)	15 (6–30)
Positive paragonimus immunoblot result†	5 (71.4)
Positive sputum ova and parasite test result†	1 (14.2)
Radiographic findings	
Pleural effusion	9 (100.0)
Nodule	4 (44.4)
Pericardial effusion	4 (44.4)

*Values are no. (%) unless otherwise indicated.
†n = 7.

Paragonimiasis can be difficult to diagnose in its early stages because of the nonspecific nature of initial symptoms. In some regions, paragonimiasis may be mistakenly diagnosed as tuberculosis. In this series of patients, initial diagnoses included pneumonia, bronchitis, influenza, gastroenteritis, acute cholecystitis, and pulmonary embolism. The median time between crayfish ingestion and the onset of clinical signs and symptoms was 4 weeks (range 2–12 weeks). The median interval between the onset of symptoms and the initial visit to health care facilities was 2 weeks (range 2–8 weeks). However, the median time from symptom onset to the correct diagnosis was 12 weeks (range 3–83 weeks). Before diagnosis of paragonimiasis, patients received multiple unnecessary medications and treatments, and these were sometimes associated with serious illness. All patients were treated with antimicrobial drugs.

Clostridium difficile infection developed in 1 patient after multiple courses of antimicrobial drug therapy. Six (67%) patients were treated with ≥l course of corticosteroids. One patient also underwent multiple thoracentesis procedures, and 1 of these procedures resulted in pneumothorax that required chest tube replacement. This patient also underwent decortication because of recurrent pleural effusions. One patient underwent laparoscopic cholecystectomy after having right upper quadrant pain. This finding may have been related to parasite migration across the diaphragm because the gallbladder did not show any pathologic changes.

Laboratory Test Results

Patients with paragonimiasis often have abnormal laboratory test results that are useful for making a diagnosis. Eosinophilia has been reported in 62%–66% of patients with infection caused by *P. westermani* flukes (*20,21*) and in 75% of patients with paragonimiasis in North America (*19*). All patients in this series had eosinophilia at initial examination (absolute eosinophil count range 600 cells/mm^3–2,300 cells/mm^3, % range 5.6%–21%). Pleural fluid analysis showed eosinophilia in 3 patients. Chest radiographic findings were abnormal for all patients with paragonimiasis in North America (*19*). Pleural effusions were present in 37% of paragonimiasis patients in Asia and in 60% of previously described patients in North America (*19,22*). All patients in this series had pleural effusions. Other chest radiographic findings included nodules, opacities, and infiltrates. Chest computed tomography scans showed pleural thickening, pericardial thickening, pericardial effusions, and worm nodules (*23*).

Four of 6 patients in the current series had pericardial effusions documented by either computed tomography or echocardiography. Although most pericardial effusions were small and did not cause hemodynamic compromise, 1 patient had cardiac tamponade that required emergency pericardiocentesis and drain placement. Analysis of pericardial fluid showed marked eosinophilia. Eosinophilic pericardial effusions were documented in 3 children with paragonimiasis caused by *P. mexicanus* flukes in Costa Rica (*24,25*). Pericardial effusion has also been reported for 1 patient with paragonimiasis in Asia (*26*). Pericardial effusions have not been reported for patients with *P. kellicotti* flukes infection, although various *Paragonimus* spp. flukes have been reported to invade soft tissue (*19,20,27,28*) and the central nervous system (*19,29*).

Serologic analysis can be useful for confirming a diagnosis of paragonimiasis. However, available serologic tests have limitations. An immunoblot for *P. westermani* flukes performed at the Centers for Disease Control and Prevention (Atlanta, GA, USA) has been reported to be highly sensitive (96%) and specific (99%) (*30*). However, this assay has not been validated for *P. kellicotti* flukes. In our series, 2 patients had negative immunoblot results at the Centers for Disease Control and Prevention for samples that had been positive by Western blot with *P. kellicotti* fluke antigen at Washington University (G.J. Weil, et al., unpub. data). These patients had symptoms and abnormal laboratory test results suggestive of paragonimiasis after ingestion of raw crayfish, and their symptoms resolved after therapy with praziquantel. Diagnosis by identification of ova in sputum specimens is specific, but has low sensitivity

(30%–40%) (*1*). Ova were present in sputum from only 1 patient in our series (*5*). Examination of stool for ova has low sensitivity (11%–15%) (*31,32*).

Response to Therapy

Praziquantel (75 mg/kg in 3 divided doses for 2 days) is the treatment of choice for paragonimiasis in the United States (*33*). Cure rates of 71%–75%, 86%–100%, and 100% have been reported with 1-, 2-, and 3-day courses, respectively (*1,34*). All patients in this series were treated with praziquantel for 2–3 days, and 7 (77.8%) experienced rapid clinical improvement or cure after treatment. One patient had some residual dyspnea and chest tightness 4 weeks after treatment. These findings may have been related to the protracted time between onset of his symptoms and initiation of appropriate therapy. He was asymptomatic at the 6-month follow-up visit. One atypical patient with chronic paragonimiasis who also had preexisting chronic obstructive pulmonary disease did not notice much improvement in his chronic dyspnea after praziquantel treatment, but defervescence and a weight gain of 30 pounds represented a clear clinical response to therapy.

Public Health Interventions

Control of this organism in the wild is not feasible because of the wide geographic distribution of crayfish and mammalian intermediate hosts that eat crayfish and serve as definitive hosts for the parasite. *P. kellicotti* flukes are highly prevalent among crayfish in rivers that are used for recreation in Missouri (*5*). Effective prevention strategies should focus on physician education to improve awareness of this disease and education targeted at the general population. We worked with public health officials to help improve awareness of this disease in physicians and in the general public. For example, we assisted the Missouri Department of Health and Senior Services in creating a health advisory (www.health.mo.gov/emergencies/ert/alertsadvisories/pdf/HAd4-30-10.pdf) for physicians in Missouri with the goal of educating physicians on the risk factors, clinical signs and symptoms, and treatment for this infection. In September 2009, we collaborated with the Missouri Department of Health and Senior Services and the Missouri Department of Natural Resources to create a warning poster (www.health.mo.gov/living/environment/fishadvisory/pdf/crayfish.pdf) that was posted at canoe rental facilities and campgrounds along rivers in Missouri. This poster warned the general public about the risk for consuming raw crayfish.

In addition, during the spring of 2010, four of the authors (M.A.L., L.M.D., T.C.B., G.J.W.) provided information to local and national print, radio, and television media to increase awareness of this infection. Three cases were identified after this media campaign. One patient sought care at our medical facility after his mother, a nurse, saw an article about paragonimiasis in her local newspaper. One patient was referred to our clinic by a friend who had seen a report on paragonimiasis on a local television station. Another patient had atypical features, but increased physician awareness helped to establish the diagnosis in this patient.

Discussion

Although only a small number of cases of human paragonimiasis have been described in the medical literature since 1984, we have seen 9 patients with this disease in St. Louis since 2006. Five other patients with this disease in Missouri have been reported to the Missouri Department of Health and Senior Services since 2009 (P. Lo, pers. comm.). *P. kellicotti* flukes are believed to be widely distributed throughout the North America. In addition, outdoor activities such as camping and float trips when combined with alcohol consumption are not uniquely confined to Missouri. It is likely that there are case-patients in other regions who have not been given a diagnosis or treated. Although most patients reported to date have been adults, this series shows that children are also at risk for infection if they ingest uncooked crayfish.

As this patient series demonstrates, delayed diagnosis can lead to unnecessary medical treatments and procedures that can cause serious illness. Clinicians should consider the diagnosis of paragonimiasis in all patients with cough, fever, and pleural effusion with peripheral eosinophilia. We are developing a new antibody assay that may help clinicians identify and treat patients with this infection. Additional efforts to raise awareness of this parasite among physicians will potentially help appropriately identify and treat currently infected persons. These efforts should also target the general public to warn them of the dangers of consuming raw crayfish.

Dr Lane is an assistant professor of medicine at Washington University School of Medicine in St. Louis. His research interests are clinical outcomes, patient safety, and quality improvement.

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Hepatitis E Virus Genotype 3 in Wild Rats, United States

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The role of rodents in the epidemiology of zoonotic hepatitis E virus (HEV) infection has been a subject of considerable debate. Seroprevalence studies suggest widespread HEV infection in commensal *Rattus* spp. rats, but experimental transmission has been largely unsuccessful and recovery of zoonotic genotype 3 HEV RNA from wild *Rattus* spp. rats has never been confirmed. We surveyed *R. rattus* and *R. norvegicus* rats from across the United States and several international populations by using a hemi-nested reverse transcription PCR approach. We isolated HEV RNA in liver tissues from 35 of 446 rats examined. All but 1 of these isolates was relegated to the zoonotic HEV genotype 3, and the remaining sequence represented the recently discovered rat genotype from the United States and Germany. HEV-positive rats were detected in urban and remote localities. Genetic analyses suggest all HEV genotype 3 isolates obtained from wild *Rattus* spp. rats were closely related.

Hepatitis E virus (HEV) is a major cause of acute hepatitis in developing countries, in which outbreaks arise most often through fecal contamination of drinking water or after flooding (*1*). Major outbreaks have been reported in India, Southeast Asia, Africa, and Mexico, and mortality rates are considerable (20%–30%) among pregnant women (*1*). In industrialized countries, HEV infections are reported sporadically and contamination of drinking water is an unlikely source, but cases are increasing as diagnostic tests are being performed more frequently (*2*). Moreover, zoonotic transmission of HEV through consumption of undercooked pork and deer meat has been confirmed (*3,4*), and detection of HEV in many mammalian hosts suggests the potential for multiple zoonotic sources of HEV infection in industrialized countries (*5*).

There are currently at least 4 genotypes of HEV known to infect humans. Genotypes 1 and 2 have been identified only from humans and are responsible for most outbreaks in developing countries (*6*). Genotypes 3 and 4 are believed to be involved in zoonotic transmission and have been isolated from swine (domesticated pig and wild boar), deer, mongoose, rabbits, cattle, and humans (*5*). Additional strains not known to infect humans have also been identified in rats and chickens, and the genetic diversity of HEV is only beginning to be understood.

Within the United States, HEV infections have been identified in travelers who have visited developing countries (*7*), and for several at-risk groups in the United States (i.e., swine veterinarians and farmers), the high number of reported seropositive persons is caused by swine–human contact (*8,9*). However, seroepidemiologic examinations of blood banks in the United States and other industrialized countries have shown high proportions of samples positive for antibodies against HEV (excluding persons who had traveled to HEV-endemic countries), but this finding was true in urban areas in which swine–human contact is absent (*8,10,11*).

HEV RNA has been detected in livers from commercially raised pigs (*12*) and represents an additional potential reservoir of infection. However, consumption of raw pork and wild game is uncommon in the United States, although it is a common practice in other industrialized nations in which high HEV seroprevalence has been reported (i.e., France) (*13*). This finding suggests that in addition to travel to HEV-endemic regions and swine–human contact, additional reservoirs of HEV infection exist in the United States, and evidence has accumulated indicating rodents as a potential HEV reservoir (*14–18*). In

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a survey of 26 rodent species in the United States, Favorov et al. (*14*) found 14 species of rodents seropositive for antibodies against HEV. Urban populations had ≈2× the proportion of seropositive rats relative to rural populations, and commensal *Rattus* spp. (*R. rattus* and *R. norvegicus*) rats had the highest proportion of seropositive animals (*14*).

The role of wild *Rattus* spp. rats as reservoirs in the epidemiology and transmission of HEV is unclear, but their ubiquity in urban environments and unparalleled propensity for carrying zoonotic pathogens makes them an obvious target of investigation. Multiple studies have reported finding IgG and IgM against HEV in *R. norvegicus* and *R. rattus* rat populations across the United States and Asia (*14*–*18*). Shukla et al. (*19*) successfully infected cell lines from *Mus musculus* mice, murid rodents closely related to *Rattus* spp. rats, with HEV genotype 3. In addition, Maneerat et al. (*20*) experimentally infected laboratory *R. norvegicus* rats with HEV isolated from infected humans, although the genotype of the infecting virus was unclear. After infection, the human virus strain effectively replicated in multiple tissues, and HEV RNA was detected in feces and serum for >30 days postexposure, suggesting that human strains of HEV can replicate in and be transmitted by *R. norvegicus* rats. However, recent discovery of a rat-specific strain of HEV not known to infect humans (*21*–*23*) suggests that high seroprevalence of antibodies against HEV may be caused by cross-reactivity rather than widespread infection with a human-infecting HEV genotype.

We used a reverse transcription PCR (RT-PCR) approach to survey *R. rattus* and *R. norvegicus* rats for HEV RNA. Our analysis detected HEV RNA in liver tissues from *R. rattus* and *R. norvegicus* rats at many localities across the United States. Sequencing of DNA from RT-PCR–positive samples indicated widespread infection with zoonotic HEV genotype 3: one rat in California was positive for the rat-specific strain. These findings suggest that wild *Rattus* spp. rats are competent hosts for genotype 3 HEV.

Materials and Methods

Rat Tissues

We obtained liver tissue samples from 446 *R. rattus* and *R. norvegicus* rats from museum collections (online Technical Appendix, wwwcdc.gov/eid-static/spreadsheets/12-0070-Techapp.xls) covering localities primarily in the United States (15 states) plus additional samples from China, Honduras, Madagascar, Mexico, Nicaragua, Peru, Russia, and Vietnam (Table). To maximize the likelihood of intact viral RNA, all liver samples selected were dissected from recently euthanized animals, immediately frozen, and maintained at −80°C until thawed for extraction.

Table. *Rattus* spp. rats tested for hepatitis E virus RNA*

Location	Species and sample size		
	R. norvegicus	*R. rattus*	No. positive
United States			
Aleutian Islands, Alaska	18	7	6
San Francisco Bay Area, California	19	112	12
Gainesville, Florida	NA	21	4
Oklahoma City, Oklahoma	1	NA	1
Memphis, Tennessee	16	NA	6
San Angelo, Texas	2	11	2
Little Rock, Arkansas	2	6	0
San Diego, California	8	5	3
Panama City, Florida	NA	24	0
Key Largo, Florida	NA	5	0
Spencer, Indiana	NA	10	0
Baton Rouge, Louisiana	NA	12	0
Prentiss, Mississippi	NA	1	0
Bernalillo, New Mexico	2	NA	0
Union County, Pennsylvania	40	NA	1
Corvallis, Oregon	4	NA	0
Houston, Texas	NA	8	0
Austin, Texas	NA	14	0
Kerns, West Virginia	1	NA	0
Seattle, Washington	1	5	0
Vietnam	NA	18	0
China	NA	5	0
Honduras	NA	2	0
Madagascar	NA	5	0
Mexico	1	2	0
Nicaragua	1	11	0
Peru	NA	16	0
Russia	30	NA	0

*NA, no samples were available.

Hemi-nested RT-PCR

Total RNA was extracted from ≈30 mg of liver tissue by using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). We used a modification of the broad-spectrum RT-PCR approach of Johne et al. (*22*) to amplify a 334-bp fragment of HEV open reading frame 1 (ORF1). Primers were selected for their ability to amplify ORF1 from all known HEV genotypes, and all primer sequences are reported by Johne et al. (*22*). Attempts to amplify the ORF1 fragment from total extracted RNA resulted in amplification of a portion of an unidentified transcript in all *R. rattus* rat samples. When we sequenced the amplicon, it was clear that spurious amplification was caused by nonspecific binding of primer HEV-cas. To circumvent this problem, we used a hemi-nested approach, with the initial RT-PCR using the HEV-cs/HEV-casN primer combination and the nested PCR using the HEV-csN/HEV-casN primer combination. With the exception of the change in primer combinations, all other aspects of amplification followed the protocol of Johne et al. (*22*). Positive PCR amplicons (verified by agarose gel electrophoresis) were purified by using the Wizard SV Gel PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced in both directions by using nested PCR primers (HEVcsN/HEVcasN).

Given the high sensitivity of a nested PCR approach, contamination can be a major issue and has been cited as problematic in investigations of HEV in rodents (*24*). Exceptional effort was made to ensure that no contamination occurred. All PCR steps were conducted in a sterile environment, under a laminar flow hood, and all surfaces, tubes, and equipment were UV irradiated between each PCR. This study was conducted in a newly constructed laboratory in which no HEV samples (or any other animal samples) had been handled, extractions were conducted in a room separate from that used for PCR amplifications, and all steps (extraction, RT-PCR, and nested PCR) included negative controls. In addition, a single HEV genotype 3 isolate was used as a positive control in PCRs, and we sequenced this isolate for the same locus targeted for the *Rattus* spp. rat samples. Any *Rattus* spp. rat HEV isolate exhibiting 100% nt identity to this positive control sequence was excluded as a contaminant.

Phylogenetic Analyses

In addition to the sequences we generated, we downloaded all complete HEV genome sequences from GenBank (accession numbers are shown in the online Appendix Figure, wwwnc.cdc.gov/EID/article/18/8/12-0070-FA1.htm) and extracted the ≈334-bp homologous portion of ORF1 from each genome. Total sequences were aligned by using the MAFFT aligner (*25*) implemented in Geneious version 5.5 (*26*). We conducted Bayesian, maximum-parsimony, and maximum-likelihood phylogenetic analyses on the combined alignment by using the avian HEV strain as an outgroup. For Bayesian analysis conducted in MrBayes version 3.2 (*27*), we partitioned the alignment by codon position and used a generalized time reversible + invariant sites + Γ substitution model, which Modeltest version 3.7 (*28*) indicated to be most appropriate. The analysis was run for 15,000,000 generations sampled every 1,000 generations, and burn-in values were determined empirically by evaluating likelihood scores. For maximum-parsimony analysis, we used tree bisection/reconnection branch swapping, 25 random additions of input taxa, and 1,000 bootstrap replicates to assess node support. For maximum-likelihood analysis, we used a generalized time reversible + invariant sites + Γ substitution model as indicated above, nearest-neighbor interchange branch-swapping, and 500 bootstrap replicates to assess node support. We generated a haplotype network for sequences generated in this study by using TCS software (*29*).

Results

We excluded 7 isolates sequenced from 1 PCR batch that matched the positive control sequence. No subsequent matches with the positive control were detected, and no contamination was detected in negative controls. We identified 35 (7.85%) *Rattus* spp. rats positive for HEV by PCR from 446 rats examined. Most positive samples were from California (15 rats), but some were from rats in Tennessee, Florida, Oklahoma, Pennsylvania, Texas, and Alaska (Table). Phylogenetic analysis placed 34 of these positive rat samples in a closely related group within the

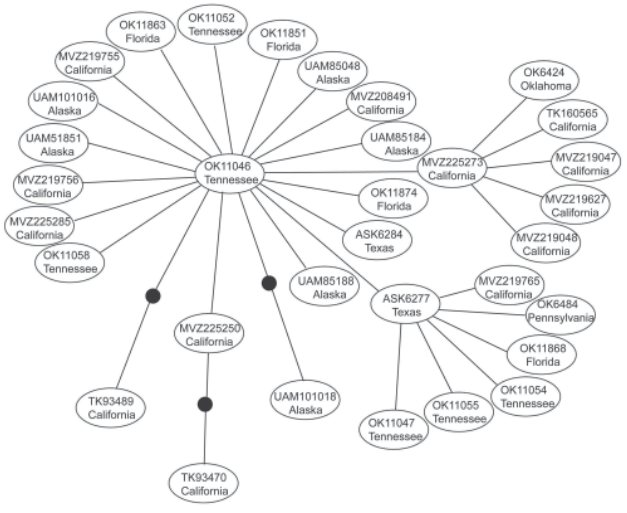

Figure. Genetic network showing the relationship among all hepatitis E virus genotype 3 sequences obtained in this study from isolates from wild rats collected in the United States. Each line represents a single mutational event and closed circles represent extinct or unsampled sequences. Each oval represents a single isolate, and the label corresponds to the tissue number shown in the online Technical Appendix (wwwcdc.gov/eid-static/spreadsheets/12-0070-Techapp.xls) and the general sampling locality.

HEV genotype 3 clade (online Appendix Figure) termed subclade 3a by Lu et al. (*6*). This placement was supported in all analyses. Mean pairwise uncorrected genetic distances between HEV genotype 3 sequences and other known HEV genotypes were 36.19%, 24.12%, 24.91%, 24.05%, and 33.52% compared with the avian genotype, genotype 2, genotype 1, genotype 4, and rat genotype, respectively. Network analysis showed that HEV genotype 3 sequences from *Rattus* spp. rats formed a tight cluster (Figure), differed by only a few mutations, and represented a single strain. Mean pairwise sequence divergence within *Rattus* spp. rat HEV genotype 3 sequences was 0.51%. A single sequence (AF082843) isolated from an HEV-infected pig was also in this group.

The single sequence not nested within the genotype-3 clade was isolated from an *R. norvegicus* rat from the San Francisco Bay area of California. Phylogenetic analyses placed it in a strongly supported clade with 2 other sequences isolated from *R. norvegicus* rats in Germany (online Appendix Figure). Uncorrected genetic distances indicated that the California rat HEV sequence is ≈2× as divergent from the 2 sequences isolated in Germany (California vs. GU345042 = 13.98%; California vs. GU345043 = 14.86%) as the 2 Germany sequences are from each other (GU345042 vs. GU345043 = 7.78%). These findings suggest a degree of distinction between rat HEV strains from the United States and Europe.

Discussion

A major conflict has surrounded the role of *Rattus* spp. rats (and other rodents) in HEV epidemiology since seroprevalence studies in the 1990s identified multiple species of rats positive for HEV antibodies in the United States and Asia (*15–18*). Maneerat et al. (*20*) infected 3 Wistar laboratory rats (*R. norvegicus*) with HEV (viral RNA was detected intermittently in feces for ≤30 days), but which genotype was used is unclear, and this result has not been duplicated (*9*). In addition, He et al. (*24*) isolated HEV genotype 1 from *R. rattus* and *Bandicota bengalensis* rats, but the study was later retracted because the authors were unable to rule out contamination as a source of detected viral RNA (*30*). More recently, Shukla et al. (*19*) successfully infected multiple *M. musculus* mouse cell cultures (in addition to infecting cow, rabbit, cat, dog, and chicken cultures) with HEV genotype 3, supporting the hypothesis that rodents may be competent hosts. However, there was substantial variation among different strains of HEV genotype 3 in the ability to infect cells derived from different hosts, including swine and human.

A recent attempt to infect adult Sprague-Dawley laboratory rats (*R. norvegicus*) with HEV genotypes 1, 2, and 3 failed (*23*). In this same study, infection of laboratory rats with the divergent rat genotype had limited success; only 25% of intravenously infected Sprague-Dawley rats and only 15.8% of nude rats seroconverted. This result is unexpected given that ≈80% of wild *R. norvegicus* rats from Los Angeles, California, where the study was conducted, were positive for IgG or IgM against HEV, suggesting that infection occurs in the wild (*23*). Johne et al. (*22*) also were unsuccessful in infecting rat liver cell lines with rat genotype HEV isolated from wild *R. norvegicus* rats from Germany. In contrast, we provide evidence of HEV genotype 3 infection in wild *R. rattus* and *R. norvegicus* rats.

Spread of HEV-positive rats indicates that infection in wild *Rattus* spp. rats is not restricted to any area of the United States or to urban areas. Our positive samples included both *Rattus* species of rat tested, and included the relatively remote Aleutian Islands in Alaska, and the urban San Francisco Bay area in California. Given the commensal nature of wild *Rattus* spp. rats and their ability to use human transportation vectors (i.e., commercial shipping) in dispersal, the prevalence of HEV in remote populations is not surprising. Recent work examining the genetic structure of *R. rattus* rats has shown that 2 mtDNA haplotypes have rapidly spread from their origin in India to every continent except Antarctica (*31,32*). Given the presence of HEV in domesticated animals (i.e., pigs) and human commensals (i.e., wild *Rattus* spp. rats), widespread domestication has likely enabled HEV to spread worldwide, potentially through interactions between humans, domesticated animals, and commensal rats. Furthermore, because *R. rattus* and *R. norvegicus* rats are sympatric over their contemporary range, lack of genetic distinction between strains infecting these 2 species is not unexpected (online Appendix Figure, Figure).

In terms of infection rates, variation in handling of tissues from field-collected animals should be considered. Although we attempted to limit our analysis to the most well-preserved tissues, there is considerable variation among collection protocols and collectors in length of time between euthanasia and dissection, time between dissection and freezing, number of times tissues were thawed and frozen (i.e., in sorting, subsampling, shipping), and consistency of storage temperature. These factors can lead to nucleic acid degradation and negatively affect the ability to detect viral RNA. Therefore, our infection rate is likely not indicative of HEV infection rates in wild *Rattus* spp. rat populations.

Recent studies have reported major variation in diversity of competent mammalian hosts for various strains of HEV genotype 3 (*19*). Although seroprevalence studies have suggested infection rates ≈80% for HEV in US *Rattus* spp. rat populations (*15,23*), attempts to infect different laboratory strains of *R. norvegicus* rats with a genotype isolated from wild *R. norvegicus* rats have shown limited

success; most attempts also failed in immunocompromised nude rats (*22,23*). These patterns, and the low genetic diversity of HEV-positive samples detected in this study (Figure), suggest that only a limited number of HEV genotype 3 strains may be capable of infecting *Rattus* spp. rats and other rodents (i.e., *Mus* spp.), possibly because of an HEV genotype 3/rat genotype recombinant.

Reduced genetic diversity of ORF1 sequences obtained from *Rattus* spp. rats requires further study, including sequencing genomes of these isolates to identify sequence diversity at other loci. Difficulty in transmitting virus from infected wild *R. norvegicus* rats into laboratory strains also indicates that certain life history or genetic characteristics may be essential for infection. Purcell et al. (*23*) reported a positive correlation between antibody prevalence and animal age in their study of seroprevalence of HEV in *Rattus* spp. rats, suggested that rats are readily infected in the wild, and that infection occurred in juvenile rats. This pattern is consistent with HEV infection in humans and swine (*33,34*), and suggests that infections should be attempted in wild and laboratory juvenile rats. Lending further support to this suggestion, the only report of major long-term infection (>30 days) of rats with HEV used weanling rats (*20*), and all other attempts we are aware of have used only adult rats (*23*). In addition, extreme variation in host specificity that Shukla et al. (*19*) observed among different HEV genotype 3 strains indicates the need for future transmission studies to include as many strains as possible.

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Hepatitis E Virus Strains in Rabbits and Evidence of a Closely Related Strain in Humans, France

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Hepatitis E virus (HEV) strains from rabbits indicate that these mammals may be a reservoir for HEVs that cause infection in humans. To determine HEV prevalence in rabbits and the strains' genetic characteristics, we tested bile, liver, and additional samples from farmed and wild rabbits in France. We detected HEV RNA in 7% (14/200) of bile samples from farmed rabbits (in 2009) and in 23% (47/205) of liver samples from wild rabbits (in 2007–2010). Full-length genomic sequences indicated that all rabbit strains belonged to the same clade (nucleotide sequences 72.2%–78.2% identical to HEV genotypes 1–4). Comparison with HEV sequences of human strains and reference sequences identified a human strain closely related to rabbit strain HEV. We found a 93-nt insertion in the X domain of open reading frame 1 of the human strain and all rabbit HEV strains. These findings indicate that the host range of HEV in Europe is expanding and that zoonotic transmission of HEV from rabbits is possible.

Hepatitis E virus (HEV) is a major cause of acute hepatitis in many developing countries in Asia and Africa, where it is transmitted by the fecal–oral route because of poor sanitation practices (*1*). Acute hepatitis E is also increasingly reported in industrialized countries, where the transmission is mainly zoonotic (*2*). The initial discovery of HEV transmission from domestic pigs (*3*) has been followed by evidence that other mammals, such as wild boars and deer, are also potential reservoirs of HEV (*4*). Although the course of HEV infection is generally self-limiting and asymptomatic (or symptomatic with acute hepatitis), fulminant hepatitis can occur in pregnant women and in persons with underlying liver disease (*5*–*7*). HEV infections can also become chronic in immunocompromised patients, such as recipients of solid-organ transplants (*8*–*10*), those with hematologic diseases (*11*,*12*), and patients infected with HIV (*13*–*15*).

HEV, genus *Hepevirus*, family *Hepeviridae*, is a positive-sense, single-stranded, nonenveloped RNA virus (*16*). The HEV genome is ≈7.2 kb long and contains 3 open reading frames (ORFs) as well as 5′ and 3′ untranslated regions: ORF1 encodes nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a small phosphoprotein. Phylogenetic analysis of HEV sequences has led to the recognition of 4 major genotypes that infect mammals from a variety of species. HEV1 and HEV2 are restricted to humans and transmitted through contaminated water in developing countries. HEV3 and HEV4 infect humans, pigs, and other mammals and are responsible for sporadic cases of hepatitis E in developing and industrialized countries (*2*). HEV3 is distributed worldwide, whereas HEV4 largely is found in Asia. Although HEV3 and HEV4 infections have been linked to the consumption of raw or undercooked meats, such as pig liver sausages or game meats (*17*,*18*), the full spectrum of animals that are reservoirs of HEV is still unknown.

Recent studies have characterized new HEV genotypes in isolates from rats in Germany (*19*), wild boars in Japan (*20*), and farmed rabbits in the People's Republic of China (*21*,*22*). Because the potential risk for zoonotic transmission

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of HEV from rabbits in France is unknown, and cases of autochthonous hepatitis E are commonly reported in this country (*23*,*24*), we investigated the prevalence of HEV in farmed and wild rabbits. We also looked for a genetic link between HEV strains circulating in rabbits and HEV strains circulating in humans in France.

Materials and Methods

Specimens from Farmed Rabbits

Bile specimens (n = 200) were collected in September 2009 from rabbits raised on 20 farms in western France, in the departments of Maine et Loire (n = 6), Vendée (n = 6), Deux-Sèvres (n = 4), Calvados (n = 2), and Loire Atlantique (n = 2), the main geographic areas of rabbit farming in France. We sampled 10 rabbits from each farm when they were slaughtered at 70–90 days of age (Table 1). All rabbits were healthy and intended for human consumption. All samples were immediately stored at −80°C.

Specimens from Wild Rabbits

Liver specimens (n = 205) were collected during September 2007–November 2010 from 18 populations of wild rabbits, established in warrens; each population was considered epidemiologically independent. The populations were located in several departments of mainland France: Dordogne (n = 7), Finistère (n = 3), Deux-Sèvres (n = 2), Loire-Atlantique (n = 2), Haute-Garonne (n = 1), Charentes (n = 1), Morbihan (n = 1), and Pyrénées-Orientales (n = 1) (Table 1). The number of rabbits sampled in a given warren ranged from 1 to 44. They were >6 months of age, apparently healthy, and intended for human consumption. Each rabbit was eviscerated within a few hours of its death, and a sample of its liver was taken and immediately frozen at −80°C. Necropsies were performed on a group of 12 rabbits from the same warren in Haute-Garonne (W3), and samples of their intestine and cecum were taken, in addition to samples from the liver.

Specimens from Humans

Serum specimens were collected from immunocompetent and immunocompromised patients who had received a diagnosis of hepatitis E from the department of virology at Toulouse University Hospital. All samples were stored at −80°C (*23*,*24*).

RNA Extraction

Samples (140 µL of rabbit bile and 50 mg of liver, intestine, and cecum) were disrupted with TRIzol (Invitrogen, Saint Aubin, France). RNA was extracted with QIAamp Viral RNA Mini Kits (QIAGEN, Courtaboeuf, France).

Table 1. Detection of hepatitis E virus RNA in farmed and wild rabbits, France

Source	Location/department	No. tested	No. (%) HEV RNA positive
Farmed rabbits			
F20	Calvados	10	0
F4	Calvados	10	1 (10)
F6	Deux-Sèvres	10	1 (10)
F7	Deux-Sèvres	10	1 (10)
F12	Deux-Sèvres	10	0
F16	Deux-Sèvres	10	0
F3	Loire Atlantique	10	2 (20)
F8	Loire Atlantique	10	0
F1	Maine et Loire	10	5 (50)
F5	Maine et Loire	10	1 (10)
F10	Maine et Loire	10	0
F15	Maine et Loire	10	0
F17	Maine et Loire	10	0
F19	Maine et Loire	10	0
F2	Vendée	10	3 (30)
F9	Vendée	10	0
F11	Vendée	10	0
F13	Vendée	10	0
F14	Vendée	10	0
F18	Vendée	10	0
Wild rabbits			
W9	Charentes	26	3 (12)
W5	Deux-Sèvres	44	13 (30)
W14	Deux-Sèvres	3	0
W2	Dordogne	5	3 (60)
W6	Dordogne	8	3 (38)
W8	Dordogne	4	1 (25)
W11	Dordogne	1	0
W12	Dordogne	5	0
W15	Dordogne	4	0
W16	Dordogne	17	0
W1	Finistère	10	10 (100)
W4	Finistère	10	4 (40)
W7	Finistère	15	4 (27)
W3	Haute-Garonne	12	6 (50)
W13	Loire Atlantique	11	0
W18	Loire Atlantique	1	0
W10	Morbihan	10	0
W17	Pyrénées Orientales	19	0

*F, farm; W, warren.

Real-time Reverse Transcription PCR

We used 1-step real-time reverse transcription PCR on the Light Cycler 480 instrument (Roche Diagnostics, Meylan, France) to amplify a 70-bp fragment. The primers and probes targeted the ORF3 region: forward primer HEVORF3-S: 5′-GGTGGTTTCTGGGGTGAC-3′, reverse primer HEVORF3-AS: 5′AGGGGTTGGTTGGATGAA -3′, and probe 5′-Fam-TGATTCTCAGCCCTTCGC-Tamra-3′ (*25*). Each 50-µL reaction mix contained 1 µL of SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), 15 µL of RNA, primers (200 nmol/L) and probes (150 nmol/L), and 40 U of RNase Out (Invitrogen). Reverse transcription was carried out at 50°C for 15 min, followed by denaturation at 95°C for 1 min. DNA was amplified with 50 PCR cycles at 95°C (20 s) and 58°C (40 s). HEV RNA was quantified by using a transcribed RNA standard constructed from a genotype 3f

HEV strain (GenBank accession no. EU495148). The limit of detection was 100 copies/mL.

DNA Sequencing

Two fragments, one within ORF2 (189 bp) and the other within ORF1, encompassing the hypervariable region and X domain (≈1,400 bp), were amplified and sequenced in both directions by the dideoxy chain termination method (PRISM Ready Reaction Ampli Taq Fs and Dye Deoxy primers; Applied Biosystems, Paris, France) on an ABI 3130XL capillary DNA analyzer (Applied Biosystems, Foster City, CA, USA). The primers used for the ORF2 fragment were the following: forward primer HEVORF2-S: 5′-GACAGAATTRATTTCGTCGGCTGG-3′ and reverse primer HEVORF2-AS: 5′-TGYTGGTTRTCATAATCC TG-3′. The primers used for the ORF1 fragment were the following: forward primer HEVORF1-S: 5′-TGACGGCYACYGTKGARCTTG-3′ and reverse primer HEVORF1-AS: 5′-ACATCRACATCCCCCTGY TGTATRGA-3′.

The whole genomes of 2 rabbit strains (W1–11 and W7–57) and 1 human strain (TLS-18516-human) were amplified by overlapping RT-PCR. The primers are listed in Table 2.

Phylogenetic Analysis

The genotype was determined by using reference strains as previously described (*26*). Phylogenetic analyses were performed with genotype information on reference sequences based on the HEV classification proposed by Lu et al. (*27*). Sequences were aligned by using ClustalW (MEGA5, www.megasoftware.net; BioEdit version 7.0, www.mbio.ncsu.edu/bioedit/bioedit). Phylogenetic trees were created by the neighbor-joining (Kimura 2-parameter) method with a bootstrap of 1,000 replicates.

The 2 partial sequences of ORF1 and the 5 full-length sequences reported in this study have been deposited in GenBank. The accession numbers are JQ013789 and JQ013790 for ORF1, and JQ013791 to JQ013795 for the full-length sequences of W1–11, W7–57, TLS-18516-human, TR19 (genotype 3c), and TR02 (genotype 3e), respectively.

Results

HEV RNA

All bile specimens from the 200 farmed rabbits and the liver specimens from the 205 wild rabbits were tested for HEV RNA (Table 1). Samples from 7 farms (35%) and 9 warrens (50%) tested positive for HEV RNA. HEV RNA was found in a 14 bile samples (7%) from farmed rabbits. The median HEV RNA concentration in the bile samples was 2.3×10^7 copies/mL (range 100 copies/mL–10^9 copies/mL). A total of 47 liver samples (23%) from wild rabbits were positive for HEV RNA; median HEV RNA concentration was 1.9×10^6 copies/g (range 1,400 copies/g–5.8×10^7 copies/g).

We tested the liver, intestine, and cecum samples from 12 wild rabbits from the same warren (W3) in triplicate to obtain a clear picture of the tissue distribution of HEV in infected rabbits. HEV RNA was detected in all the tissues from 4 rabbits (nos. 4, 7, 9, 12), in the liver and intestine of 1 rabbit (no. 5), and in the liver only of 1 rabbit (no. 6) (Table 3). The virus loads in the liver (mean 4.8 log copies/g), intestine (mean 4.0 log copies/g), and cecum (mean 3.6 log copies/g) were not significantly different.

ORF2 Sequences

Phylogenetic analyses, conducted on the basis of a 189-nt fragment within ORF2 of the 37 HEV strains from rabbits, HEV3 strains from humans circulating in France, and HEV reference sequences (HEV1, HEV2, HEV3, HEV4, rabbit HEV, rat HEV, wild-boar HEV) indicated that the 37 new ORF2 sequences from rabbit HEVs were clustered. One cluster contained 3 ORF2 sequences from previously characterized HEV from farmed rabbits from China, 2 ORF2 sequences from HEVs from farmed rabbits in France, and 13 ORF2 sequences from HEVs from wild rabbits in France (Figure 1). This cluster also contained an ORF2 sequence from a strain from a person in France (TLS-18516-human) (Figure 1). This strain was found in a serum sample from a 46-year-old man with an elevated alanine aminotransferase level (400 IU/L, reference <35 IU/L).

Table 2. Primers used to amplify and sequence the whole genome of HEV strains, France*

Fragment size, bp	Nucleotide position†	Primer	Sequence, 5′ → 3′
225	15–238	Sense	ATGTGGTCGATGCCATGGAGGCCCA
		Antisense	CTCATTATGTATAACACGTTGAATAG
2,900	152–3937	Sense	AGACAGATATTCTTATCAATTTAATGCAACCCCGC
		Antisense	GCCGCAAGTAACACGGGCGGCCGTGTGAGGTGTGAA
2,000	3207–5212	Sense	AAGTCTAGGTCTATACAGCAGGG
		Antisense	GCCGGTGGCGCGGGCAGCATAGGCA
2,160	5060–7208	Sense	AATGTYGCYCAGGTYTGTG
		Antisense	TTTTTTTTTTTTCCYGGGRGCGC

*HEV, hepatitis E virus.
†Nucleotide position refers to Burma strain, M73218.

Table 3. Detection of HEV RNA by real time RT-PCR in the 12 wild rabbits from warren W3 according to tissue sampled, France*

	Liver		Intestine		Cecum	
Rabbit no.	No. positive	Mean viral load†	No. positive	Mean viral load†	No. positive	Mean viral load†
1	0	<LOD	0	<LOD	0	<LOD
2	0	<LOD	0	<LOD	0	<LOD
3	0	<LOD	0	<LOD	0	<LOD
4	3	7.3	3	5.3	2	3.4
5	3	3.7	1	3.1	0	<LOD
6	3	4.4	0	<LOD	0	<LOD
7	1	3.1	2	3.6	1	3.8
8	0	<LOD	0	<LOD	0	<LOD
9	3	6.6	3	5.2	3	4.6
10	0	<LOD	0	<LOD	0	<LOD
11	0	<LOD	0	<LOD	0	<LOD
12	3	3.7	2	3.6	1	3.8

*Three samples were obtained of each tissue type from each rabbit. HEV, hepatitis E virus; RT-PCR, reverse transcription PCR; LOD, limit of detection.
†log copies/g.

ORF1 Sequences

Phylogenetic analysis based on a 1,400-nt fragment within ORF1, indicated that the ORF1 sequences from HEV strains from rabbits in France (n = 4) or China (n = 3) and the ORF1 sequence from the human strain TLS-18516-human formed a distinct genetic group among sequences of HEV genotypes 1–4 (data not shown). The cluster of rabbit HEV sequences was also distinct from the HEV sequences from wild-boar and rat HEV genotypes that were characterized recently.

Comparison of the ORF1 sequences from rabbit HEV strains with reference ORF1 sequences from HEV genotypes 1–4 showed an insertion of 93 nt in the X domain of the ORF1 of all the rabbit HEV strains. This insertion was also found in the TLS-18516-human strain. The deduced amino acid sequences corresponding to this insertion, located between amino acids 938 and 939 (Burmese strain, M73218), were not very similar, except for 2 conserved amino acids at the C-terminal end.

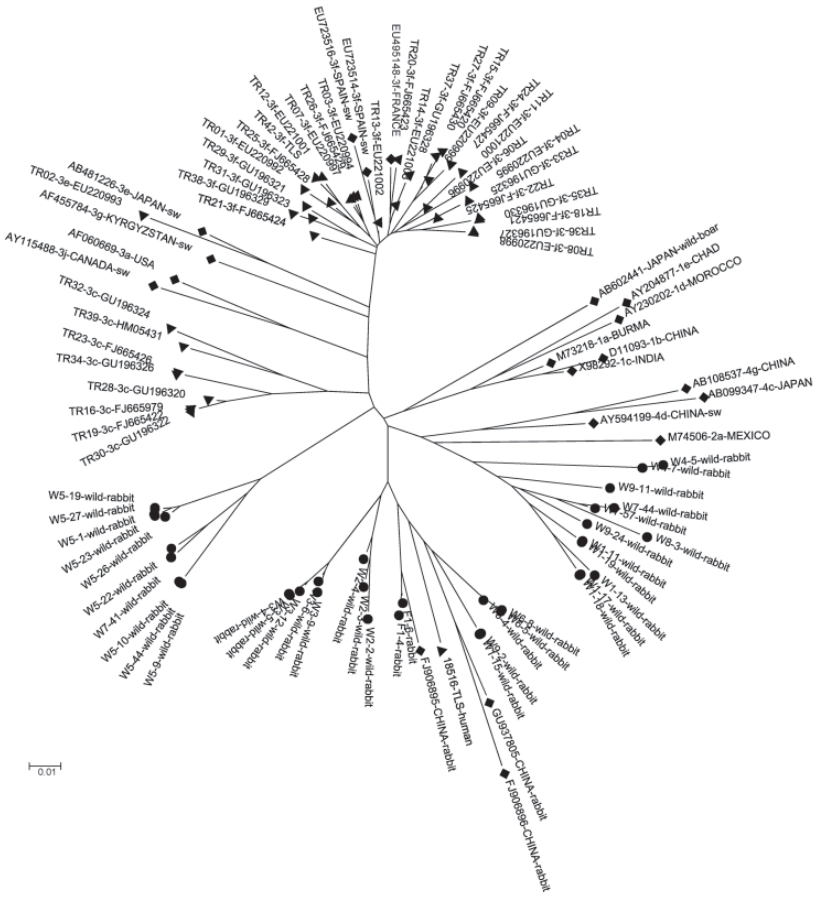

Figure 1. Phylogenetic tree for the 189-bp sequence of open reading frame 2 of the capsid gene of rabbit hepatitis E virus (HEV) strains (circles), human strains circulating in France (triangles), and reference strains (diamonds). GenBank accession numbers are shown for each HEV strain used in the phylogenetic analysis. Scale bar indicates nucleotide substitutions per site.

Genome Sequences

We obtained the full-length genomic sequences of HEV strains from 2 wild rabbits in France and the TLS-18516-human strain. The phylogenetic tree, constructed by the neighbor-joining method using the full-length genomic sequences (including the sequences of genotypes 3f, 3e, and 3c, which were circulating in France), revealed that the HEV genomes from the rabbit strains and the TLS-18516-human strain belonged to the same clade. This clade was clearly separated from genotypes 1–4, found in other mammals and from the new HEV genotypes found in wild boars and rats (Figure 2).

The length of the rabbit strain W1–11 genome, excluding the poly(A) tract at the 3′ terminus, was 7,262 nt. The length of the rabbit strain W7–57 was 7,231 nt, and that of the TLS-18516-human strain was 7,259 nt. The nucleotide sequences of the rabbit strains and the TLS-18516-human strain were 80.3%–85% identical (Table 4). The nucleotide sequences of the rabbit or TLS-18516-human strains were 76.1% to 78.2% identical to those of genotype 3, 72.7% to 73.7% identical to those of genotype 1, 72.2% to 73.5% identical to those of genotype 2, and 72.9% to 74.9% identical to those of genotype 4. These comparisons therefore indicate that the sequences of the rabbit HEV strains and the TLS-18516-human strain are distinct from all known strains of HEV genotypes 1–4 and from the newly described HEV genotypes from wild boars and rats.

Discussion

We found that farmed and wild rabbits in France are naturally infected with HEV. We also characterized a human HEV strain that is closely related to rabbit HEV strains; this finding thus supports the potential of zoonotic transmission from rabbits to humans.

The HEVs found in farmed rabbits in several geographic areas of China have been identified (*21*,*22*). HEV was also recently found in farmed rabbits in Virginia, USA (*28*). Our study results show that rabbits in Europe are infected with HEV and that some farmed rabbits and wild rabbits in France are infected. We found HEV RNA in 7% of the farmed rabbits and in 23% of the wild rabbits. However, the ages of the rabbits and the tissues tested (bile samples from farmed rabbits and liver samples from wild rabbits) may explain the observed difference in HEV prevalence. Nevertheless, previous studies have shown that the virus loads in liver and bile samples from swine infected with HEV are similar (*29*,*30*). Although the greater prevalence of HEV in wild rabbits could be linked to their older age, we could not test for a relationship between the prevalence of HEV and rabbit age because we did not know the rabbits' precise ages.

Our analysis of the distribution of HEV in the tissues of infected wild rabbits showed HEV RNA not only in the liver, but also in the intestine and cecum; our analysis also showed that the virus loads from these organs were not significantly different. This finding suggests that

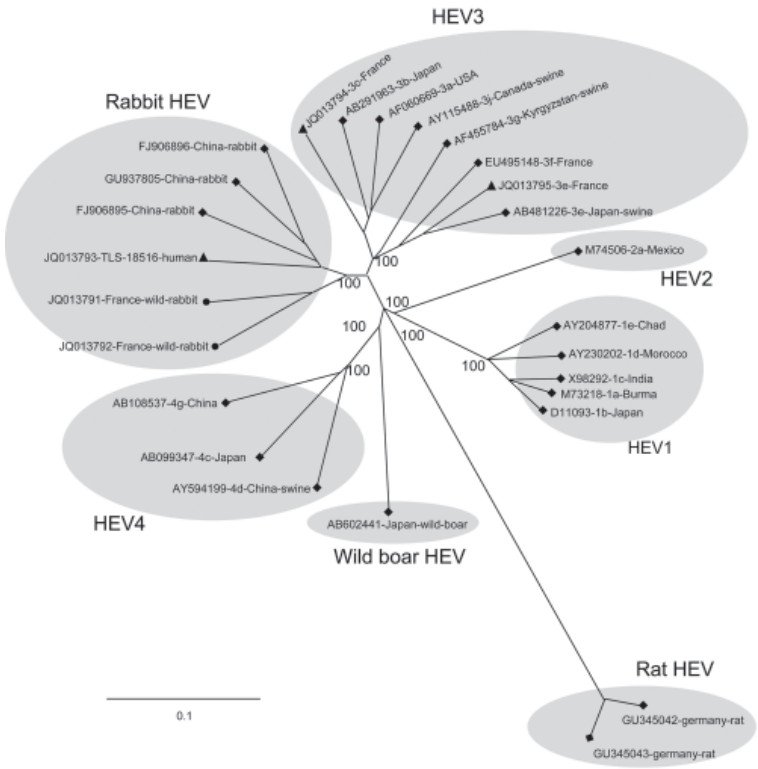

Figure 2. Phylogenetic tree based on full-length sequences of hepatitis E virus (HEV) rabbit strains (circles), the human strain TLS-18516 (triangles) and reference strains (diamonds). GenBank accession numbers are shown for each HEV strain used in the phylogenetic analysis. Scale bar indicates nucleotide substitutions per site.

Table 4. Percent identities of full-length sequences among HEV strains from rabbits and other HEV strains, France*

Virus strain	HEV strains, % identity				
	TLS-18516	HEV1	HEV2	HEV3	HEV4
GU937805-China-rabbit	85.0	73.0–73.7	72.2	76.3–78.2	72.9–73.9
W7–57-wild-rabbit	80.3	72.7–73.4	73.5	76.2–77.6	73.2–73.6
W1–11-wild-rabbit	80.6	73.2–73.7	73.4	76.1–78.0	74.2–74.9

*HEV, hepatitis E virus; HEV1 (M73218–1a-Burma, D11093–1b-Japan, X98292–1c-India, AY204877–1e-Chad, AY230202–1d-Morocco); HEV2 (M774506–2a-Mexico) ; HEV3 (AF060669–3a-USA, AY115488–3j-Canada-Sw, AB291963–3b-Japan, TLS-TR19–3c, TLS-TR02–3e, AB481226–3e-Japan-Sw,EU495148–3f-France,AF455784–3g-kyrgyzstan-Sw); HEV4 (AB099347–4c-Japan, AB108537–4g-China, AY594199–4d-China-Sw).

extrahepatic sites of HEV replication exist in rabbits, as has been demonstrated for HEV3 in pigs (*31*). However, because the intestine and cecum samples may have been contaminated with blood, our results need to be confirmed in future studies using methods that ensure that tissues other than the liver are not contaminated with blood.

To determine whether rabbits could be a reservoir for viruses that cause human infection, we analyzed partial and complete nucleotide sequences of the rabbit HEV strains and compared these sequences with those of human HEV strains circulating in France. Analysis of ORF2 showed that the sequences from rabbit HEV strains formed clusters, one of which included the sequences of HEV genotypes 2 and 4. The bootstrap values were very low because the fragments analyzed were small. In contrast, phylogenetic analyses based on ORF1 and the full-length genome indicated that all the rabbit strains from China and France belong to the same clade. One human strain, TLS-18516-human, clustered with the rabbit strains and appeared to be somewhat different from the 4 major HEV genotypes found in mammals and the newly described HEV genotypes from rats and wild boars. Although the full-length sequences of the genomes of the rabbit strains and the TLS-18516-human strain are more similar to that of HEV3 than to those of HEV1, HEV2, and HEV4, they do not seem to belong to the established HEV genotype 3 found in humans and swine, as recently suggested (*20*,*32*). Differences in the classification of rabbit HEV could be because the full-length genomic sequences were used as the reference for phylogenetic analyses. Genotype 3 is highly diverse, with 10 identified subtypes (*27*). We included in our analysis the full-length genomes of subtypes 3f, 3c, and 3e, which account for ≈74%, 13%, and 5% of the human and swine HEV strains circulating in France (*26*,*33*). We also included the other full-length genomes representative of HEV3 subtypes, but subtypes 3d, 3h, and 3i are not yet available in GenBank. Our data indicate that the genomes of rabbit HEV strains or TLS-18516-human were <80% identical with HEV3, regardless of which method was used to align the sequences. This finding is compatible with the definition of a new genotype, as previously proposed (*21*,*22*).

We found a 93-nt insertion in the X domain of the ORF1 of the human strain TLS-18516-human and of all the rabbit HEV strains. This insertion, also found in the rabbit HEV strains from China (*34*), is not present in any known strain of HEV genotypes 1–4 or in the new HEV genotypes from rats and wild boars. The X domain corresponds to a macro domain found in the nonstructural polyproteins of several positive-stranded viruses such as togaviruses and coronaviruses (*35*–*37*). This domain can bind polyadenosine diphosphate–ribose regions and could play a role in the replication or transcription of virus RNA. Whether the insertion in the X domain influences the function of the HEV macro domain warrants further investigation. Several determinants, including this insertion, could be essential for specifying the host range, zoonotic transmission, and pathogenesis of rabbit HEV strains (*34*).

What rabbit HEV strains contribute to the epidemiology of hepatitis E in humans is not clear. HEV is endemic to southwestern France, and the annual incidence of locally acquired HEV infections has been estimated as 3.2% (*38*,*39*). A case–control study found that the only factor independently associated with HEV infection was the consumption of game meat, mostly wild boar, deer, and wild rabbit (*23*). However, molecular data from various studies in France indicate that most HEV strains identified belong to genotypes 3f, 3c, or 3e, which are prevalent in pigs and wild boars (*23*,*26*,*40*). A recent study showed the same proportions of genotypes 3f, 3c, and 3e in human and pig populations (*33*). Although this finding could indicate that rabbit HEV strains are less readily transmitted to humans than HEV genotype 3 strains, the primers used for PCR amplification were not specifically designed for rabbit HEV strains. Therefore, the true prevalence of HEV RNA among rabbits and humans may have been underestimated. In addition, genotyping rabbit HEV may have been difficult because reference sequences have become available only recently.

The immunocompetent or immunocompromised status of the patient that became infected with a rabbit HEV strain, as well as the source of his contamination, is unknown because of the lack of medical follow-up. Molecular and epidemiologic studies are needed to determine the prevalence of rabbit HEV strains among immunocompetent and immunocompromised patients.

In conclusion, we have shown that in France, farmed and wild rabbits can be infected with HEV. Phylogenetic analysis, based on full-length genomes and a molecular signature in the X domain of ORF1, indicates that rabbit HEV strains could be a new genotype. Our identification of

a human HEV strain that is closely related to rabbit HEV strains reinforces the potential zoonotic risk for infection with this virus. Further studies are needed to demonstrate cross-species transmission directly and to evaluate the contribution of the rabbit reservoir to human HEV infection and disease.

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Dr Izopet is chief of the virology laboratory of Toulouse University Hospital. His research interests include the molecular virology of HEV and its pathogenesis.

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Hepatitis E Virus in Pork Production Chain in Czech Republic, Italy, and Spain, 2010

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We evaluated the prevalence of hepatitis E virus (HEV) in the pork production chain in Czech Republic, Italy, and Spain during 2010. A total of 337 fecal, liver, and meat samples from animals at slaughterhouses were tested for HEV by real-time quantitative PCR. Overall, HEV was higher in Italy (53%) and Spain (39%) than in Czech Republic (7.5%). HEV was detected most frequently in feces in Italy (41%) and Spain (39%) and in liver (5%) and meat (2.5%) in Czech Republic. Of 313 sausages sampled at processing and point of sale, HEV was detected only in Spain (6%). HEV sequencing confirmed only g3 HEV strains. Indicator virus (porcine adenovirus) was ubiquitous in fecal samples and absent in liver samples and was detected in 1 slaughterhouse meat sample. At point of sale, we found porcine adenovirus in sausages (1%–2%). The possible dissemination of HEV and other fecal viruses through pork production demands containment measures.

Human hepatitis E is endemic worldwide, particularly in Asia, where large waterborne outbreaks have been reported (*1*). Seroprevalence of hepatitis E virus (HEV) is >60% in rural southern People's Republic of China (*2*) and 4%–10% in western Europe (*3*) and the United States (*4*). In these areas, hepatitis E occurs mostly as sporadic cases (*5*–*7*), but epidemics also have been described (*8*). Most cases in Europe have been linked to genotype 1 (g1) virus and associated with travel to g1-endemic areas. However, autochthonous human infections are increasing in Europe and in other industrialized countries (*5*,*6*,*9*). Of the 4 genotypes affecting humans, genotype 3 (g3) is the main HEV genotype also circulating among pigs in Europe (*10*) and human infections are observed sporadically worldwide (*11*,*12*).

Several reports indicate that HEV can be transmitted through zoonotic and foodborne pathways, including through consumption of raw and undercooked liver, meat, or sausages from domestic pigs, wild boar, and deer (*8*,*13*,*14*). Several investigations have shown that farmed domestic pigs are widely infected with and shed g3 HEV in Europe. Studies conducted in Spain (*8*,*13*,*14*), Italy (*15*), and France (*16*) have detected HEV genomic RNA in livers of pigs of slaughtering age, indicating that HEV-contaminated food might reach supermarkets (*17*). In butcher shops in the Netherlands (*18*) and Germany (*19*), ≈6.5% and ≈4%, respectively, of pork livers contained HEV, which raises concern about the potential for direct transmission through contact with or consumption of contaminated food.

Despite the large widespread distribution of HEV-shedding pigs and the possible role of farmed pigs as the main virus reservoir, the number of human hepatitis E cases in Europe remains low, suggesting inefficient virus transmission or lower pathogenicity of swine g3 strains than of g1 strains for humans. Because g3 HEV is common in pigs but rare in humans, humans are postulated to not be a main host for g3 virus replication (*11*,*20*). Nonetheless, a possibly large underestimation of HEV spread in humans cannot be excluded because of asymptomatic cases, inadequate diagnostics, and scarce medical attention (*21*). Mansuy et al. suggested inadequacy of previous diagnostic methods and recently found unprecedentedly high HEV seroprevalence among blood donors in France (*22*).

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Relatively few studies of foodborne human hepatitis E are available (*8*,*21*,*23*), making evaluation of HEV-associated risks difficult. Investigation of HEV throughout the pork production chain from farm to point of sale is needed to highlight areas of risk and proper control. A recent report from European Food Safety Authority biohazard experts (*24*) underscored an urgent need for integrated studies on HEV circulation, performing farm-to-table integrated risk assessment. For other foodborne pathogens, such studies comprise quantitative microbial risk assessment on the basis of exposure and dose-response models (*25*). Unfortunately, for HEV, quantitative approaches are hardly accessible because of the absence of reliable cell culture systems for viral infectivity titration.

We aimed to assess HEV prevalence in the pork production chain from slaughterhouse to point of sale in Czech Republic, Italy, and Spain during 2010 in the framework of the FP7 VITAL (Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains) project (www.eurovital.org/). This systematic multicountry investigation of domestic swine HEV was conducted by using standardized molecular approaches, including reverse transcription quantitative PCR (RT-qPCR) detection, process, and internal amplification controls (IACs) and proper fecal viral indicators (porcine adenovirus [PAdV]).

Materials and Methods

Sampling Strategy

Samples were taken at perceived critical points for virus contamination. They were identified from Hazard Analysis and Critical Control Point System audit principles-based questionnaires (K. Willems and R. Moloney, pers. comm.) completed in each premise and analyzed by VITAL food-safety management and risk assessment experts (M. Bouwknegt and A. De Roda Husman, pers. comm.).

Samples

A total of 113 fecal, 112 liver, and 112 meat (lingual muscle) samples from 113 healthy pigs (*Sus scrofa* subsp. *domestica*) were collected in slaughterhouses from Czech Republic, Italy, and Spain during 2010 (Table 1). Samples originated from 4 pig farms per country. Packaged sausages were sampled in processing sites and supermarkets in Italy and Spain (128 and 93 samples, respectively) and in 8 supermarkets in Czech Republic (92 samples).

Additional ad hoc samples were collected during fact-finding visits to production farms, processing plants, and points of sale (Table 2). Briefly, 73 samples were collected from working surfaces and cutting tools (swabs from knife, belt surface, and meat mincer) from slaughtering areas (10 samples), processing areas (19 samples), and points of sale (12 samples) and from workers' hands (20 samples) and workers' toilets (12 samples). In Czech Republic, 6 effluent water samples from slaughterhouses also were examined.

Sample Process Control Virus

Murine norovirus 1 (MNV-1) was used as sample process control virus (SPCV). A single batch with MNV-1 at the concentration of 4.7×10^7 PFU/mL was prepared and used by all collaborating institutes throughout the study (*26*).

Virus Concentration and Nucleic Acid Isolation

Pig Feces

Feces (>1 g) were collected aseptically. A total of 250 mg of sample in 15-mL centrifuge tubes were suspended in 2.25 mL phosphate-buffered saline containing gentamycin (10 mg/mL), and 10 μL SPCV (4.7×10^5) was added. Suspensions were vortexed for 60 s and centrifuged at 3,000 × *g* for 15 min. Supernatants were immediately used for nucleic acid isolation or stored at –70°C. Nucleic acid was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Final elution was performed 2× with 50 μL elution buffer, resulting in a 100-μL nucleic acid extract, for immediate testing or –70°C storage.

Pork Liver, Meat, and Sausages

Liver and meat or sausage samples were collected (1 cm^3 from 3 different locations) and stored in sterile plastic bags. According to the method of Bouwknegt et al. (*18*), samples were finely chopped and homogenized in an RNase-free mortar with 4 mL of Buffer RLT (RNeasy Midi Kit, QIAGEN) containing 1:100 β-mercaptoethanol. A total of 250 mg homogenate was transferred into microcentrifuge tubes containing 1 mL RLT buffer, 2.5 g sterile 1-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA), and 10 μL SPCV (4.7×10^5). Tubes were applied to a mechanical disruptor (Ribolyser-Cell-Disrupter, Hybaid Ltd., Ashford, UK) for two 40-s/4-m/s cycles. After centrifugation (10,000 × *g*, 20 min, 2×), 800 μL of resulting supernatants were immediately processed by RNeasy Midi Kit or freeze-stored. Nucleic acid extracts (300 μL) were assayed immediately or freeze-stored.

Workers' Hands and Surfaces

Workers' hands and surfaces were sampled by using sterile moistened swabs, and samples were stored in 5 mL of 10 mg/mL gentamicin-containing phosphate-buffered saline in plastic tubes. Unwashed hands were sampled immediately before lunch or afternoon coffee break. For surfaces, 10-cm^2 areas were rubbed. Liquids were decanted from swab containers into 50-mL centrifuge

Table 1. Detection of HEV and indicator virus PAdV in samples from the pork production chain, Czech Republic, Italy, and Spain, 2010*

Production stage and sample source	Virus	Czech Republic		Italy		Spain		All	
		No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive
Slaughterhouse									
Feces	HEV	40	1 (3)†	34	14 (41)	39	15 (38)†	113	30 (27)
	PAdV	40	39 (98)	34	31 (91)	39	35 (90)	113	105 (93)
Liver	HEV	40	2 (5)†	33	2 (6)	39	1 (3)†	112	5 (4)
	PAdV	40	0	33	0	39	0	112	0
Meat	HEV	40	1 (3)	33	2 (6)	39	0	112	3 (3)
	PAdV	40	0	33	1 (3)‡	39	0	112	1(1)
Processing/points of sale: sausage	HEV	92	0	128	0	93	6 (6)	313	6 (2)
	PAdV	92	1 (1)	128	1 (1)	93	2 (2)‡	313	4 (1)

*HEV, hepatitis E virus; PAdV, porcine adenovirus.
†Samples originated from the same animal.
‡Sample negative for HEV.

tubes containing 10 µL SPCV (4.7×10^5). Suspensions were vortexed and centrifuged (3,000 × *g*, 5 min), and supernatants were used immediately or freeze-stored. Nucleic acids were extracted by NucliSENS miniMAG Kit (bioMérieux, Marcy l'Etoile, France) and eluted 2× with 50 µL elution buffer.

RT-qPCR

Nucleic acids were assayed undiluted and diluted 10-fold by performing RT-qPCRs in duplicate. All reaction mixes included an IAC (*27*). All RT-qPCRs were in duplex format, targeting specific viruses (MNV-1, HEV, PAdV) and IACs labeled with FAM (6-carboxy fluorescein) and VIC (Applied Biosystems, Foster City, CA, USA) probes, respectively. All tests included virus- and IAC-negative controls.

PAdV RT-qPCR

A duplex RT-qPCR was used as described (*28*), including IACs and a carryover contamination prevention system using uracil-N-glycosylase (Roche Molecular Diagnostics, Manheim, Germany). Reactions contained 1× TaqMan Universal PCR Master-Mix (Life Technologies, Branchburg, NJ, USA), 0.9 µM primers, 0.225 µM PAdV TaqMan probe (FAM-labeled), 50 nM IAC probe (VIC-labeled), and 100 copies of PAdV IAC. Ten microliters of nucleic acid extract were added to 25-µL final reaction volumes. Thermocycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

HEV RT-qPCR

A 1-step duplex RT-qPCR was used (*29*) and included IACs. Reactions contained 1× RNA UltraSense reaction mix (Life Technologies), 0.25 µM primers, 0.1 µM probe HEV-P (FAM-labeled), 50 nM IAC probe (VIC-labeled), 1× ROX reference dye, 1 µL RNA UltraSense enzyme mix, and 300 HEV IAC copies. Ten microliters of nucleic acid extracts were added to 20 µL final volumes. Thermocycling conditions were 15 min at 50°C and 2 min at 95°C, followed by 45 cycles of 10 s at 95°C, 20 s at 55°C, and 15 s at 72°C.

MNV-1 RT-qPCR

A 1-step duplex RT-qPCR was adopted (*30*), including IACs. Reaction contained 1× RNA UltraSense reaction mix, 0.2 µM primers, 0.2 µM probe minor groove binder–open reading frame (ORF) 1/ORF2 (FAM-labeled), 50 nM IAC probe (VIC-labeled), 1× ROX reference dye, 1 µL RNA UltraSense enzyme mix, and 600 MNV-1 IAC copies. Ten microliters of nucleic acid extract were added

Table 2. Detection of HEV and indicator virus PAdV in swabs in the pork production chain, Czech Republic, Italy, and Spain, 2010*

Production stage (area), sample type	No. tested	Positive, no. (%)	
		HEV	PAdV
Production (slaughterhouse: carcass dissection and liver removal)			
Water effluents	6	0	0
Workers' hands and aprons	7	4 (57)	5 (71)
Working surfaces	10	6 (60)	6 (60)
Processing (skin removal and sausage preparation)			
Workers' hands	7	2 (29)	1 (14)
Working surfaces	19	4 (21)	0
Points of sale			
Workers' hands and gloves	6	1 (17)	0
Working surfaces	12	1 (8)	0
Hand wash basin tap and toilet edge	12	1 (8)	1 (8)
All samples	79	19 (24)	13 (16)

*HEV, hepatitis E virus; PAdV, porcine adenovirus.

(final reaction volume 20 μL). Thermocycling conditions were 15 min at 50°C and 2 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Data Reporting and Interpretation

For proper results interpretation, we considered 4 signals: 1) target virus, 2) SPCV, 3) target IAC, and 4) SPCV IAC (*31*). With cycle threshold (C_t) ≤45, independently of corresponding IAC C_t, the PCR result was considered positive. With C_t≥45 and corresponding IAC C_t≤45, results were interpreted as negative. When both targets and corresponding IACs showed C_t≥45, reactions were considered failed. When ≥1 replicate target assay (HEV or PAdV) was positive, the sample was considered positive. Absence of SPCV and its IAC signals indicated preamplification processes (virus concentration and extraction) failure (*31*). In the presence of SPCV, SPCV IAC, and target IAC signals, target virus signal absence was conclusively indicating test negative result.

HEV Genotyping

Positive HEV samples were sequence-analyzed amplifying 2 ORF2 regions (348- and 121-bp fragments) (*32*,*33*). Sixteen sequences obtained were examined in GenBank (www.ncbi.nlm.nih.gov/genbank). The 5 shorter 100-bp fragments (3 fecal samples in Italy and 1 liver and meat sample in Czech Republic) were used only to identify genotype or confirm longer sequences. The 11 longer sequences (300 bp) from 4 fecal samples in Italy (GenBank accession nos. JN861803, JN861804, JN861805, JN861806) and 7 sequences from 5 sausages and 2 environmental swabs in Spain (GenBank accession nos. JN903913, JN903914, JN903915, JN903916, JN903917, JN903918, JN903919) also were used for HEV genotyping and subgenotyping. We performed phylogenetic analyses with Bionumerics v6 (Applied Maths, Kortrijk, Belgium) by using the neighbor-joining method with 1,000 replicates with Kimura-2 correction factor.

Results

HEV in Pork Products

We detected HEV RNA in all pork production chain sites in investigated countries, with some differences (Table 1). Overall, HEV RNA was detected in ≥1 samples (feces, liver, meat) from 36 (32%) of 113 pigs examined at slaughterhouses for which all sample types were collected (Table 1). HEV RNA was detected frequently in slaughterhouse samples in Italy and Spain, i.e., 18 (53%) positive samples from 34 animals and 15 (38%) of 39, respectively (Table 1), whereas in Czech Republic, HEV RNA prevalence at slaughterhouses was remarkably lower, i.e., 3 (8%) positive samples from 40 animals. Pig feces showed highest HEV RNA presence (27%), followed by liver (4%) and meat (3%) (Table 1).

Sausage samples from Italy and Spain were collected from processing plants of the same company slaughtering animals or from same company products in local supermarkets. Sausages sampled in Czech Republic were obtained from randomly chosen supermarkets. HEV was detected in 6 (6%) of 93 samples in Spain, whereas 0 of 220 sausages in Czech Republic or Italy were positive.

PAdV in Pork Products

To evaluate possible fecal contamination, PAdV DNA presence (*34*) was determined for all samples assayed for HEV. PAdV was highly prevalent in feces (90%–98%) in investigated countries (Table 1). None of 112 liver samples were PAdV positive, and only 1 of 112 meat samples was PAdV positive, in Italy. In addition, 4 (1%) of 313 sausages (2 from Spain, 1 each from Czech Republic and Italy) were positive for PAdV (Table 1).

Environmental Samples

We collected 41 surface swabs from working surfaces, meat mincers, knives, and other working items at the 3 pork production chain sites. Overall, swab samples were positive for either HEV (11 [27%] of 41) or PAdV (6 [15%] of 41) (Table 2). HEV-positive samples were found more frequently at slaughterhouse (6 of 10) than at processing and points of sale (4 [21%] of 19 and 1 [8%] of 12, respectively) sites, and PAdV was found only in slaughterhouse samples (6 of 10). At slaughterhouses, positive swabs (3 knives, 2 floor, 1 belt surface) contained both HEV and PAdV, indicating potential fecal contamination during slaughtering steps, whereas 0 of 5 HEV-positive samples at processing and points of sale sites was positive for PAdV, disproving possible fecal cross-contamination during later production phases (Table 2). A total of 20 swab samples were taken from workers' hands, gloves, or aprons along the production chain. Overall results were similar to those for working surfaces in slaughtering premises; in fact, 5 (71%) of 7 samples were positive for both HEV and PAdV. Moreover, PAdV was detected in 1 of 2 HEV-positive samples at processing sites (Table 2). Finally, HEV or PAdV was detected in 1 (8%) of 12 toilet swab samples collected at points of sale. The 6 Czech Republic slaughterhouse effluent samples were negative for both PAdV and HEV.

Sequence Analysis

HEV-positive samples were genotyped and sequenced to determine possible animal or human origin of the virus. A total of 9 samples (4 from Italy, 5 from Spain) yielded ≈300-bp sequences and were compared with HEV sequences in public databases. All HEVs belonged to g3.

The 4 HEV-positive samples (HEVSwITFAE09BO10, HEVSwITFAE18BO10, HEVSwITFAE22BO10, HEVSwITFAE11BO10) in feces from slaughterhouses in Italy originated from the same herd and belonged to subtype g3c, sharing 99.4%–100% identical nucleotides. Three of 5 sequences from sausage in Spain belonged to subtype g3f (HEVSwESSAU56, HEVSwESSAU57, HEVSwESSAU60), whereas 2 additional g3 strains (HEVSwESSAU64, HEVSwESSAU66; 99.5% identity) could not be assigned to specific subtypes (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/8/11-1783-FA1.htm), although their sequences were closer to subtype g3c. Two sequences from swabs collected in Spain (HEVSwESADHOC4A, HEVSwESADHOC5A) also belonged to g3f, showing 100% reciprocal nucleotide identity and 89% identity with g3f strains from sausages. Sequences for g3 subtypes from Italy and Spain exhibited ≤85% nucleotide identity, suggesting circulation of different strains in these countries.

Shorter sequences (121 bp, ORF2) also were obtained (*33*) from 3 fecal samples in Italy (100% identity), and 2 additional identical sequences were obtained from liver and meat at a slaughterhouse in Czech Republic. All were confirmed as g3 swine HEV, but further subtyping was not possible because of short sequence length.

Discussion

Pork is a major food source worldwide (*10*), and HEV is widespread among farmed swine and can be transmitted zoonotically, including through pork products (*5*,*10*). We investigated HEV presence throughout the pork production chain in 3 European countries from pigs entering slaughterhouses through processing to retail stores.

To optimize detection sensitivity, in our sampling strategy we assumed low HEV prevalence in pork products and environmental surfaces (*17*,*35*) and involved 3 laboratories. In addition to liver, we selected sausage because it is handled by consumers and is a blend of different meat and slaughtered animals. To maintain consistent results among countries and sample treatment, we validated standardized sampling and molecular procedures by ring test (*36*), including IAC and sample process controls (*26*,*27*,*31*).

Samples analyzed throughout the pork production chain in Italy and Spain were from the same herds from farm to retail sale. In Czech Republic, more points of sale were sampled, thus representing a larger animal population. HEV prevalence in pig feces was similar in Italy and Spain (41% and 38%, respectively), reflecting previous data in these and other European countries (*10*). Conversely, only 3% of pigs from Czech Republic shed HEV. Because of shared protocols and controls, this difference cannot be attributed to different diagnostic sensitivity among partners, which otherwise detected HEV in similar numbers of liver and meat samples.

Lower HEV shedding by pigs in Czech Republic might reflect different farming methods, such as animal housing and separation, herd size, slaughtering age, and/or environmental factors that possibly influence infectious HEV persistence, spread, and transmission. Previous data from Czech Republic (*37*) showed up to 40.0% HEV-positive bile samples from piglets, suggesting infection rates close to shedding rates reported for Italy and Spain. However, that study did not examine HEV fecal shedding, and pigs were only 2–3 months of age. Furthermore, varying prevalence of HEV in pig feces also has been reported in Italy and Spain (*15*,*38*), possibly reflecting differences in farm selection.

The absence of fecal HEV in pigs with HEV-positive liver or bile in Czech Republic suggests that bile concentration in the fecal mass was lower when samples were taken, as might be expected if pigs were fed long before reaching the slaughterhouse. This finding might also help explain the different fecal HEV positivity among countries.

We confirm broad HEV circulation within pig farms and HEV RNA in livers and other pork products (*8*,*17*,*18*). We found HEV prevalence in 3%–6% of liver samples at slaughter, similar to findings in the Netherlands (*18*) but somewhat less than in the United States (11%) (*17*). HEV RNA was present in meat samples only in Czech Republic and Italy (3% and 6%, respectively), whereas sausages were HEV positive only in Spain (6%). This finding might result from low sample numbers but also could reflect different methods for final product preparation by using different meat blends, fat, or liver intentionally or after unintentional cross-contamination.

HEV positivity markedly decreased from feces (27%) to liver (4%), meat (3%), and sausage (2%) but never disappeared during production. However, detection of HEV by RT-qPCR did not conclusively demonstrate viable virus and thus risks to consumers.

PAdV has been confirmed as a suitable indicator of swine fecal contamination during pork production (*28*). Although most pig feces in our study were PAdV positive (90%–98%), PAdV was never detected in liver and detected only occasionally in pork meat (1/33 samples in Italy) or sausage (4/313 samples, all 3 countries). Comparing HEV and PAdV findings, risks for cross-contamination of pork products with swine feces during preparation appear to be low but not absent.

Three of 112 pork meat samples tested were positive for HEV and 1 for only PAdV (Table 1). We have no proof of HEV replication in muscle, and finding HEV RNA in pork products probably reflects endogenous HEV particles in infected liver and/or viremic blood (*39*). Although liver

and bile are usually removed before processing, the HEV genome sporadically detected in meat most likely represents cross-contamination of carcasses during slaughtering, which suggests a need for worker training.

PAdV detection in 1 meat sample and 4 sausages also indicates some fecal contamination during slaughtering, which was, however, similarly low in all countries. PAdV and HEV were not present in the same sausage samples from Spain, and PAdV was detected in fewer samples than was HEV (2 vs. 6), which argues against potential higher risks for fecal contamination in the food chain in Spain. The higher HEV prevalence in sausage in Spain than in Italy or Czech Republic is unclear and deserves further investigation.

The samples from food handlers and the environment in Italy and Spain also identified areas where procedures and information could be implemented. Detection of HEV and PAdV in 60% of floor and working surfaces and 57%–71% of hands and aprons of workers dissecting pigs indicates that the initial production areas (bleeding to evisceration) are at higher risk for fecal contamination and highlight possible hazards to workers.

In the cutting/slicing/chopping areas, we did not detect PAdV in fecal samples. However, HEV detection on hands and surfaces indicates that endogenous HEV can be spread during cutting of liver and meat in industrial premises, requiring cross-contamination control measures. Limited handling might instead explain the single detection of HEV on a butcher's bench at point of sale.

The HEV detected from a supermarket personnel toilet was not genotyped. Thus, its possible origin, i.e., pig versus human, cannot be confirmed.

Our analysis of short sequences confirms presence of only g3 HEV. Sixteen sequences from Italy and Spain were subtyped; g3c was identified as the prevalent strain in Italy, and the less common g3f was noted only in Spain. Two identical HEV sequences in sausage from Spain might represent a novel g3 subtype, similar to a deer g3 HEV strain found in Spain in 2010 (*40*).

In conclusion, our study indicates that HEV is present throughout the pork production chain and that processing does not substantially abate endogenous virus. Consequently, consumers might purchase pork products that contain detectable HEV genome in up to 6.0% of instances, independent of source and country of origin, probably unrelated to fecal contamination during pork processing.

We cannot exclude the possibility that in some pork products HEV was infectious. However, HEV infectious dose for humans is unknown, and viral load in pork might not be sufficient to infect humans efficiently. Storage, processing, and blending of meat from HEV-positive and -negative animals (e.g., sausage) might substantially decrease risks for foodborne infection, possibly explaining why HEV food transmission in Europe seems relatively inefficient. However, consumers should eat only pork that has been thoroughly cooked, particularly liver, and avoid cross-contamination of surfaces and other food by handling pork products, especially offal.

This study addressed only fresh meat or sausage sold within few days of preparation. Future studies should be extended to other pork products, such as salami, which are eaten after short periods of curing and might still contain residual infectious virus.

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Factors Related to Increasing Prevalence of Resistance to Ciprofloxacin and Other Antimicrobial Drugs in *Neisseria gonorrhoeae*, United States

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Learning Objectives

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

- Describe overall patterns of drug resistance stratified by sexual orientation, based on an analysis of data from GISP
- Describe the association of recent travel with drug resistance in MSM and heterosexuals, based on an analysis of data from GISP
- Describe the first appearance of drug resistance in heterosexuals and MSM, based on an analysis of data from GISP.

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Using data from the Gonococcal Isolate Surveillance Project, we studied changes in ciprofloxacin resistance in *Neisseria gonorrhoeae* isolates in the United States during 2002–2007. Compared with prevalence in heterosexual men, prevalence of ciprofloxacin-resistant *N. gonorrhoeae* infections showed a more pronounced increase in men who have sex with men (MSM), particularly through an increase in prevalence of strains also resistant to tetracycline and penicillin. Moreover, that multidrug resistance profile among MSM was negatively associated with recent travel. Across the surveillance project sites, first appearance of ciprofloxacin resistance in heterosexual men was positively correlated with such resistance for MSM. The increase in prevalence of ciprofloxacin resistance may have been facilitated by use of fluoroquinolones for treating gonorrhea and other conditions. The prominence of multidrug resistance suggests that using other classes of antimicrobial drugs for purposes other than treating gonorrhea helped increase the prevalence of ciprofloxacin-resistant strains that are also resistant to those drugs.

Gonorrhea is the second most frequently reported communicable disease in the United States (*1*). Following implementation of a national gonorrhea control program in the mid-1970s, gonorrhea incidence in the Unites States declined by 74.3% from 1975 to 1997 (*2*). However, during this time, the treatment and control of gonorrhea have been complicated by the appearance and spread of antimicrobial drug resistance in *Neisseria gonorrhoeae* (*3*,*4*). Cephalosporins and fluoroquinolones became recommended for treating gonorrhea in the United States in 1993 (*5*), prompted by the rise in resistance to penicillins and tetracyclines, the antimicrobial agents previously recommended for treatment. In the past decade, several countries reported a sharp rise in the proportion of *N. gonorrhoeae* strains resistant to fluoroquinolones (QRNG) (*1*,*4*,*6*–*9*). Increasing QRNG prevalence in the United States led to a series of changes in treatment recommendations away from fluoroquinolones. In 2002, the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) recommended that cephalosporins be used instead of fluoroquinolones as first-line treatment for gonorrhea acquired in Hawaii or California (*8*); in 2004, fluoroquinolones were no longer recommended as a first line of treatment for infected men who have sex with men (MSM) and, as of 2007, fluoroquinolones were no longer recommended, and cephalosporins were the only recommended class of drugs for treatment of gonococcal infections in the United States (*10*). As treatment failures for oral cephalosporins are documented in Asia (*3*,*11*,*12*), and strains with reduced susceptibility to cephalosporins have begun to appear in the West, including the United States (*3*,*13*,*14*), the origins and causes of increased drug resistance in *N. gonorrhoeae* need to be understood in order to improve control measures for emerging resistant strains and thereby maintain the utility of the few existing antimicrobial drug options for treatment of gonorrhea.

We examined several hypotheses to explain the increased prevalence of QRNG during 2002–2007, with the objective of identifying principles that may be informative for predicting and preventing the spread of resistance to cephalosporins or other drug classes. We considered the role of travel as a contributing factor for the growth of resistance, in heterosexual men and MSM. Having observed a difference in travel patterns between men of differing sexual orientation, we hypothesized about a potential role that multidrug resistance may play in the propagation of that resistance profile. Additionally, we studied the association between the times of first appearance of ciprofloxacin resistance in heterosexual men and in MSM in several different Gonococcal Isolate Surveillance Project (GISP) sites, examining the possibility that resistance spread in persons of one sexual orientation led to the appearance of resistance in persons of another sexual orientation.

Methods

Data

GISP is a national sentinel surveillance system for detection of gonococcal antimicrobial drug resistance that includes sexually transmitted disease (STD) clinics in 25–30 US cities each year, 4–5 regional laboratories, and CDC. Each month, the first 25 urethral gonococcal isolates were collected from men attending the clinics and submitted to regional laboratories for antimicrobial drug susceptibility testing. Patient data were abstracted from medical records.

Methods for *N. gonorrhoeae* susceptibility testing have been described (*15*). Briefly, Difco GC base medium (Becton Dickinson, Sparks, MD, USA) was inoculated with 10^4 CFU. Antimicrobial drug susceptibilities were determined by the agar-dilution technique with GC-II base medium (Becton Dickinson). Control strains (F-18 [ATCC 49226], F-28, P681E, CDC 10328, CDC 10329, SPJ-15, and SPL-4) were included with each susceptibility run.

Def nitions

Sexual orientation is a standard GISP data element and is specified as follows: heterosexual men (82.9% of GISP patients during 1998–2007), MSM (13.8%), or bisexual men (3.4%). Temporal analyses of antimicrobial drug resistance, stratified by the infected person's sexual orientation were largely restricted to heterosexual men and MSM. Travel history has been collected in GISP since mid-2002, with the aim of exploring whether acquisition of gonorrhea was local or occurred elsewhere. Until January 2004, travel history was defined as self-reported travel

to Hawaii or outside the United States within the past 60 days; after that, travel history pertained to travel outside the state where the clinic was located. The above change was introduced because the aim of the travel variable was to assess the scope of nonlocal acquisition of gonorrhea. By 2004, QRNG was established in the United States, and it appeared reasonable to combine all out-of-state travel (both outside and within the United States) into the category of potential nonlocal acquisition.

A strain's resistance to ciprofloxacin was defined as an MIC ≥1μg/mL, per Clinical and Laboratory Standards Institute criteria (*16*). Temporal patterns of ciprofloxacin resistance among heterosexual men, MSM, and bisexual men are summarized in Figure 1. For strains resistant to penicillin and tetracycline, resistance was defined as an MIC ≥2 μg/mL. The term "triply resistant gonococci" was defined as isolates having resistance to ciprofloxacin, penicillin, and tetracycline. We use the word "type" to mean a defined pattern of susceptibility or resistance to the drugs of interest; by this definition, for penicillin, tetracycline, and ciprofloxacin, there are 2^3 (8) different types.

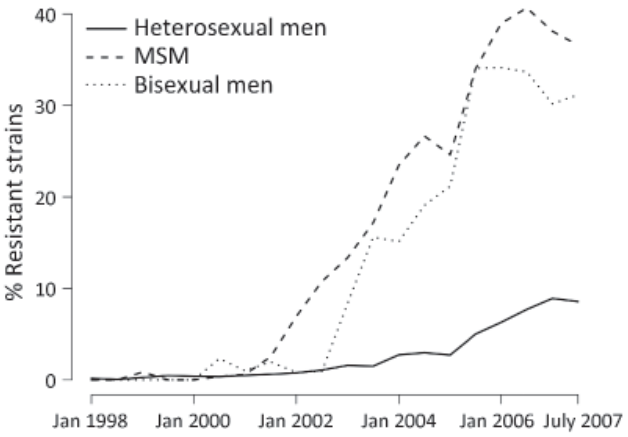

Figure 1. Semiannual prevalence of resistance to ciprofloxacin in *Neissseria gonorrhoeae* isolates from the Gonococcal Isolate Surveillance Program for heterosexual men, men who have sex with men (MSM), and bisexual men. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/11-1202-F1.htm).

Prevalence of Resistance Phenotypes by Patient's Sexual Orientation, Time, and Site

The proportion of isolates with various resistance phenotypes (sensitive or resistant to ciprofloxacin, penicillin, and tetracycline) was computed by half-year, separately for heterosexual men and MSM. Among GISP patients in each group who were infected with particular types, the proportion with a travel history was plotted for each half-year period.

Travel History and Drug-Resistance Prof les

For certain gonococcal types (the triply resistant type and the monoresistant [ciprofloxacin resistant, tetracycline- and penicillin-sensitive] type), we studied the association between the presence of this type and the report of recent travel. We constructed 4 separate multivariate logic regression models: by sexual orientation of patient (MSM or heterosexual) and resistance type (multi- or single-resistant types); thus, 2 × 2 = 4 models. In each model (applied to all GISP specimens from persons with a given sexual orientation), the binary outcome Y is the presence of that resistance type (1 if the specimen is of that type), and the covariates are M (month of sample collection) and RTH (presence of recent travel history) (1 if travel history is present). Thus,

$$P(Y=1)=\frac{e^{\beta_0+\beta_{RTH}\cdot RTH+\beta_M\cdot M}}{1+e^{\beta_0+\beta_{RTH}\cdot RTH+\beta_M\cdot M}}$$

The regression coefficient β_{RTH} is therefore the logarithm of the odds ratio for carrying a strain of the selected type for a person with recent travel history versus that of a person with no recent travel history. The regression coefficient β_M is the logarithm of the odds ratio for carrying a strain of the selected type on month $M + 1$ versus month M. The latter can also be interpreted as the relative monthly growth rate for the selected type (*17*). Using the model in (*17*), suppose that the selected type grows exponentially at a monthly rate of r_1, and the other types combined grow at a monthly rate r_2 in the population. Thus the population presence of the selected type on month M is $c_1 exp(r_1 \times M)$, and the presence of other types is $c_2 exp(r_2 \times M)$. The probability that a random specimen on month M is of the selected type is therefore

$$\frac{c_1 exp(r_1\cdot M)}{c_1 exp(r_1\cdot M)+c_2 exp(r_2\cdot M)}$$

The odds ratio for carrying a strain of the selected type on month $M + 1$ versus month M is then $exp(r_1 - r_2)$, where $r_1 - r_2$ is the relative growth rate for the selected type.

Because travel information was missing from some sites, we restricted the model to sites from which travel information was available for >50% of GISP patients. We conducted the regression analysis under the scenario of omitting the persons with missing travel information and through multiple imputations for those persons (*18*). Because missing values in various sites were largely found in consecutive temporal batches, we assumed that missing information was not correlated with presence of the chosen type or recent travel history during those periods at those sites. We imputed history of recent travel at random, using available data to calculate the proportion of GISP patients that were recent travelers by site and sexual orientation

within a 1-year interval centered at the time of each missing sample. We performed 500 joint imputations, and the mean and variance of the regression coefficients were estimated as described (*18*, p. 86). The Hosmer and Lemeshow goodness-of-fit test was applied to the subset of the data for which travel information was available (>83% of data in each model).

Because data on travel history were lacking before mid-2002, the period we selected for the logistic regression analysis was October 2002–December 2006. Fifteen GISP sites, representing 64.6% of all MSM GISP patients during that period, had travel information on >50% of MSM patients (83.5% of patients from those sites). Sixteen GISP sites, representing 47.6% of all heterosexual GISP patients during that period, had travel information on >50% of heterosexual patients (89.8% of patients from those sites). For the monoresistant type of *N. gonorrhoeae* in MSM, the period selected was October 2002–December 2004 (by which point the growth in prevalence of that type appears to have stopped; Figure 2, panel A). Thirteen GISP sites, representing 45.9% of all MSM GISP patients during that period, had travel information for >50% of MSM patients (92.9% of patients from those sites).

First Appearance and Persistence of Resistance in Heterosexual Men and MSM

We examined the time of first appearance of ciprofloxacin-resistant *N. gonorrhoeae* strains in heterosexual men and in MSM at GISP sites during 1998–2006. Only sites that reported continuously during those years were examined. Because low levels of ciprofloxacin resistance, particularly in strains from heterosexual men, were detected in the 1990s (and those are possibly attributable to different genetic lineages than those that caused the rise in resistance levels in the 2000s), we only looked at sites where resistance first appearance after 2000; thus, the first appearance of resistance in GISP was more likely to correspond to appearance of the new lineage at that site, rather than persistence of the old strain.

Results

Overall Patterns of Drug Resistance Stratif ed by Patient's Sexual Orientation

Figure 1 shows the prevalence of ciprofloxacin resistance in GISP isolates during 1998–2007 for heterosexual men, MSM, and bisexual men. Resistance increased faster and reached higher levels among MSM (42.5% at peak) compared with patterns for heterosexual men (9.1% at peak), with resistance levels among bisexual patients being consistently somewhat lower than among MSM (peaking at 37.7%).

During the period of rapid expansion of fluoroquinolone resistance of *N. gonorrhoeae* among MSM, the type resistant to ciprofloxacin, tetracycline, and penicillin (hereafter, triply resistant) was the fastest growing class of ciprofloxacin-resistant types among MSM (Figure 2, panel A), reaching 25.6% of all MSM GISP specimens at the peak. The type resistant to ciprofloxacin and sensitive to tetracycline/penicillin (monoresistant type) also experienced substantial growth, on par with the type resistant to ciprofloxacin and tetracycline (peaking at 6.7% and 8.5%, respectively, for all MSM GISP specimens), though the growth in the prevalence of those 2 types appears

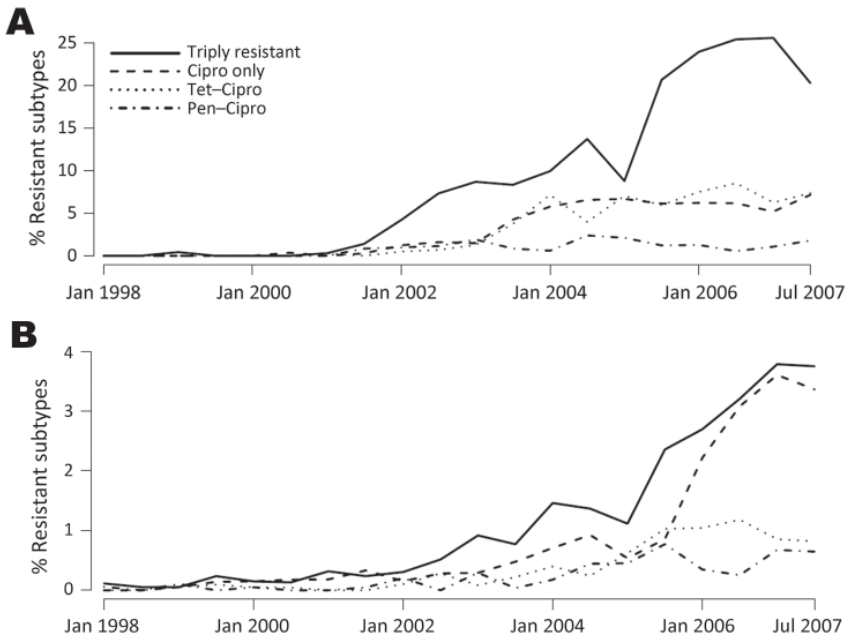

Figure 2. Semiannual prevalence of the ciprofloxacin (Cipro)–resistant type of *Neisseria gonorrhoeae* for men who have sex with men (A) and heterosexual men (B), stratified by resistance characteristics to tetracycline (tet) and penicillin (pen). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/11-1202-F2.htm).

to have stalled earlier than the growth in the prevalence of the triply resistant type.

Triple resistance has been the most common form of ciprofloxacin resistance since mid-2001 among heterosexual men (Figure 2, panel B), peaking at 3.8% of GISP specimens from heterosexual men, and it was followed closely by the monoresistant type, peaking at 3.6% of GISP specimens from heterosexual men.

Recent Travel and Resistance among MSM and Heterosexual Men

To test the hypothesis that travel contributed to the increase in resistance to ciprofloxacin, we calculated the proportion of persons in the GISP dataset who reported recent travel (see Methods), stratified by the resistance pattern of their isolate and by patient's sexual orientation (Figure 3).We performed multivariate logistic regression analysis to determine the association between particular types (triply resistant and monoresistant, for MSM and for heterosexual men), recent travel, and time. The Table gives the estimates of the regression coefficients (with 95% confidence bounds) for each choice of a resistance type and patient's sexual orientation; interpretation of those coefficients is described in Methods.

Overall, triple resistance prevalence in isolates infecting MSM grew rapidly despite negative association with recent travel (odds ratio [OR] 0.72 for a GISP specimen to be triply resistant from MSM with a recent travel history versus those without a recent travel history, 95% CI (0.52–0.99)), while travel contributed to the growth of triple resistance levels in heterosexual men (corresponding OR 3.22, 95% CI [2.24–4.62]). The positive association between monoresistance and recent travel suggested by Figure 3 was borderline statistically significant for heterosexual men (corresponding OR 1.72, 95% CI [0.98–3.03]) and not statistically significant for MSM.

First Appearance of Resistance in Strains from Heterosexual Men and MSM

To assess the extent to which the ciprofloxacin resistant type may bridge (i.e., spread across from) populations that differ in sexual orientation, we compared the month of first appearance for ciprofloxacin resistance in heterosexual men and MSM (Figure 4). Sharing of the resistant strains would be expected to result in correlated timing of the appearance of the resistant type, with sites where resistance that appeared early in MSM also appeared early in heterosexual men. Assuming that sampling was identical for MSM and heterosexual men, then the ordering within a city would provide evidence about the possible route of transmission; however, different proportions of MSM and heterosexual men at each site mean that correlated timing is interpretable as evidence of transmission, but the direction of transmission is difficult to determine.

The months of first appearance for ciprofloxacin resistance in 12 cities by patient's sexual orientation are documented in Figure 4. The Spearman rank correlation coefficient for the times of first appearance among MSM and heterosexual men at different GISP sites is 0.79, p = 0.002. This finding suggests that for a pair of GISP sites A and B, if resistance in isolates from persons of one sexual orientation appeared earlier at site A than at site B,

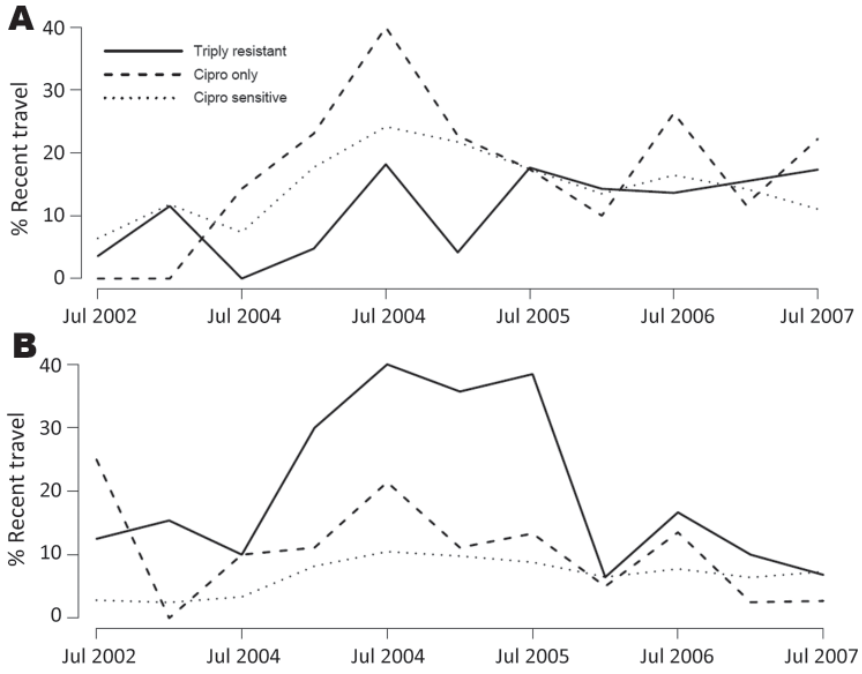

Figure 3. Semiannual prevalence of recent travel history in different type carriers for men who have sex with men (A) and heterosexual men (B). Ciprofloxacin (Cipro) sensitive, MIC ≤0.0625 μm/L. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/11-1202-F3.htm).

Table. Regression coefficients and the p values for 4 groups, according to resistance type and patient's sexual orientation

Model*	β_0 (95% CI)	β_{RTH} (95% CI)	β_M (95% CI)	Hosmer-Lemeshow p value
Men who have sex with men				
Triply resistant	–2.48 (–2.72 to –2.24)	–0.328 (–0.650 to 0.005)	0.03 (0.023 to 0.037)	0.19
Monoresistant	–3.75 (–4.42 to –3.08)	0.51 (–0.18 to 1.2)	0.046 (0.006 to 0.086)	0.18
Heterosexual men				
Triply resistant	–4.72 (–5.08 to –4.36)	1.17 (0.81 to1.53)	0.022 (0.011 to 0.033)	0.35
Monoresistant	–5.05 (–5.49 to –4.60)	0.543 (–0.02 to 1.11)	0.018 (0.005 to 0.032)	0.69

*Logistic model given by equation 1.

resistance in persons of another sexual orientation was also likely to appear earlier at site A than at site B.

Discussion

A common explanation for the rise in QRNG is repeated importation (*9*,*19*); some evidence that importation played a role in the initial growth of resistance in the United Kingdom can be seen in data from the Gonococcal Resistance to Antimicrobials Surveillance Programme (*20*). However, genetic evidence for a combination of importation, followed by internal proliferation of fluoroquinolone resistance in *N. gonorrhoeae,* has been reported (*21*,*22*). Similarly, GISP data suggest that whereas importation may play a substantial role when resistance levels are relatively low, the prevalence of resistance for certain types increases further for certain types without the aid of importation. Indeed, for heterosexual men, for whom levels of ciprofloxacin resistance were relatively low, resistance of the GISP gonococcal isolates was positively associated with patient's recent travel. Nevertheless, prevalence of QRNG was much higher in MSM, with the triply resistant type being the most prominent component in the rise of ciprofloxacin resistance in GISP isolates. Starting at the end of 2002, when GISP travel data became available and 7% of GISP isolates from MSM were triply resistant, prevalence levels for that type of isolate from MSM further increased along with negative association with recent travel. Given the lack of travel data before mid-2002, we cannot, however, exclude the possibility that the initial growth in the prevalence of the triply resistant type in MSM was aided by importation.

In addition, using ciprofloxacin for gonorrhea treatment may have contributed to the increase in ciprofloxacin resistance. Indeed, ciprofloxacin resistance has declined significantly since 2007 when CDC indicated that fluoroquinolones were no longer a recommended treatment for gonorrhea (*23*). At the same time, although CDC stopped recommending fluoroquinolones for gonorrhea treatment in California and Hawaii in 2002 and nationally for MSM in 2004, ciprofloxacin resistance continued to increase in California and among MSM for years after those recommendations were made. However, adherence to these recommendations might have been poor in non-GISP settings because the oral cephalosporins recommended for gonorrhea treatment were not marketed in the United States during the study period (*12*). Community usage of fluoroquinolones for other indications might have also contributed to the rise in ciprofloxacin resistance in *N. gonorrhoeae*. Fluoroquinolones were the most commonly prescribed class of antimicrobial agents in the United States around the study period (*24*).

The prominence of multidrug resistance, particularly among MSM, combined with the negative association with recent travel in MSM starting in late 2002, suggests that this type has proliferated without the aid of repeated importation. Resistance to a variety of antimicrobial agents for that type raises the possibility that usage of nonfluoroquinolone antimicrobial drugs could have played a role in propagation of multidrug resistance. Data reported by Kent et al. (*25*) show high rates of asymptomatic *N. gonorrhoeae* infection among MSM attending STD clinics in San Francisco. Some of those patients likely received antimicrobial drugs to treat conditions other than gonorrhea. For such persons, multidrug resistant *N. gonorrhoeae* strains are more likely

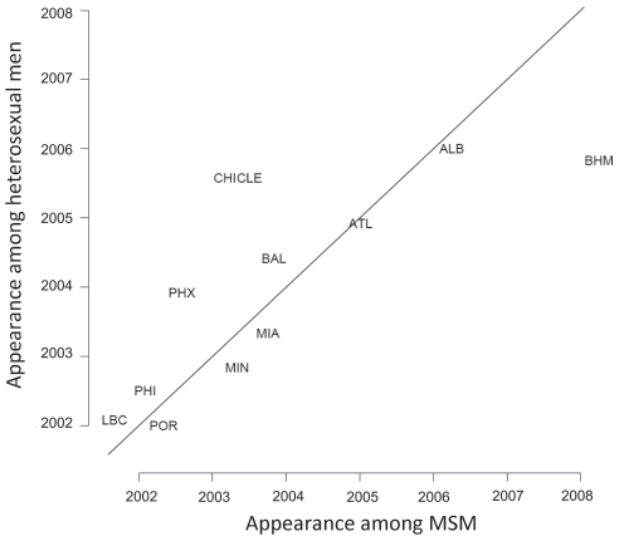

Figure 4. First month of appearance of ciprofloxacin resistance in *Neisseria gonorrhoeae* isolates from heterosexual men and from men who have sex with men (MSM). ALB, Albuquerque, NM; ATL, Atlanta, GA; BAL, Baltimore, MD; BHM, Birmingham, AL; CHI, Chicago, IL; CLE, Cleveland, OH; LBC, Long Beach, CA; MIA, Miami, FL; MIN, Minneapolis, MN; PHI, Philadelphia, PA; PHX, Phoenix, AZ; POR, Portland, OR.

to survive the antimicrobial drug treatment, giving the multidrug-resistant type a selective advantage over other *N. gonorrhoeae* types. Further studies are needed to assess the scope of antimicrobial drug use for various indications, including STD treatment, in asymptomatic *N. gonorrhoeae* infections and the magnitude of the possible selective advantage.

The presence of bisexual men in the GISP data suggests a possibility for passage of resistant strains between the MSM and heterosexual communities. To address this question, we explored whether associations could be shown between the time of first appearance of ciprofloxacin resistance in heterosexual men and in MSM at different GISP sites. The timing of first appearance is highly correlated between heterosexual men and MSM, with a median delay (in either direction) of 5.5 months, despite a 5-year range of times of first appearance across different sites. Moreover, this correlation was observed in different geographic regions at different times, suggesting that resistant strains spread from 1 group to the other within each city. Given that heterosexual men are much more numerous than MSM among the GISP patients, the fact that resistance was detected first in MSM in half of the cities may reflect the more rapid rate of increase in MSM than in heterosexual men (described previously) and possibly also that the resistant type truly appeared first in MSM in most cities but was first recorded in heterosexual men in some places; our data do not enable us to disentangle these 2 contributions.

Our study had several limitations. The use of travel data to infer the role of out-of-site acquisition of resistance is complicated by several factors: travel data go back only to mid-2002; until 2004 "travel" signified the presence of travel abroad or to Hawaii within the past 60 days, and later it was changed to include any out-of-state travel. Moreover, during the periods for which our logistic regression analysis was performed, travel data were missing for many GISP patients. For periods when travel data were missing, we note that our regression analysis included only clinics that had travel information available for >50% of patients of the selected sexual orientation. Moreover, multiple imputations were used to deal with the missing travel data (*18*). Of course, many gonorrhea cases are not captured by GISP, and travel by these infected persons may have been critical in the introduction of the resistant strains.

Although gonorrhea patients with recent travel history may not have acquired their infection during travel, the negative association between recent travel history and triple resistance argues against acquisition through travel as a driver of increasing prevalence of that type among MSM. Positive association with recent travel for other types argues that acquisition through travel played a role in the increase in resistance level of the corresponding type, although travel data do not enable us to quantify the extent of that role.

Our study on the rise of ciprofloxacin resistance sheds some light on the possible mechanisms that might contribute to the emergence of cephalosporin resistance. Importation of resistance and acquisition through domestic travel are likely to play a substantial role in the initial rise in resistance levels. Analysis of the rise in triple resistance levels in isolates infecting MSM suggests that resistance levels for some types, particularly the multidrug resistant type, may increase through selective advantage in domestic transmission. This increase is probably associated with antimicrobial drug usage; repeated importation is not required. Several studies (*13*,*14*,*26*) have shown that decreased *N. gonorrhoeae* susceptibility to cephalosporins such as cefotaxime is associated with resistance to other antimicrobial agents. This finding suggests that proliferation of multidrug resistance is also possible for cephalosporin-resistant *N. gonorrhoeae*. Correlation between appearance times in persons of the 2 sexual orientations further strengthens the argument that prevention efforts should be geared toward both groups, particularly when the appearance of resistance is detected in either population.

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Comparison of Enzootic Risk Measures for Predicting West Nile Disease, Los Angeles, California, USA, 2004–2010

Jennifer L. Kwan, Bborie K. Park, Tim E. Carpenter, Van Ngo, Rachel Civen, and William K. Reisen

In Los Angeles, California, USA, 2 epidemics of West Nile virus (WNV) disease have occurred since WNV was recognized in 2003. To assess which measure of risk was most predictive of human cases, we compared 3 measures: the California Mosquito-Borne Virus Surveillance and Response Plan Assessment, the vector index, and the Dynamic Continuous-Area Space-Time system. A case–crossover study was performed by using symptom onset dates from 384 persons with WNV infection to determine their relative environmental exposure to high-risk conditions as measured by each method. Receiver-operating characteristic plots determined thresholds for each model, and the area under the curve was used to compare methods. We found that the best risk assessment model for human WNV cases included surveillance data from avian, mosquito, and climate sources.

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) is amplified within a mosquito–bird cycle, with tangential transmission to equids and humans (*1*). Since the introduction of WNV into Los Angeles, California, USA, in 2003, our research (*2–5*) has focused on surveillance indicators for enzootic WNV transmission and prediction of human cases. The Greater Los Angeles County Vector Control District (GLACVCD) serves >6 million of the ≈10 million residents of Los Angeles County and conducts year-round surveillance for WNV activity (*6*). In addition to having a robust surveillance dataset, Los Angeles County is a suitable location for evaluating environmental risk because the large human population enables the sensitive detection of dead birds (*7*), increases opportunities for human–vector contact, and experienced 2 outbreaks during the study period (*6*).

We compared the predictive ability of 3 measures of human risk by using time-series graphs, sensitivity, specificity, positive predictive value (PPV), and concordance between human case onset and states of high risk based on enzootic transmission during 2004–2010. We believed that for operational decision support a successful risk measure should correctly 1) identify periods of low risk when few or no cases occur, 2) predict high or increased risk before human cases occur, and 3) identify periods of high risk concurrent with the occurrence of human cases.

The 3 measures of risk we compared were the California Mosquito-Borne Virus Risk Assessment (CMVRA), the vector index, and the Dynamic Continuous-Area Space-Time (DYCAST) system. The CMVRA (*8*) calculates risk on the basis of ranks of environmental variables for enzootic transmission and is used by health agencies throughout California to measure risk. At its inception, the CMVRA was evaluated retrospectively for its ability to detect cases of Western equine encephalomyelitis virus (family *Togaviridae*, genus *Alphavirus*) and St. Louis encephalitis virus (family *Flaviviridae*, genus *Flavivirus*) in California during low-, medium- and high-risk seasons (*9*). Additional assessment of the ability of CMVRA to track WNV cases in Bakersfield, California, produced impressive results during 2004 and 2007 (*10,11*).

The second method was the vector index, an estimate of the number of infected mosquitoes collected per trap-night. This index successfully determined human risk in Colorado (*12*,*13*) and is used by the Colorado Department of Public Health and Environment (www.cdphe.state.co.us/dc/zoonosis/wnv/wnvsentinel.html).

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The third method was the DYCAST (*14*) system, which provides an assessment of risk in time and space by using reports of dead birds from the California Department of Public Health Dead Bird Hotline. This risk estimate differs from the previous 2 in that the spatial scale is fine (0.44 km^2 grid cells), it is computationally more complex, and it does not rely on laboratory test results (*15*).

Understanding the characteristics of risk estimates to determine the best predictive measure for human cases is needed for several reasons. First, reducing the rate of false-positive results will reduce message fatigue associated with repeated false warnings of high-risk conditions. Second, increasing the proportion of high-risk areas correctly identified (sensitivity) can reduce the costs associated with emergency mosquito control by correctly focusing timely intervention. Third, a qualitative assessment of risk estimates that incorporates different variables for enzootic transmission enables understanding of the ability of different assemblages of surveillance data for predicting human risk. Overall, a better understanding of the tools used in decision support for emergency intervention can only improve the protection of human health.

Materials and Methods

The epidemiology of WNV in Los Angeles has been described in detail (*6*). Methods used for data collection for each risk assessment tool are summarized briefly below and in detail (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1558-Techapp.pdf).

CMVRA

The CMVRA (*8*) calculated risk on the basis of average daily temperature, mosquito abundance and infection, counts of WNV RNA–positive dead birds, and sentinel chicken seroconversions over successive 2-week periods. Each variable was assigned to quintile ranks, and these categorical values were averaged to calculate a final risk estimate. Thresholds ≤2.5 were considered low-risk (normal season) conditions; those 2.6–4.0 were considered medium-risk (emergency planning) conditions; and those ≥4.1 were considered high-risk (epidemic) conditions.

Details of sampling, laboratory testing, and risk calculation are summarized in the online Technical Appendix. In the current study, temperature data were aggregated from the National Aeronautics and Space Administration Terrestrial Observation and Prediction System (*16*) at a 1-km^2 scale for the GLACVCD jurisdiction. Abundance anomalies for *Culex pipiens quinquefasciatus* mosquitoes collected by gravid traps (*6*) were calculated by comparing current 2-week estimates to 5-year averages for the same period. WNV infection incidence in *Cx. p. quinquefasciatus* mosquitoes was calculated from mosquito pool data by using the Excel (Microsoft, Redmond, WA, USA) add-in developed by Biggerstaff (*17*). Dead birds reported by the public and testing positive for WNV RNA and sentinel chicken seroconversions were ranked according to frequency and scale of occurrence for the broad region (Los Angeles County) and the specific region (within GLACVCD jurisdiction). Reports of sentinel chicken seroconversions from Los Angeles County outside the GLACVCD boundary were found on the California West Nile virus Web site (www.westnile.ca.gov). Human cases, recorded by the Los Angeles County Department of Public Health, Acute Communicable Disease Control, were excluded from the current risk calculations because they were used as an outcome measure.

Vector Index

The vector index also was calculated for 2-week time steps by using abundance (numbers per gravid trap per night) and infection incidence for *Cx. p. quinquefasciatus* mosquitoes collected by gravid traps by using the bias-corrected maximum-likelihood estimate (*6*) (online Technical Appendix). Usually the species-specific maximum-likelihood estimate is multiplied by female mosquito abundance measured by CO_2 trap counts to yield an arbovirus equivalent of the entomologic inoculation rate in malaria epidemiology (*18*). Vector index estimates were stratified into frequency percentiles by using SAS version 9.1 software (SAS Institute Inc., Cary NC, USA), and the percentiles were assessed individually for their efficacy for predicting human cases.

DYCAST

For DYCAST, 0.44-km^2 grid cells were overlaid onto the Los Angeles County study area. There were 22,687 grid cells in Los Angeles County, but only 6,666 grid cells were within the GLACVCD boundary. We assessed the DYCAST risk estimates by using a predetermined Knox test significance threshold of ≈0.10 = high risk. The Knox test statistically delineated significantly positive groups of grid cells into clusters or hot spots. Unlike the other 2 methods, the DYCAST model assessed risk on a daily basis, providing a time and location of high risk on the basis of the spatial grouping of the number of reports of dead birds; data were independent of a predetermined spatial allocation of sampling assets and laboratory diagnostics. To make this method comparable with the previous 2 methods, we selected the minimum DYCAST value by grid for each 2-week period. The DYCAST model then was assessed by using daily and 2-week aggregations.

Another unique feature of the DYCAST model is the spatial resolution. The other 2 methods provide an assessment of high-risk conditions that can be anywhere within the GLACVCD boundary, whereas DYCAST delineates high-risk conditions within a defined space.

Again, to make our assessments comparable, we aggregated DYCAST high- and low-risk cells spatially by week, up to the spatial limit imposed by the GLACVCD boundary (6,666 cells). The new spatial aggregates were compared with human case occurrence to determine an optimal number of grid cells needed to establish a high-risk area. This comparison was performed by constructing a receiver operator characteristic (ROC) curve of the plotted sensitivity versus 1 – specificity for all aggregated cell counts.

Reports of Human Cases

Reports of laboratory-confirmed human cases, compiled by the Acute Communicable Disease Control program of the Los Angeles County Department of Public Health and occurring within GLACVCD, included West Nile fever (WNF) and West Nile neuroinvasive disease (WNND) diagnoses and asymptomatic viremic blood donors. Onset dates for symptomatic persons were adjusted backward 10 days to account for the intrinsic incubation period (*19*,*20*). Seven blood donors with viremia later became symptomatic for WNV disease and were added as WNV cases; the mean time from donation to symptom onset was 6.2 days (SD ±6.14, median 3.5). To account for earlier detection, the infection dates for all viremic blood donors were adjusted backward 4 days (10 latent days minus 6 induction days). As reported for Los Angeles County (*6*), the percentage of WNND among all reported WNV infections increased significantly over time because of reduced physician requests for laboratory testing for febrile illness, thereby reducing the total number of human cases reported. In addition, unpublished data from elsewhere in California also indicate that relatively few persons hospitalized with neuroinvasive disease are tested for WNV, which possibly further reduced recent estimates of WNV-associated human disease. Because mosquito and public health agencies respond to reports of human cases regardless of diagnosis, we chose to use these data in the current analyses. The Institutional Review Board at the University of California, Davis, approved protocols for using human surveillance data (approval no. 201018171-1).

Analysis

Time-series graphs of the CMVRA and the vector index were plotted with human cases to depict which attained high-risk thresholds before human cases occurred. A true or false-positive finding was a time period identified as high risk during which ≥1 or 0 human infections occurred, respectively. A true-negative period was a period identified as low risk and during which no human infections occurred; conversely, false-negative periods were identified as low-risk periods when human infections occurred. Sensitivity was calculated as the proportion of high-risk periods correctly identified; specificity was the proportion of low-risk periods correctly identified (*21*). The PPV, likelihood ratio positive, and likelihood ratio negative were calculated as measures of relative precision (*22*).

ROC curves were plotted to define optimum response thresholds. The area under the curve (AUC) was calculated to compare the 2 methods. ROC and AUC calculations were performed by using SAS version 9.1 and the Macro %ROC (http://support.sas.com/kb/25/addl/fusion25017_5_roc.sas.txt).

With the above analyses providing information about the accuracy of each risk assessment, a separate case-crossover study was performed by using the known onset information to create an estimate of the relative risk of acquiring WNV during high-risk periods. Illness onset dates for case-patients and asymptomatic viremic blood donors were lagged backward as described above. Mantel-Haenszel relative risks were calculated to determine whether high-risk values were significantly associated with human infection (*23*–*25*). Mantel-Haenszel relative risks were calculated by using the proportion of high-risk periods before estimated infection as the expected frequency of exposure and the concordance odds of disease transmission occurring during a high-risk period by each model and threshold. Data aggregation and zonal statistics were performed by using PostgreSQL 8.3.7 and PostGIS 1.3.1.

Results

CMVRA

Risk estimates (Figure 1, panel A) consistently reached emergency planning thresholds (threshold ≥2.6) before human case detection. In 2004, epidemic thresholds (≥4.1) were reached by mid-August (Table 1) after 39 human cases had been reported. During the second epidemic in 2008, risk assessments reached epidemic thresholds after 8 human cases were identified. Using the epidemic threshold, we identified 13 true-positive intervals, 0 false-positive intervals, 151 true-negative intervals, and 28 false-negative intervals. Estimates using this method were driven by ranks for environmental conditions and infections in dead birds, followed by mosquito infection rates and abundance. Antecedent sentinel chicken seroconversions consistently ranked lowest on the 5-point scale until human cases occurred because they were temporally concordant (*26*).

Using the emergency planning threshold, we identified 40 true-positive intervals, 28 false-positive intervals, 123 true-negative intervals, and 1 false-negative interval. Although there were more false-positive intervals, they represented high-risk periods before the onset of human cases because the threshold reached ≥2.6 at least 2 weeks before human cases occurred in all study years except 2008

(Table 1). On the basis of the advance warning that this risk estimate provided and the increase in sensitivity (Table 2), the 2.6 threshold was a better threshold for epidemic prediction.

We calculated sensitivity and specificity separately for each study year by using the 2.6 emergency planning threshold (Table 3). Use of this test validity revealed that sensitivity, i.e., correctly identified high-risk periods, dipped in 2005, whereas specificity, i.e., proportion of correctly identified low-risk periods, was lowest in 2006 and 2008.

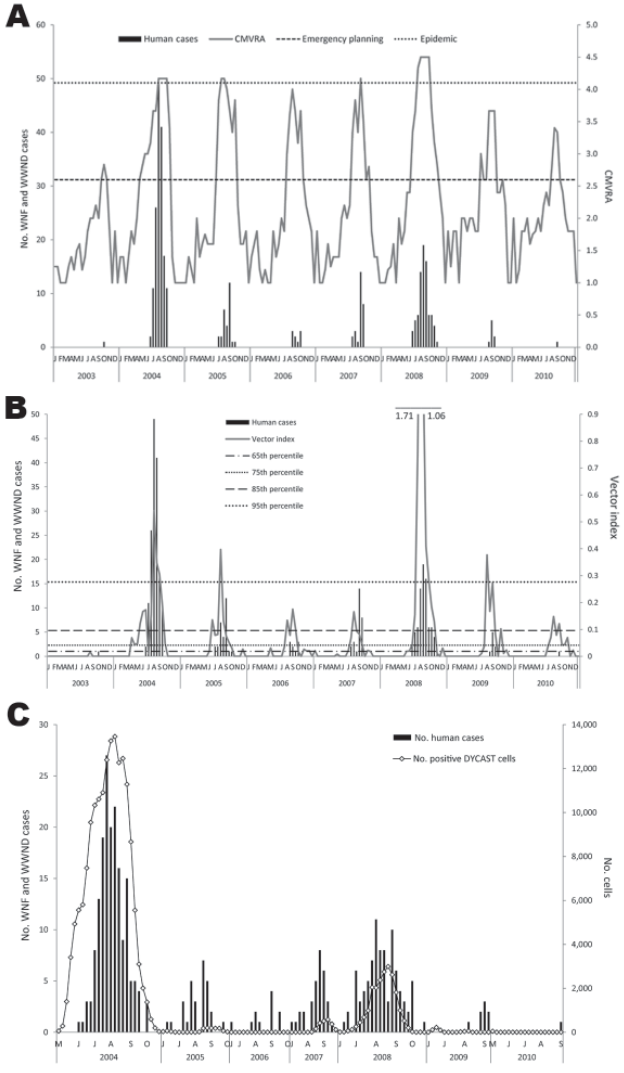

Figure 1. Comparison of risk estimates to human case occurrence for WNF and WWND, Los Angeles, California, USA. A) CMVRA estimates by using *Culex pipiens quinquefasciatus* mosquitoes collected by gravid traps. Dashed lines refer to the risk assessment thresholds of emergency planning at 2.6 and epidemic at 4.1. B) Vector index calculated with *Cx. p. quinquefasciatus* collected by gravid traps. Lines show risk levels discussed in text. C) Weekly counts of positive DYCAST grid cells compared with human case counts. WNF, West Nile fever; WWND, West Nile neuroinvasive disease; CMVRA, California Mosquito-Borne Virus Risk Assessment; DYCAST, Dynamic Continuous-Area Space-Time system.

Vector Index

Vector index estimates (Figure 1, panel B) were calculated biweekly for the entire study period for *Cx. p. quinquefasciatus* mosquito collections and were driven exclusively by mosquito infection incidence. Using the 65th percentile (0.018) as the threshold, we identified 38 true-positive, 37 false-positive, 116 true-negative, and 1 false-negative intervals. The frequency distribution of the vector index was highly right skewed and could not be evaluated at lower percentiles because all other percentiles were 0. The vector index increased and remained >0.095 (85th percentile) 4 weeks before the onset of human cases in 2004, 2009, and 2010 and 2 weeks before case onset in 2005 and 2006 (Figure 1, panel B). The sensitivity and specificity of the vector index, calculated annually (Table 3), demonstrated that sensitivity was lower than for the CMVRA in all study years except 2009 and 2010, with the lowest value (0.500) in 2007. The specificity of the vector index was consistently better than that of the CMVRA, except for 2009 and 2010, when only 2 human cases occurred.

DYCAST

Positive DYCAST cells were observed before human case occurrence in 5 of the 7 study years (Table 1). Counts of positive DYCAST grid cells compared with human case onset is presented in Figure 1, panel C. The DYCAST risk estimate, calculated by grouping the biweekly estimates, was used in the yearly comparisons of sensitivity and specificity (Table 3). Temporal changes in sensitivity and specificity showed the impact of reduced reporting of dead birds over time because the values for both measures of validity were highest in 2004 and declined to 0 or near 0 in all subsequent years.

Human Case Reports

A total of 389 cases of WNV disease were reported during the study period. Of these, 14 reports were missing onset date information and were not used to evaluate the risk estimates.

Analysis

The proportion of high-risk intervals correctly identified (sensitivity) was greatest in the CMVRA when the 2.6 emergency planning threshold was used (Table 2). The vector index provided the second highest sensitivity by using values just >0 (65th percentile). The greatest specificity, i.e., proportion of low-risk intervals correctly identified, was observed in the CMVRA at the epidemic threshold of 4.1, followed by the vector index at the 95th

Table 1. First dates for risk assessment thresholds and onset of human West Nile disease, Los Angeles, California, USA, 2004–2010*

Model	Threshold	Year	Date	
			Threshold met	First case
CMVRA	2.6, emergency planning	2004	Apr 30	Jun 21
		2005	Jun 30	Jul 5
		2006	Jul 31	Jul 10
		2007	Jul 15	Jul 20
		2008	Jun 15	Jun 24
		2009	Jul 15	Aug 18
		2010	Jun 30	Sep 14
	4.1, epidemic	2004	Aug 15	Jun 21
		2005	Jul 31	Jul 5
		2006	Aug 31	Jul 10
		2007	Sep 15	Jul 20
		2008	Jul 31	Jun 24
		2009	Not observed	Aug 18
		2010	Not observed	Sep 14
Vector index	>0.018, 65th percentile	2004	Apr 15	Jun 21
		2005	Jun 15	Jul 5
		2006	May 15	Jul 10
		2007	May 15	Jul 20
		2008	May 30	Jun 24
		2009	Jul 15	Aug 18
		2010	Jul 15	Sep 14
	>0.069, 80th percentile	2004	Apr 15	Jun 21
		2005	Jun 30	Jul 5
		2006	Aug 15	Jul 10
		2007	Jul 31	Jul 20
		2008	Jul 15	Jun 24
		2009	Aug 15	Aug 18
		2010	Jul 31	Sep 14
DYCAST	Daily	2004	May 4	Jun 21
		2005	Jun 12	Jul 5
		2006	Oct 4	Jul 10
		2007	Aug 13	Jul 20
		2008	Jun 4	Jun 24
		2009	Jun 20	Aug 18
		2010	Apr 5	Sep 14
	Weekly, wk. no.	2004	18	26
		2005	24	28
		2006	40	28
		2007	33	29
		2008	23	26
		2009	24	34
		2010	19	37

*CMVRA, California Mosquito-Borne Risk Assessment; DYCAST, Dynamic Continuous-Area Space-Time system.

percentile; the PPV followed this finding. The likelihood ratio positive, i.e., the likelihood that a high-risk condition was identified correctly when a human case occurred, was greatest for the vector index at the 95th percentile. The likelihood ratio negative, i.e., how much the odds of a human case decrease during low-risk conditions, was lowest in the emergency planning threshold of the CMVRA.

Discriminatory ability, as measured by the AUC, was greatest for the CMVRA (0.982), followed by the vector index (0.845) (Figure 2). Ideal response level cutoffs for the CMVRA as indicated in the ROC plots would be 1.8 and 2.6. The ideal response level for the vector index was more difficult to identify because of the obvious tradeoff between the sensitivity and specificity as evidenced in the ROC plot. The DYCAST cell aggregates performed no better than chance with an AUC of 0.468, with worst performance occurring when a single positive cell was used to assess risk.

A case-crossover study was conducted for all cases and asymptomatic blood donors with a known illness onset or donation date. The relative risk, i.e., risk for WNV infection given exposure to high-risk conditions, was greatest when detected by the CMVRA by using the emergency planning threshold (Table 2).

Discussion

Since the introduction of WNV into the United States in 1999, WNF and WNND have caused at least 31,365 illnesses and 1,250 deaths (*27*). Once considered to be a mild influenza-like illness, WNF is now understood to be an acute viral infection, often followed by months of illness associated with depression, altered moods,

Table 2. Comparison of CMVRA, vector index, and DYCAST for predicting risk for West Nile disease by the calculation threshold applied, validation method, and associated risk, Los Angeles, California, USA, 2004–2010*

Model	Sensitivity	Specificity	PPV	LRP	LRN	Mantel-Haenszel RR (95% CI)
CMVRA						
2.6	0.976	0.815	0.588	5.261	0.03	403.453 (70.506–2,308.659)
4.1	0.317	1	1	UND	0.683	38.255 (29.425–49.736)
Vector index (percentile)						
0.018 (65)	0.974	0.758	0.507	4.029	0.034	25.251 (18.120–35.033)
0.041 (75)	0.846	0.902	0.688	8.631	0.171	25.383 (18.350–35.112)
0.095 (85)	0.564	0.954	0.759	12.33	0.457	24.284 (17.503–33.692)
0.276 (95)	0.246	0.993	0.909	36.231	0.748	23.253 (16.878–32.036)
DYCAST						
Daily	0.268	0.165	<0.001	0.321	4.443	10.112 (7.367–13.880)
Biweekly	0.361	0.045	0.006	0.378	14.242	9.756 (7.764–12.258)

*CMVRA, California Mosquito-Borne Virus Risk Assessment; DYCAST, Dynamic Continuous-Area Space-Time system; PPV, positive predictive value; LRP, likelihood ratio positive; LRN, likelihood ratio negative; RR, relative risk; UND, undefined due to the high specificity.

headaches, and fatigue (*28*–*30*). The illness associated with WNND, including meningitis, encephalitis, and acute flaccid paralysis, has been associated with persistent motor and cognitive deficits and incomplete recovery (*28*,*31*). Reported cases of WNF and WNND underrepresent the actual number of WNV cases in the U.S. population (*32*,*33*), and symptomatic persons represent only a fraction of those infected. In addition to individual suffering, the medical and public health costs associated with WNV average >$40,000 per case (*34*) at a time when many health agencies are facing serious budgetary shortfalls.

The individual health toll and associated medical costs present a strong case for active intervention. Current means to prevent WNV infection include integrated vector management by larval mosquito control to arrest viral amplification and, in an outbreak, ground or aerial adulticide applications to eliminate infectious female mosquitoes and personal protection to avoid mosquito bites. Emergency application of adulticides became particularly controversial in California (*35*), even though it is the only method that targets mosquitoes capable of transmitting virus and is cost-effective for preventing human cases (*36*). In light of this controversy, mosquito control agencies in California are often hesitant to apply adulticides until epidemics appear imminent on the basis of available risk estimates or the occurrence of human cases. Our study comparatively evaluated 3 risk measures currently used as decision support tools for intervention and for predicting human cases.

By using only indicators of enzootic transmission, the CMVRA consistently produced estimates in the emergency planning range before human case occurrence; however, epidemic thresholds were not reached until after human cases had been detected. Risk assessment by this method required a robust arboviral surveillance program, with regular sampling for multiple surveillance indicators. The specificity and PPV when the epidemic threshold of 4.1 was used were excellent; however, this was at the expense of adequate lead time for initiating intervention efforts before some human cases. Additionally, the sensitivity of the risk estimate was less than desirable at 0.317, meaning that fewer than one third of the high-risk periods were correctly identified. The CMVRA using the 4.1 threshold was poor at predicting high-risk intervals but good at predicting low-risk intervals.

The 2.6 emergency planning threshold for the CMVRA increased sensitivity and provided a predictive indication of human cases before their onset. The likelihood ratio positive was better than the DYCAST risk estimates, and the likelihood ratio negative was the best of all methods. In addition, the associated risk for human cases, measured by the Mantel-Haenszel relative risk, was the greatest. At the 2.6 emergency planning threshold, the CMVRA was excellent at predicting high-risk periods and good at predicting low-risk periods.

The vector index was simple to calculate and required only a mosquito surveillance and testing program, thereby saving costs associated with sentinel chicken maintenance and sampling and dead bird reporting and testing programs. Unfortunately, this measure did not have preestablished risk thresholds. In our study, it appeared that setting the threshold to >0 (i.e., whenever mosquito infection was detected) would be adequate for predicting human cases in urban settings, such as Los

Table 3. Comparison of the sensitivity and specificity of CMVRA calculated at the emergency planning threshold of 2.6, the vector index calculated at the 80th percentile, and DYCAST risk estimates aggregated weekly for detecting risk for West Nile disease, Los Angeles, California, USA*

	2004		2005		2006		2007		2008		2009		2010	
Model	Sen	Spe	Sen	Spe	Sen	Spe	Sen	Spe	Sen	Spe	Sen	Spe	Sen	Spe
CMVRA	1	0.667	0.857	0.647	1	0.556	1	0.778	0.9	0.571	1	0.857	1	0.913
Vector index	0.778	0.867	0.714	0.941	0.667	1	0.5	1	0.8	1	1	0.714	1	0.652
DYCAST	0.517	0.268	0.034	0.143	0	0	0.063	0	0	0.013	0	0	0	0

*CMVRA, California Mosquito-borne Virus Risk Assessment; DYCAST, Dynamic Continuous-Area Space-Time system; sen, sensitivity; spe, specificity.

Angeles, where *Cx. p. quinquefaciatus* mosquitoes are the primary vectors and temperatures generally permit viral amplification. The estimates of the vector index increased before case occurrence in 5 of the 7 years. The sensitivity and specificity were comparable with those of the CMVRA, but the likelihood ratio positive was the greatest of all risk estimates. The likelihood ratio negative was better than that of the DYCAST but not as good as that of the CMVRA. Therefore, the vector index was moderate at predicting high-risk periods and very good at predicting low-risk periods. The measure of risk associated with a high-risk value, assessed by the Mantel-Haenszel relative risk, was also better than the DYCAST risk estimate but not as good as either CMVRA threshold.

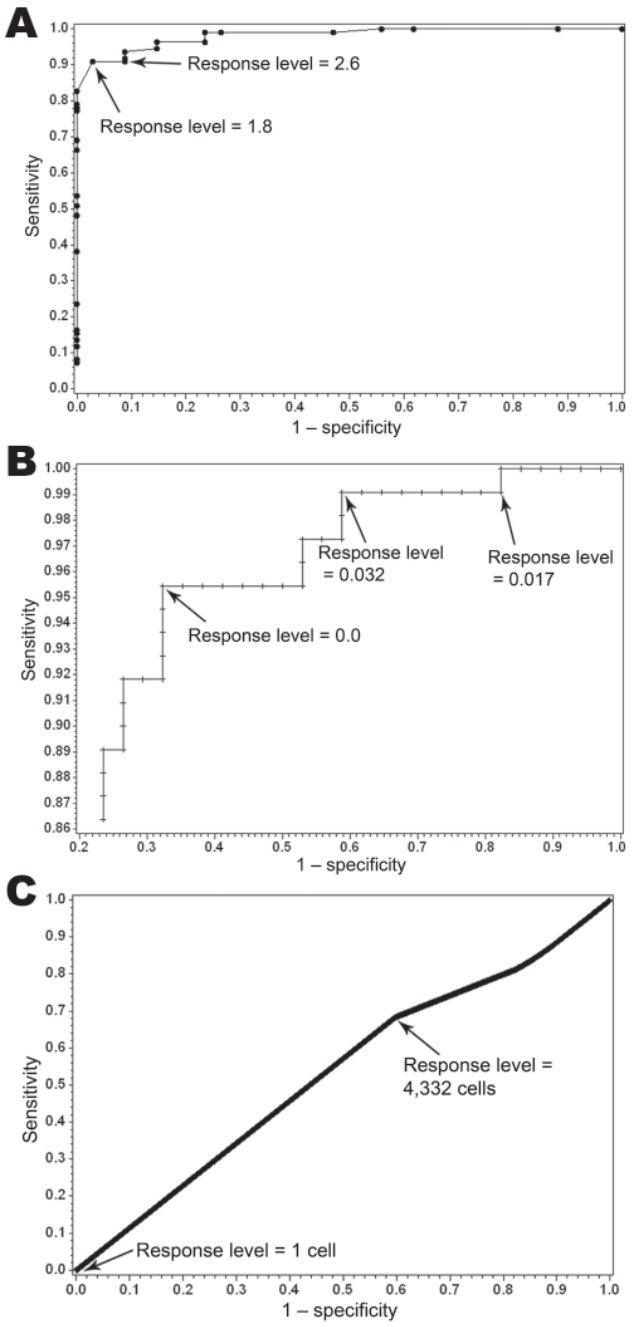

Figure 2. Receiver operator characteristic curves for California Mosquito-Borne Virus Risk Assessment (A), vector index (B), and Dynamic Continuous-Area Space-Time system (C), with labeled cutoff points for 2004–2008 data, Los Angeles, California, USA.

The DYCAST risk estimate was useful in years with amplified enzootic transmission, when dead birds were considered the primary WNV surveillance indicator (*4*,*37*–*39*). However, after the initial epidemic, WNV activity has been progressively more difficult to predict by using DYCAST because of reduced reporting to the California Dead Bird Hotline. Whether this decrease resulted from truly decreased numbers of dead birds as bird populations became progressively more resistant to infection or to public apathy/decreased awareness was not possible to ascertain. Losing time precision by aggregating estimates clearly increased measures of validity, which considering the uncertainty regarding time between WNV exposure and disease onset seemed appropriate to improve predictive power. The sensitivity of the weekly DYCAST risk estimate was similar to that of the CMVRA, but the specificity, PPV, likelihood ratio positive, and likelihood ratio negative were all uniformly worse than the other 2 methods, even when aggregated spatially. Additionally, the measure of relative risk associated with risk estimates was less than that of the CMVRA and the vector index.

In conclusion, critical decisions on intervention by using risk estimates require knowledge of the strengths and weaknesses of the selected method to respond in an adequate and timely manner to prevent human cases while reducing unnecessary response and costs associated with falsely identified high-risk periods. The goals we set for a good WNV risk estimate were a balance of these attributes and were achieved best in urban and suburban Los Angeles by the CMVRA by using the 2.6 epidemic planning threshold. In light of this finding, an evaluation of the CMVRA should be done in other ecologic settings with transmission driven by other vector species to determine whether the threshold should be adjusted to provide better antecedent estimates of human risk.

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At the time of this study, Dr Kwan was a Postdoctoral Scholar at the Center for Vectorborne Diseases at the School of Veterinary Medicine, University of California, Davis. She currently works as an analyst with the National Institutes of Health. Her research interests emphasize geographic information system data management and analysis, health delivery systems, and policy.

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Molecular Epidemiologic Investigation of an Anthrax Outbreak among Heroin Users, Europe

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In December 2009, two unusual cases of anthrax were diagnosed in heroin users in Scotland. A subsequent anthrax outbreak in heroin users emerged throughout Scotland and expanded into England and Germany, sparking concern of nefarious introduction of anthrax spores into the heroin supply. To better understand the outbreak origin, we used established genetic signatures that provided insights about strain origin. Next, we sequenced the whole genome of a representative *Bacillus anthracis* strain from a heroin user (Ba4599), developed Ba4599-specific single-nucleotide polymorphism assays, and genotyped all available material from other heroin users with anthrax. Of 34 case-patients with *B. anthracis*–positive PCR results, all shared the Ba4599 single-nucleotide polymorphism genotype. Phylogeographic analysis demonstrated that Ba4599 was closely related to strains from Turkey and not to previously identified isolates from Scotland or Afghanistan, the presumed origin of the heroin. Our results suggest accidental contamination along the drug trafficking route through a cutting agent or animal hides used to smuggle heroin into Europe.

Bacillus anthracis is a gram-positive endospore-forming bacterium that causes the disease anthrax in livestock, wildlife, and humans. Because of its hardy spores, *B. anthracis* can survive for extended periods in the environment, a trait that likely contributed to the successful global spread of this organism (*1*). The mostly dormant life cycle of *B. anthracis* and its relatively recent emergence as a pathogen have resulted in a genome that is highly clonal, with little genetic variation among even the most distantly related strains (*2–4*).

Anthrax is most commonly contracted by exposure to contaminated animal products, such as skins, wool, or meat; its symptoms vary in severity depending on the route of infection. Cutaneous anthrax, the most common manifestation of disease, accounts for 95% of cases, whereas pulmonary and gastrointestinal anthrax are much less common and follow inhalation or ingestion of spores, respectively. Inhalational anthrax is rare but particularly deadly, with up to a 90% fatality rate (*5*).

In 2000, a novel form of cutaneous anthrax, termed injectional anthrax, was proposed after anthrax was diagnosed in a heroin "skin popper" (one who injects the drug beneath the skin, rather than into a vein) from Norway on postmortem examination (*6*). Injectional anthrax symptoms are more severe than those of cutaneous anthrax and are typified by severe soft tissue infection at the injection site, which can progress to septic shock, meningitis, and death (*7*). The origin of anthrax in the Norwegian heroin user was never identified, although contaminated heroin was suspected (*6*). This case was the first to demonstrate this previously unrecognized route of *B. anthracis* infection.

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In December 2009, two cases of injectional anthrax were diagnosed in heroin users in Scotland after *B. anthracis* was detected in blood cultures (*8*). These cases marked the beginning of an emerging anthrax outbreak among European heroin users. Over the following months, 14 anthrax deaths were confirmed and 119 anthrax cases were suspected (*9*), leading to increasing media attention as the severity of this outbreak became more apparent. This attention was spurred by 3 factors. First, *B. anthracis* is not found naturally in Scotland, and human cases of anthrax in Europe are extremely rare, with only 3 cases of anthrax notified in Europe in 2008 (*10*). Second, the pathology of injectional anthrax is especially devastating (*11*,*12*). Third, anthrax cases appeared to befall only an ostensibly targeted population of persons, leading to initial suggestions of deliberate contamination of the heroin supply. Although investigations were unable to show nefarious intent, the mode of contamination with *B. anthracis* spores remained elusive because of an inability to culture *B. anthracis* from, or detect *B. anthracis* DNA in, suspected contaminated heroin (*9*).

In the current study, we applied a molecular phylogeographic approach to identify the likely origin of the *B. anthracis* spores responsible for the 2009–2010 outbreak in Europe. We used canonical single-nucleotide polymorphism (canSNP) genotyping against heroin anthrax samples and an extensive collection of diverse worldwide samples (*1*,*13*) and whole genome sequencing techniques to determine a possible origin for the *B. anthracis* spores responsible for this outbreak.

Materials and Methods

B. anthracis–containing Heroin Samples

We obtained 36 samples, positive for *B. anthracis* by culture or PCR, from 34 injecting heroin users throughout the duration of the epidemic (December 2009–November 2010) (Table). Most samples were from users in Scotland (n = 29), with 5 samples from England, 1 sample from Germany, and 1 sample of unknown origin.

DNA Extraction

A *B. anthracis* isolate obtained from a Scottish heroin user in December 2009 (Ba4599) was cultured for 18 hours on 5% sheep blood agar, and genomic DNA was obtained by using phenol/chloroform extraction. All other *B. anthracis* isolates obtained during the outbreak were extracted by using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). We extracted 337 geographically diverse *B. anthracis* isolates from the Trans-Eurasian (TEA) group of the *B. anthracis* phylogeny (*1*,*4*,*15*) by using 5% Chelex 100 (Bio-Rad, Hercules, CA, USA) (*16*). Strains for whole genome sequencing were extracted by using the DNeasy Blood and Tissue kit (QIAGEN).

SNP Genotyping

Several previously established canSNP real-time PCR assays for *B. anthracis* were used to determine the approximate phylogenetic placement of Ba4599 on the *B. anthracis* phylogenetic tree (*1*,*15*,*17*). This approach enabled us to identify a subset of isolates that matched the Ba4599 genotype according to canSNP genotyping. Novel assays within the A.Br.008/009 group were then run against Ba4599 and other A.Br.008/009 samples in our collection to refine the phylogenetic placement of Ba4599 within A.Br.008/009 and to identify its closest known relatives.

To divide the A.Br.008/009 TEA group into 2 lineages (A.Br.008/011 and A.Br.011/009; Figure), we converted the canSNP A.Br.011 assay (*17*) into a TaqMan MGB dual-probe real-time PCR (Applied Biosystems, Foster City, CA, USA). These A.Br.011 primers and probes (5′ → 3′) were used: ABr011_For: CTAAAAAGAAACGAATTCCCGCTGA, ABr011_Rev: CGATAAAAATCGGAATTGAAGCAGGAG, ABr011_Anc: VIC-CGCCCCCATTATTT, and ABr011_Der: 6-FAM-CGCCCCTATTATTT. The SNP position is underlined in the TaqMan probe sequences.

The *B. anthracis* isolate from heroin and 114 other isolates from our collection clustered within the A.Br.008/011 lineage. We therefore focused on further resolving the positions of members of this group. Comparison of the A0897 genome (see below) with previously published genomes (*1*,*3*) yielded 67 SNPs within this lineage (Figure). Three of these SNPs were incorporated into allele-specific real-time PCR assays (*18*) and used to reduce the list of closest relatives to Ba4599 (Figure). The A.Br.008/011 SNP assays are named according to their location on the Ames Ancestor chromosome (GenBank accession no. AE017334) and are listed in order of increasing proximity to the terminal genome on this branch, *B. anthracis* strain A0897. Primers for these 3 A.Br.008/011 lineage SNPs are shown in 5′ → 3′ orientation and as ancestral, derived, and common primers, respectively: SNP5013862 (ATTGAAATGATGATTTTTCACgA,GATTGAAATGATGATTTTTCACgT, TGGTTTATACCATTGTATTGCCCG), SNP1967560 (AATCATCAACATGGTCTTCTGTAAaC, AATCATCAACATGGTCTTCTGTAAgA, GAAAAACCAGAAGTAGTGTGCGGTG), and SNP1118831 (CTCGCTCTGCGTACGTTTG, CTCGCTCTGCGTACGTTcA, TATCAATCTGAAGAAGGTAGCGATAACG). Underlined nucleotides indicate the SNP position, and lowercase nucleotides indicate deliberately incorporated penultimate mismatches for enhanced allele specificity (*19*). These 3 SNPs grouped 2 samples (A0149 and A0264) from our collection with Ba4599.

Whole Genome Sequencing

We sequenced 3 genomes (Ba4599, A0897, and A0149) to place Ba4599 into a broader phylogenetic context and to accurately establish the evolutionary relationships among these strains. We also sought to identify heroin strain–specific SNPs that could be used to rapidly determine whether *B. anthacis* that caused infections in heroin users was from the same point source as Ba4599. Because of the highly clonal nature of *B. anthracis* and its low mutation rate, phylogenetic analysis using small numbers of SNPs derived from whole genome sequencing is a highly accurate method for determining qualitative and quantitative patterns of relatedness (*3*,*20*). With the exception of A0149 and previously sequenced strains, isolates were subjected to paired-end whole genome sequencing on the Illumina Genome Analyzer IIx instrument (Illumina Inc., San Diego, CA, USA). Additional sequencing was performed for Ba4599 on the 454 GS FLX instrument (454 Life Sciences, Branford, CT, USA) to provide complementary validation of whole genome sequencing SNP calls and improve contiguity of the genome assembly. De novo hybrid assembly of the Ba4599 Illumina and 454 data was performed by using Celera Assembler version 6.1 (*21*). Ba4599 contigs are available in DDBJ/EMBL/GenBank (accession no. AGQP00000000 and AGQP01000000).

A0149 was sequenced with a shotgun Sanger library approach by using pUC19 and M13 reads before electrophoresis on an AB3730*xl* instrument (Applied Biosystems). Read mapping was performed by using BWA and BWA-SW 0.5.9 (*22*,*23*). Ames Ancestor (GenBank accession nos. AE017334–AE017336) was used as the reference genome for assembly and WesternNA USA6153 (GenBank accession no. AAER00000000) as a related but distinct genome for the TEA group. Processing and data filtering were performed with SAMtools 0.1.12a (*24*) by using an in-house java script that filtered out results, providing <10× coverage. For in-house genomes, SNP calls were then made by using SolSNP version 1.1 with

Table. Genotyping results of 36 culture- or PCR-confirmed cases of anthrax in heroin users, 2009–2010, Europe*

Sample no.	Status†	Collection date	Location	SNP1053700	SNP1173928
Ames‡	NA	1981	Texas, USA	A	G
Ba4599§	C+/PCR+	2009 Dec 16	Glasgow, Scotland	G	C
A112	C+/PCR+	2009 Dec 18	Germany	G	C
4622	C+/PCR+	2009 Dec 19	Glasgow, Scotland	G	C
4646	C-/PCR+	2009 Dec 22	Airdrie, Scotland	G	C
4670	C+/PCR+	2009 Dec 23	Glasgow, Scotland	G	C
4745	C-/PCR+	2009 Dec 31	Glasgow, Scotland	G	C
0002	**C+/PCR+**	**2010 Jan 4**	**Glasgow, Scotland**	**G**	**C**
0007	**C+/PCR+**	**2010 Jan 4**	**Glasgow, Scotland**	**G**	**C**
0001	C+/PCR+	2010 Jan 4	Dundee, Scotland	G	C
0046	C-/PCR+	2010 Jan 6	Stirling, Scotland	G	C
0074	**C+/PCR+**	**2010 Jan 7**	**Kirkcaldy, Scotland**	**G**	**C**
0075	**C+/PCR+**	**2010 Jan 7**	**Kirkcaldy, Scotland**	**G**	**C**
0117(2)	C+/PCR+	2010 Jan 9	Dundee, Scotland	G	C
0142	C+/PCR+	2010 Jan 9	Glasgow, Scotland	G	C
0271	C-/PCR+	2010 Jan 15	Glasgow, Scotland	G	C
0393	C+/PCR+	2010 Jan 20	Kilmarnock, Scotland	G	C
0426	C+/PCR+	2010 Jan 21	Glasgow, Scotland	G	C
0491	C+/PCR+	2010 Jan 22	Glasgow, Scotland	G	C
0773	C+/PCR+	2010 Feb 4	London, England	G	C
0871	C+/PCR+	2010 Feb 9	Glasgow, Scotland	G	C
0874	C+/PCR+	2010 Feb 9	Glasgow, Scotland	G	C
0844	C-/PCR+	2010 Feb 9	Blackpool, England	G	C
1297	C+/PCR+	2010 Feb 26	London, England	G	C
1060	C+/PCR+	2010 Mar 5	Fife, Scotland	G	C
1134	C+/PCR+	2010 Mar 5	Dumfries, Scotland	G	C
1320	C+/PCR+	2010 Mar 5	Dumfries, Scotland	G	C
0981	C-/PCR+	2010 Mar 5	Airdrie, Scotland	G	C
1458	C-/PCR+	2010 Apr 1	Dumfries, Scotland	G	C
1927	C-/PCR+	2010 Apr 1	Paisley, Scotland	G	C
2145	C-/PCR+	2010 Apr 16	Livingston, Scotland	G	C
2199	C-/PCR+	2010 Apr 16	Glasgow, Scotland	G	C
2506	C+/PCR+	2010 Apr 27	Edinburgh, Scotland	G	C
2728	C+/PCR+	2010 May 7	Glasgow, Scotland	G	C
3739	C+/PCR+	2010 Jul 16	Glasgow, Scotland	G	C
4936/5011	C-/PCR+	2010 Aug 26	Leicester, England	G	C
6696	C+/PCR+	2010 Nov 1	Maidstone, England	G	C

*ID, identification; SNP, single nucleotide polymorphism; NA, not applicable; C, culture. **Boldface** indicates pairs of isolates from the same patient.
†PCR+ samples were determined by using assays targeting *cap*, *lef* (Special Pathogens Reference Unit (UK), pers. comm.) and *bagC* loci (*14*).
‡Ancestral allele DNA control.
§Derived allele DNA control.

the following alignment limits: minimum coverage of 20, minimum mapping qualities of 20, and a filter call of 0.95. MUMmer 3.20 (*25*) was used to determine SNP calls in public genome sequences.

Whole Genome Phylogenetic Analysis

Maximum-parsimony phylogenetic trees were inferred by conducting a heuristic search in PAUP* 4.0b10 (*26*) by using the filtered whole genome sequencing SNP data as input. The tree was rooted by using Ames Ancestor as the outgroup because this strain is not a descendant of the TEA group.

Phylogenetic Placement of Nearest Neighbors

We did not have whole genome sequencing data for isolate A0264 from Turkey. We therefore used canSNP genotyping assays to determine the approximate phylogenetic location of this strain relative to Ba4599 and A0149. CanSNPs along the branch leading to A0149 and Ba4599 were identified from whole genome sequencing data by searching for SNPs with allelic states shared only between these 2 genomes. We designed assays for 3 of these SNPs (Figure). The positions of these SNPs in the Ames Ancestor genome (NC_007530.2) are listed along with flanking sequence from Ames Ancestor. The SNP is underlined and the derived allele, found in Ba4599, is included in parentheses: SNP1530761 GGGCATTAGGATCAGCGATAA (T), SNP3287006 AGGTTGCCTTCCCCATCTATT (A), and SNP3836105 AATCGTAAAGTGGCTGTATTT (C). Primers for the first SNP (1530761) assay are listed in order of derived, ancestral, and common primers as follows (5′ → 3′): SNP1530761 (CTACTGCTTCTTACACATTTATCGCTGtA, TACTGC TTCTTACACATTTATCGCTGtT, GTTCCGCTCGGTA CGGTATC). This assay was the most robust of the 3 tested assays.

We then used whole genome comparisons to identify SNPs along the branch leading to the Ba4599 genome. These SNPs were tested against A0264 and all 36 clinical samples from the heroin outbreak. We targeted 2 of the 13 identified SNPs (Figure) for TaqMan assay design as follows: SNP1053700 (CCTCGGAAATGAAGTGGTTGAAAAT, CGGGAATGTTGACATTAAGCTCATT, VIC-CTGCA TAAATACCAGATAGTAA, FAM-CTGCATAAATACC AGGTAGTAA) and SNP1173928 (GCAGGTCTTCGA ATGATGTGTCAAT, GCTCTTCCACGATTTCAAAG TCATT, VIC-CCTGTTGTAGAATATCT, FAM-CTGT TGTACAATATCT). Ninety-three TEA isolates unrelated to the heroin outbreak and 2 non-TEA isolates belonging to the Ames (A0462) and Western North America (A0303) groups were screened to confirm specificity of these 2 SNP assays for the Ba4599 heroin genotype.

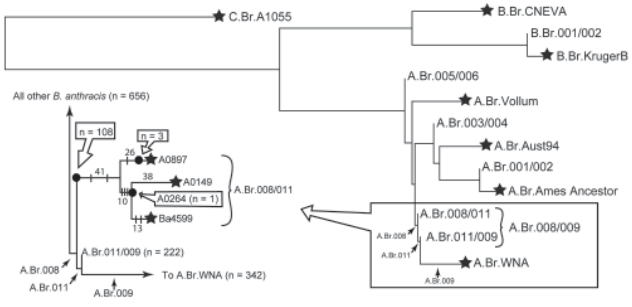

Figure. Phylogenetic location of the heroin Ba4599 genome on the global *Bacillus anthracis* phylogenetic tree (*1*). Ba4599 was isolated from a heroin user who died of anthrax at the beginning of the 2009–2010 European anthrax outbreak. Canonical single nucleotide polymorphism (SNP) typing situated Ba4599 within the A.Br.008/011 clade of the Trans-Eurasian group (arrows). Closer examination of the boxed area (inset) revealed that Ba4599 was closely related to 2 isolates from Turkey, A0149 and A0264. Solid black circles indicate the approximate position of collapsed branches (n = 3) and are labeled with the corresponding number of strains that fall within the node. Branch termini are occupied by whole genome sequenced strains (black stars). SNPs are numbered on a given branch; vertical bars along the A.Br.008/011 branches denote the phylogenetic placement of SNP assays used in our analysis of *B. anthracis* from heroin users in Europe. Consistency index = 0.923.

Results and Discussion

Phylogeographic Placement of *B. anthracis* Heroin Strain Ba4599

Anthrax is considered to be endemic to many parts of western, central and southern Asia, with large numbers of human and animal anthrax cases being reported annually in countries such as Afghanistan, Bangladesh, India, Iran, Kazakhstan, Kyrgyzstan, Pakistan, Turkey, and the Republic of Georgia. Molecular epidemiologic studies of *B. anthracis* have demonstrated that particular genotypes correspond with geographic regions, a trait made possible by the relatively recent emergence of this highly clonal pathogen (*1*,*3*,*4*). Therefore, by establishing the genotype of Ba4599, we expected to find clues regarding the geographic origin of the anthrax outbreak caused by contaminated heroin.

Afghanistan produces 90% of the world's heroin (*27*), and the Khyber Pass region of Pakistan and Afghanistan has historically been one of the major centers for staging and trafficking of heroin into western Europe (*28*). It was therefore logical to suspect that heroin trafficked into Europe during the 2009–2010 anthrax epidemic originated in Afghanistan, having become contaminated at the primary source (*7*). Indeed, some media reports speculated that the potential source of anthrax spores in the heroin supply was a cutting agent derived from *B. anthracis*–infected Afghan livestock (*29*).

We sought to phylogenetically place the representative Ba4599 heroin isolate by using previously established

(*1*,*15*) and novel canSNP signatures, thereby pinpointing the potential geographic origin of contamination. Our canSNP typing grouped Ba4599 within the A.Br.008/011 node of the TEA group (Figure). TEA strains are the most common and widespread group of *B. anthracis* in the world, being found in Europe, the People's Republic of China, Russia, Kazakhstan, and West Africa (*1*). Seventeen recent isolates from animals in Afghanistan were analyzed and proved not to be members of TEA but rather were affiliated with the evolutionarily distinct Vollum clade. These results are inconsistent with the conclusion that heroin became contaminated during primary production and transformation of opium in Afghanistan.

Precise source attribution of the outbreak caused by *B. anthracis*–contaminated heroin is complicated by the wide phylogeographic spread of the TEA group. To improve phylogenetic resolution and to deduce the closest relatives of Ba4599, we identified and characterized additional molecular targets within the A.Br.008/011 group (SNPs 5013862, 1967560, and 1118831). Ba4599 has derived alleles for the first 2 SNPs but an ancestral allele for 1118831; we were therefore interested in identifying other strains with the same canSNP profile as Ba4599. We tested these 3 SNP signatures across all 120 A.Br.008/011 strains within our *B. anthracis* collection, comprising isolates from Albania (n = 5), Argentina (1), Austria (1), China (47), Ethiopia (1), Hungary (3), Iran (1), Italy (21), Norway (1), Pakistan (2), Poland (1), Republic of Georgia (5), Russia (2), Scotland (12), Slovakia (2), Turkey (6), Ukraine (1), or unknown origin (8). Notably, Ba4599 was indistinguishable from only 2 isolates, A0149 and A0264, both of which originated from Turkey. Twelve isolates linked to a fatal case of anthrax in a drum maker in Scotland in 2006, which originated from contaminated animal skins from Ghana, did not match the Ba4599 genotype. Similarly, the bioweaponized *B. anthracis* used on Gruinard Island, Scotland, during World War II (*30*) belongs to the unrelated Vollum clade and is thus distinct from Ba4599.

Differentiation of Ba4599 and Turk A0149

Although Ba4599 and A0149 are closely related, they are not identical. By using whole genome sequencing, we identified 51 SNPs that differentiated Ba4599 from A0149, of which 12 were unique to Ba4599 and 38 unique to A0149 (Figure). A second isolate from Turkey, A0264, was tested against 3 of the 10 SNPs leading to the A0149/Ba4599 group (SNPs 1530761, 3836105, and 3287006) and the 2 Ba4599-specific SNPs (1173928 and 1053700) to approximate its phylogenetic location. Our analyses of SNPs 1530761, 3836105, and 3287006 revealed that A0264 shared the derived genotype with A0149 and Ba4599. In contrast, A0264 did not match the 2 SNPs along the Ba4599 branch, similarly to A0149 (Figure). Therefore, A0264 probably does not fall on the branch leading to Ba4599; however, precise placement would require assaying all 13 SNPs along the Ba4599 branch or whole genome sequencing of A0264. Four other isolates from Turkey in our collection fall on the A.Br.008/011 branch but possess an ancestral allele for SNPs 1530761, 3836105, and 3287006, and are thus not as closely related to Ba4599 as are A0149 and A0264.

Source Attribution of *B. anthracis* in Heroin

On the basis of our molecular typing results, we strongly suspect that *B. anthracis* spores were accidentally introduced into the heroin supply in Turkey (or surrounding regions) before being smuggled into Europe. Heroin produced in Afghanistan is thought to be trafficked through 2 major routes: the Silk Route and, more commonly, the Balkan Route. Both of these routes pass through numerous countries where anthrax is endemic and where isolates belonging to the TEA group have been found. Although we were unable to exhaustively test *B. anthracis* isolates along these routes to find a precise match to Ba4599, Turkey is definitely a possible origin, given that this country is central to the heroin smuggling trade from Afghanistan into Europe along the Balkan Route (*31*), and Turkish laboratories are believed to play a key role in conversion of the morphine base into its usable heroin form (*32*).

Thus, our genotyping results support the hypothesis that the heroin was contaminated along the trafficking route and not at its origin (Afghanistan) or destination (Scotland). How it was contaminated is highly speculative, but it may have involved the addition of an animal-derived cutting agent, e.g., bone meal, or, alternately, wrapping in animal hide contaminated with *B. anthracis* spores. Contaminated animal hide products have been implicated in several allochthonous anthrax cases in Europe and the United States (*33*–*38*), including *B. anthracis* isolates from drum skins linked to a 2006 anthrax case in Scotland. Isolates in our collection from other countries along the Balkan and Silk Routes, including Albania, Hungary, Italy, Poland, Russia, and Ukraine, did not match the Ba4599 genotype, giving greater credence to our hypothesis of Turkey as the point of origin. However, we do not possess isolates from several other anthrax-endemic countries along the Silk and Balkan Routes and therefore cannot rule out the possibility of contamination elsewhere in the region.

Genotypes of Subsequent *B. anthracis* Isolates from Heroin Users

Two Ba4599-specific SNP assays were used to rapidly screen 35 other *B. anthracis*–positive (by culture or PCR) samples from the heroin-associated outbreak, including an isolate obtained from a heroin user with anthrax in Germany (*14*). These 2 SNP assays were also screened

across a panel of 92 TEA and 2 non-TEA *B. anthracis* samples to determine assay specificity. All outbreak isolates obtained for this study shared the 2-SNP Ba4599 genotype, suggesting that the outbreak originated from a batch of heroin that was contaminated with *B. anthracis* spores from a single point source. In contrast, none of the nonoutbreak samples matched the Ba4599 genotype at either SNP, demonstrating that these SNPs were specific to Ba4599. The German patient had no history of travel to the United Kingdom, indicating acquisition of anthrax from a single batch of heroin that was disseminated to at least 3 European countries (Scotland, England, and Germany). It is therefore possible that future cases of anthrax may arise, should the contaminated batch of heroin recirculate in the user population.

Conclusions

In this study, we used a high-resolution molecular epidemiologic approach to identify the geographic source of anthrax spores responsible for the largest outbreak of injectional anthrax observed to date. Using all available clinical material, we found that the 2009–2010 anthrax outbreak that affected heroin users across Scotland, Germany, and England was caused by a single anthrax strain. Further, genetic and genomic analyses demonstrated that the anthrax spores, while not an exact match, were most closely related to isolates from Turkey. Turkey is a central country along the Balkan Route, a common route for trafficking heroin from its primary source in Afghanistan into European countries. Given the commonality of the TEA group throughout the world, and the close relationship of Ba4599 to isolates from Turkey, we do not possess any evidence to suggest that the heroin was contaminated with nefarious intent. Our results suggest accidental contamination from an animal-derived source, such as bone meal (a cutting agent used to dilute heroin) or animal hides. Although no new cases of injectional anthrax have been reported since a case was diagnosed in November 2010 in Maidstone, England, this outbreak may indicate an emerging infectious disease in drug addicts in Europe and that illicit drugs such as heroin are a novel point of entry for this pathogen into non–anthrax-endemic regions. Educating users of the potential risks of acquiring anthrax from their drug use, coupled with maintained vigilance of public health investigators toward identifying anthrax cases, would help mitigate the public health effects of future outbreaks in this susceptible population.

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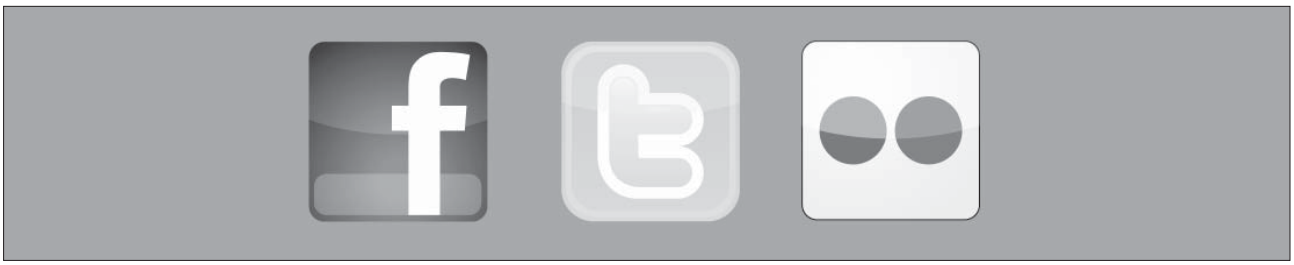

Escherichia coli O104 Associated with Human Diarrhea, South Africa, 2004–2011

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To determine the origin of >4,000 suspected diarrheagenic *Escherichia coli* strains isolated during 2004–2011 in South Africa, we identified 7 isolates as serotype O104; 5 as enteroaggregative *E. coli* O104:H4, and 2 as enteropathogenic *E. coli* O104:non-H4. Pulsed-field gel electrophoresis showed that these isolates were unrelated to the 2011 *E.* coli O104:H4 outbreak strain from Germany.

Escherichia coli is the predominant microorganism of human colonic flora (*1*). It is mostly harmless to the intestinal lumen. However, some strains (diarrheagenic *E. coli* [DEC]) can cause disease ranging from moderate-to-severe diarrhea with complications to hemolytic uremic syndrome (*1*).

During May–June 2011, an outbreak of bloody diarrhea and hemolytic uremic syndrome occurred in Germany and other parts of Europe (*2*). The Shiga toxin–producing *E. coli* (STEC) serotype O104 strain was the etiologic agent of this outbreak and accounted for >4,000 cases and 50 deaths (*3*). This outbreak strain showed an unusual combination of virulence factors of STEC and enteroaggregative *E. coli* (EAggEC). Furthermore, the outbreak strain showed extended spectrum β-lactamase (ESBL) activity (*2*,*4*).

Before the 2011 outbreak, *E. coli* O104 had been reported in parts of Europe and South Korea (*5*,*6*). In South Africa, data for *E. coli* serotypes are scarce. However, because questions arose about the ancestral origin of the 2011 outbreak strain from Germany, we investigated the occurrence of *E. coli* O104 associated with human diarrhea in 2 surveillance programs for enteric pathogens in South Africa during 2004–2011. We also investigated phenotypic and genotypic properties of *E. coli* O104 strains from South Africa and compare these with properties of the outbreak strain from Germany.

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The Study

The Centre for Enteric Diseases (CED) of the National Institute for Communicable Diseases in South Africa is a reference center for human infections involving enteric pathogens including DEC, *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae*, and it participates in national laboratory-based surveillance for these pathogens. Isolates are voluntarily submitted to the CED from ≈200 clinical microbiology laboratories across the country. The CED also recovers enteric pathogens through its involvement in the Rotavirus Surveillance Project, which started in mid-2009 and involves 5 sentinel hospital sites in South Africa. This project is involved with identification of enteric pathogens (bacterial, viral, and parasitic) associated with diarrhea in children <5 years of age.

All suspected DEC isolates received at the CED were identified by using standard microbiological identification techniques. Serotyping of O antigen was performed by using tube agglutination as described (*7*). Presence of H4 antigen was determined by PCR detection of the *fliC*$_{H4}$ gene (*8*). Resistance to antimicrobial drugs (ampicillin, amoxicillin/clavulanic acid, sulfamethoxazole/trimethoprim, chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline, kanamycin, imipenem, ceftriaxone, and ceftazidime) was determined by using Etests (bioMérieux, Marcy l'Etoile, France), and ESBL activity was investigated by using double-disk synergy methods, as described by Clinical and Laboratory Standards Institute (Wayne, PA, USA) 2009 guidelines (*9*).

PCR was used to distinguish DEC strains from nonpathogenic *E. coli* strains. The PCR consisted of 3 multiplex reactions with published primer sequences. Reactions included 0.2 μmol/L of each primer (Table 1) and 1.5 mmol/L $MgCl_2$. PCR thermal cycling included 35 cycles at 94°C for 1.5 min, 60°C for 1.5 min, and 72°C for 1.5 min.

An isolate was identified as STEC if a PCR result was positive for Shiga toxin genes 1 or 2 (*stx1* or *stx2*); as enterohemorrhagic *E. coli* if a PCR result was positive for the gene coding intimin outer membrane protein and an *stx* gene; as enteropathogenic *E. coli* (EPEC) if a PCR result was positive for the gene coding intimin outer membrane protein; as enterotoxigenic *E. coli* if a PCR result was a positive for genes coding heat-stable enterotoxin or heat-labile enterotoxin; as enteroinvasive *E. coli* if a PCR result was positive for the gene coding an invasion protein; as EAggEC if a PCR result was positive for the gene coding a transporter protein; and as diffusely adherent *E. coli* if a PCR result was positive for the gene coding an accessory

Table 1. PCR primers used for amplification of *Escherichia coli* genes, South Africa

Gene target*	Primer sequence, 5′ → 3′	Size of amplification product, bp	Multiplex PCR in which primers are included	Reference
stx1	CAGTTAATGTGGTGGCGAAGG	348	A	(*10*)
	CACCAGACAATGTAACCGCTG		A	(*10*)
stx2	ATCCTATTCCCGGGAGTTTACG	584	A	(*10*)
	GCGTCATCGTATACACAGGAGC		A	(*10*)
eae	TCAATGCAGTTCCGTTATCAGTT	482	A	(*11*)
	GTAAAGTCCGTTACCCCAACCTG		A	(*11*)
est	ATTTTTCTTTCTGTATTGTCTT	190	B	(*12*)
	CACCCGGTACAAGCAGGATT		B	(*12*)
elt	GGCGACAGATTATACCGTGC	440	B	(*12*)
	CGGTCTCTATATTCCCTGTT		B	(*12*)
ipaH	CTCGGCACGTTTTAATAGTCTGG	933	C	(*11*)
	GTGGAGAGCTGAAGTTTCTCTGC		C	(*11*)
aat	CTGGCGAAAGACTGTATCAT	630	C	(*13*)
	CAATGTATAGAAATCCGCTGTT		C	(*13*)
daaC	CAGGTCATCCGGTCAGTCGG	212	C	This study
	CAATGCCACGTACAACCGGC		C	This study

**stx*, Shiga toxin; *eae*, intimin outer membrane protein; *est*, heat-stable enterotoxin; *elt*, heat-labile enterotoxin; *ipaH*, invasion protein; *aat*, transporter prote *daaC*, accessory protein with a function in F1845 fimbriae production.

protein with a function in F1845 fimbriae production. These genes are listed in Table 1.

Genotypic relatedness of strains was investigated by using a PulseNet protocol (*14*) for pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA in a CHEF-DR III electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) and the following electrophoresis parameters: voltage 6 V, run temperature 14°C, run time 19 h, initial switch time 6.76 s, final switch time 35.38 s, and included angle 120°. PFGE patterns were analyzed by using BioNumerics version 6.5 software (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms of patterns were created by using unweighted pair group method with arithmetic averages. Analysis of band patterns incorporated the Dice coefficient at an optimization setting of 1.5% and a position tolerance setting of 1.5%.

During January 2004–May 2011, CED received >4,000 suspected DEC isolates for further laboratory characterization. Of these isolates, 7 (0.2%) were serotype O104. These isolates were collected from Gauteng (n = 3), Mpumalanga (n = 2), and Eastern Cape (n = 1) and North West (n = 1) Provinces of South Africa. *E. coli* O104 was isolated more often from children <2 years of age than from older children and adults (Table 2). There were more female patients (57%) affected than male patients (43%). Five (71%) of 7 isolates were EAggEC, and 2 (29%) of 7 isolates were EPEC (Table 2). PCR amplification of the $fliC_{H4}$ gene showed that all EAggEC isolates produced H4 antigen and that all the EPEC isolates did not produce H4 antigen. Therefore, all EAggEC were serotype O104:H4 and all EPEC were serotype O104:non-H4.

EPEC isolates were susceptible to all antimicrobial drugs tested (Table 2). EAggEC isolates were susceptible to amoxicillin/clavulanic acid, chloramphenicol, nalidixic acid, ciprofloxacin, kanamycin, imipenem, ceftriaxone and ceftazidime; were resistant to ampicillin and sulfamethoxazole/trimethoprim; and were variably susceptible to tetracycline and streptomycin (Table 2). None of the EPEC or EAggEC isolates showed ESBL activity.

Dendrogram analysis of PFGE patterns showed that EPEC isolates were diverse and clustered at a pattern similarity of 76%, and that EAggEC isolates were highly clonal and clustered at a pattern similarity of 90% (Figure). The PFGE pattern of the 2011 outbreak strain from Germany did not match those of strains from South Africa. Therefore, the outbreak strain from Germany was determined to be

Table 2. *Escherichia coli* O104 strains associated with human diarrhea, South Africa, 2004–2011*

Strain no.	Collection date	Province	Patient age/sex	*E. coli* pathotype	H antigen	Antimicrobial drug susceptibility†
1	2007 Feb 23	Eastern Cape	4 mo/F	EAggEC	H4	$R^{Amp}S^{Amc}R^{Sxt}S^{Chl}S^{Nal}S^{Cip}R^{Tet}S^{Kan}S^{Str}S^{Imi}S^{Cro}S^{Caz}$
2	2007 Nov 16	Mpumalanga	43 y/M	EAggEC	H4	$R^{Amp}S^{Amc}R^{Sxt}S^{Chl}S^{Nal}S^{Cip}R^{Tet}S^{Kan}R^{Str}S^{Imi}S^{Cro}S^{Caz}$
3	2009 Apr 12	Gauteng	10 mo/F	EPEC	Non-H4	$S^{Amp}S^{Amc}S^{Sxt}S^{Chl}S^{Nal}S^{Cip}S^{Tet}S^{Kan}S^{Str}S^{Imi}S^{Cro}S^{Caz}$
4	2010 Feb 24	Mpumalanga	7 mo/M	EAggEC	H4	$R^{Amp}S^{Amc}R^{Sxt}S^{Chl}S^{Nal}S^{Cip}S^{Tet}S^{Kan}S^{Str}S^{Imi}S^{Cro}S^{Caz}$
5	2010 Mar 3	North West	7 mo/F	EAggEC	H4	$R^{Amp}S^{Amc}R^{Sxt}S^{Chl}S^{Nal}S^{Cip}R^{Tet}S^{Kan}S^{Str}S^{Imi}S^{Cro}S^{Caz}$
6	2010 Jun 16	Gauteng	12 mo/M	EAggEC	H4	$R^{Amp}S^{Amc}R^{Sxt}S^{Chl}S^{Nal}S^{Cip}S^{Tet}S^{Kan}R^{Str}S^{Imi}S^{Cro}S^{Caz}$
7	2010 Jul 17	Gauteng	15 mo/F	EPEC	Non-H4	$S^{Amp}S^{Amc}S^{Sxt}S^{Chl}S^{Nal}S^{Cip}S^{Tet}S^{Kan}S^{Str}S^{Imi}S^{Cro}S^{Caz}$

*EaggEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*.

†R, resistant; S, susceptible. Isolates were classified as antimicrobial drug resistant at the following MIC breakpoint concentrations: ampicillin (Amp), MIC ≥16 μg/mL; amoxicillin/clavulanic acid (Amc), MIC ≥16 μg/mL; sulfamethoxazole/trimethoprim (Sxt), MIC ≥4 μg/mL; chloramphenicol (Chl), MIC ≥16 μg/mL; nalidixic acid (Nal), MIC ≥32 μg/mL; ciprofloxacin (Cip), MIC ≥2 μg/mL; tetracycline (Tet), MIC ≥8 μg/mL; kanamycin (Kan), MIC ≥32 μg/mL; streptomycin (Str), MIC ≥64 μg/mL; imipenem (Imi), MIC ≥8 μg/ml; ceftriaxone (Cro), MIC ≥16 μg/mL; ceftazidime (Caz), MIC ≥16 μg/mL.

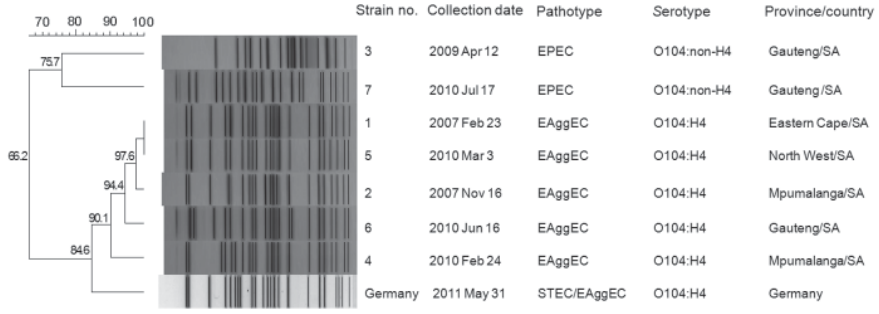

Figure. Pulsed-field gel electrophoresis profiles (*Xba*I digestion) of *Escherichia coli* O104 strains from South Africa (SA) compared with a strain from Germany. EPEC, enteropathogenic *E. coli*; EaggEC, enteroaggregative *E. coli*; STEC, Shiga toxin–producing *E. coli*. Scale bar and numbers along branches indicate percentage pattern similarity.

unrelated to strains from South Africa. However, the strain from Germany was most closely related to the EAggEC strain cluster from South Africa (pattern similarity 85%).

Conclusions

Our findings show that *E. coli* O104 is rarely associated with human diarrhea in South Africa and accounts for <1% of all DEC pathotypes identified during 2004–2011 by laboratory-based surveillance and limited sentinel surveillance. Strains of *E. coli* O104 from South Africa were mostly associated with EAggEC pathotypes; most (5/7) were identified as EAggEC. Infection with this pathotype has been associated with more persistent diarrhea (duration >14 days) (*15*). Therefore, patients infected with EAggEC are more likely to have fecal cultures tested, potentially leading to greater numbers of EAggEC isolates identified.

In South Africa, *E. coli* O104 infections were more commonly identified in children than in adults. Unlike the *E. coli* O104 strain that caused the outbreak in Germany, strains of *E. coli* O104 from South Africa did not produce Shiga toxin and did not show ESBL activity. PFGE data supported these phenotypic data, suggesting that strains from South Africa were not related to the outbreak strain from Germany. The PFGE data also showed that strains of EAggEC O104:H4 from South Africa were highly clonal. Further work is necessary to better understand the global distribution of these isolates and the role of molecular epidemiologic techniques in characterizing this newly emerging serotype.

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Vertical Transmission of *Babesia microti*, United States

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Babesiosis is usually acquired from a tick bite or through a blood transfusion. We report a case of babesiosis in an infant for whom vertical transmission was suggested by evidence of *Babesia* spp. antibodies in the heel-stick blood sample and confirmed by detection of *Babesia* spp. DNA in placenta tissue.

Babesiosis is an emerging infection in the United States, principally caused by *Babesia microti* (*1*). The most common route of infection is the bite of an *Ixodes scapularis* tick; transmission can also occur by transfusion of infected blood products, and vertical transmission in animals has been documented (*2*,*3*) and is a potential route of transmission for humans. We report a case of babesiosis in an infant for whom vertical transmission was suggested by *Babesia* spp. antibodies in a heel spot blood sample and confirmed by detection of *Babesia* DNA in placenta tissue.

The Case-Patient

A 6-week-old girl from Yorktown Heights, New York, was admitted to the hospital on September 16, 2002, with a 2-day history of fever, irritability, and decreased oral intake. The mother was asymptomatic during and after her pregnancy. The infant was delivered vaginally and full term at 3,430 g without complications. The infant's mother had visited parks in Westchester and Dutchess Counties in New York during the pregnancy but was unaware of any tick bites. The infant had no known tick exposure, and neither mother nor infant had a history of blood transfusion.

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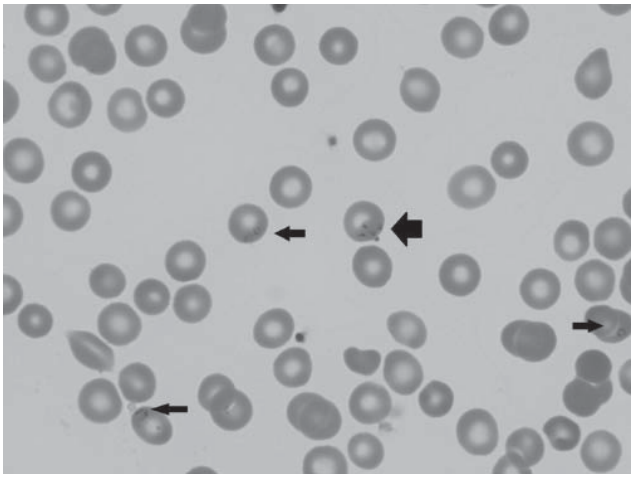

Figure. Peripheral blood smear of 6-week-old infant with suspected congenital babesiosis. Thin arrows indicate *Babesia* spp. parasites; thick arrow shows the classic tetrad formation or Maltese cross.

During examination, the infant was alert but irritable and pale. Axillary temperature was initially 36.8°C but increased to 38.1°C on the same day. Her conjunctivae were icteric, she had a palpable spleen tip, and her liver was palpable 3 cm below the costal margin. Initial laboratory findings included hemoglobin 7.1 g/dL, platelet count 100 × 10^3/μL, and leukocyte count 19.7 × 10^3 cells/μL with a differential of 4% segmented neutrophils, 80% lymphocytes, and 16% monocytes. Reticulocyte count was 5.5%. Total bilirubin concentration was 2 mg/dL with a direct fraction of 0.4 mg/dL; aspartate aminotransferase level was 66 U/L, alanine aminotransferase level was 50 U/L, and alkaline phosphatase level was 339 U/L. Cultures of blood, urine, and cerebrospinal fluid samples yielded negative results. Lyme disease serologic test result was negative.

Routine examination of a peripheral blood smear showed *B. microti* in 4% of erythrocytes (Figure); a blood sample from the infant was positive by PCR for *B. microti* DNA. Total *B. microti* antibody titer was >256 by indirect immunofluorescence assay, with a polyvalent secondary antibody (anti-IgG+IgA+IgM) (*4*) that was presumed to be principally IgG because test results for IgM were negative (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-0988-Techapp.pdf). The heel-stick blood sample obtained on the infant's third day of life as part of newborn screening was tested and found to be negative for *B. microti* by PCR (*5*) and for IgM but total antibody positive (>128) (online Technical Appendix).

Examination of the placenta showed focal basal decidual inflammation, mild chorangiosis, and villus dysmaturity. *Babesia* spp. piroplasms were not detected in

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maternal or fetal blood by histologic examination of hematoxylin and eosin–stained sections of formalin-fixed, paraffin-embedded tissue of the placenta disk, amnion/chorion, and umbilical cord. *Babesia* DNA was detected by real-time PCR testing of paraffin-embedded placenta tissue (online Technical Appendix) (*6*). Cycle threshold values were relatively high (37.1–38.2), indicating that the amount of parasite DNA in the sample was close to the limit of detection; results were reproducible on duplicate testing of DNA samples extracted from separate paraffin blocks. The real-time PCR product was of the correct size, and the melting curve demonstrated melting temperatures within 1°C from the placenta, the positive control, and a positive sample from an unrelated patient , confirming that the correct product was amplified. At time of the illness in the infant, the mother was negative for *Babesia* spp. according to PCR and smear but positive for total antibodies (>256).

Table. Comparison of selected clinical and laboratory data from reported cases of congenital babesiosis in 5 infants*

	Reference				
Clinical data	(*7*)	(*8*)	(*9*)	(*10*)	This study
Year of diagnosis/ location	Not given/Long Island, New York	Not given/Long Island, New York	Not given/New Jersey	Not given/Long Island, New York	2002/Westchester County, New York
Infant age at time of symptom onset, d	30	32	19	27	41
Clinical findings	Fever, irritability, pallor, hepatosplenomegaly	Fever, lethargy, poor feeding, pallor, scleral icterus, hepatomegaly	Fever, poor feeding, gagging, irritability, pallor, scleral icterus, hepato-splenomegaly	Fever, pallor	Fever, decreased oral intake, irritability, scleral icterus, pallor, hepatosplenomegaly
Initial babesia parasitemia level, %	5	4.4	15	2	4
Hospitalization, d	6	5	8	NA	5
Maternal tick bite	1 wk before delivery	7 wk before delivery	4 wk before delivery	None known	None known
Babesia spp. serologic and PCR results for infant	30 d after birth: IgM+/IgG+ (128/128) by IFA; 32 d after birth: IgM+/IgG+ (256/512) by IFA; PCR ND	At illness onset: IgG IFA 160; IgM/IgG immunoblot +; PCR ND	At illness onset: IgM+/IgG+ (40/256) by IFA; PCR ND	NA	Newborn screening (heel stick): IgM– (<16); total antibody + (>128) by IFA; PCR–; 6 wks after birth: IgM– (<16); total antibody + (>256) by IFA; PCR+
Babesia spp. evaluation results for mother	30 d after birth: IgM+/IgG+ (2,048/1,024); 32 d after birth: IgM+/ IgG+ (4,096/1,024); peripheral smear – at time of delivery and at 30 and 32 d after birth	7 wk before birth: IgG IFA <40; IgM/IgG immunoblot –; 2 mo after birth: IgG IFA 640; IgM/IgG immunoblot +; peripheral smear – at delivery and at infant illness onset	At infant illness onset: IgM+/IgG+ (80/>1,024) by IFA; peripheral smear negative at time of infant illness onset	At infant illness onset: PCR+	Birth: placenta PCR+; 6 wk after birth: IgM ND; total antibody + (>256) by IFA; PCR–; peripheral smear –
HGB, g/dL	9.3	10.8	8.8	NA; HCT 24.3%	7.1
Platelets, x $10^3/\mu L$	38	87	34	101	100
Leukocytes/PMN leukocytes, cells/μL	6,500/1,170	NA	9,000/1,890	NA	19,700/788
LDH, U/L	894	NA	2535	NA	NA
Bilirubin indirect, mg/dL	3.6	9.7	5.9	NA	1.6
AST, U/L	90	NA	53	NA	66
ALT, U/L	90	NA	18	NA	50
Treatment	CLI and quinine for 10 d	CLI and quinine with AZT added on day 3; on day 5 changed to AZT plus quinine for additional 7 d	AZT and ATO for 10 d	AZT and ATO, duration not given	AZT and ATO for 9 d
Follow-up	Well at 6 mo posttreatment	Improved at 2 wk	Lost to follow-up	NA	22 mo
Blood transfusion for anemia	Yes, for HCT of 18%	Yes, for HGB of 7.3 g/dL	Yes, for HGB of 7.0 g/dL	Yes, for HCT of 17.3%	Yes, for HGB of 5.2 g/dL with HCT of 15.8%

*No mothers became ill. NA, not available; +, positive; IFA, indirect immunofluorescence assay; ND, not done; –, negative; HGB, hemoglobin; HCT, hematocrit; PMN, polymorphonuclear; LDH, lactate dehydrogenase level; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CLI, clindamycin; AZT, azithromycin; ATO, atovaquone.

The infant was treated with a 9-day course of azithromycin plus atovaquone. A blood transfusion was administered when her hemoglobin concentration fell to 5.2 g/dL. The infant became afebrile by 72 hours and was discharged after a 5-day hospitalization. Repeat blood smears revealed a parasite load of 0.3% at discharge. On final evaluation at 22 months of age, physical examination revealed no abnormalities; hemoglobin level was 11.7 g/dL, *Babesia* PCR was negative, and total *Babesia* antibody level was positive at 128.

Conclusions

Congenital babesiosis has been rarely reported (Table) (*7*–*10*). This case provided convincing evidence for congenital babesiosis because of prepartum infection involving the placenta in the mother. On the basis of experience with congenital malaria, we assume that *Babesia* spp. parasites cross the placenta during pregnancy or at the time of delivery (*11*,*12*). In congenital malaria, increasing evidence suggests that the malaria parasites are most often acquired antenatally by transplacental transmission of infected erythrocytes (*12*).

Reported cases of congenital babesiosis share many similarities, including asymptomatic maternal infection and development of fever, hemolytic anemia, and thrombocytopenia in the infant detected between 19 and 41 days after birth. All of the infants responded to antimicrobial drug therapy; 3 were treated with azithromycin plus atovaquone (*9*,*10*), the preferred treatment regimen for mild babesiosis (*1*). All infants required a blood transfusion because of severe anemia. The clinical signs and symptoms for these cases of congenital babesiosis are similar to those of congenital malaria in non–disease endemic areas (*11*,*13*).

We found *Babesia* spp. antibodies on day 3 of life by analyzing the patient's heel-stick blood sample, which likely represented maternal transfer of IgG. Passive transfer of maternal antibodies is regarded as a protective factor against congenital malaria, and some newborns with malaria who are parasitemic at birth spontaneously clear the infection without ever becoming ill (*11*,*14*). The temporary presence of maternal IgG in infants has been suggested as an explanation for the typical 3–6 week incubation period of congenital malaria in non–disease endemic areas (*14*).

The real-time PCR used to find *B. microti* DNA in placenta tissue is ≈20× more sensitive than microscopic examination of Giemsa-stained blood smears (*6*). Assuming a blood sample with a parasitemia equivalent to that detected in the placental tissue, a blood smear would contain ≤10 infected cells per slide. Given the low level of *Babesia* DNA in the placenta tissue, it is not surprising that histologic examination did not reveal piroplasms. Nonetheless, limited evidence of placental abnormalities suggests a pathologic process.

In summary, babesiosis is an emerging infectious disease (*15*) that can rarely cause congenital infection. This diagnosis should be considered in the differential diagnosis of fever and hemolytic anemia in infants from disease-endemic areas.

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Klebsiella pneumoniae in Gastrointestinal Tract and Pyogenic Liver Abscess

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To determine the role of gastrointestinal carriage in *Klebsiella pneumoniae* liver abscess, we studied 43 patients. Bacterial isolates from liver and fecal samples from 10 patients with this condition and 7 healthy carriers showed identical serotypes and genotypes with the same virulence. This finding indicated that gastrointestinal carriage is a predisposing factor for liver abscess.

Klebsiella pneumoniae has emerged as the predominant cause of pyogenic liver abscess in Taiwan and other countries in eastern and Southeast Asia (*1–4*). This condition is frequently associated with severe complications, including septic endophthalmitis and other extrahepatic lesions, especially in patients with diabetes (*1*).

In Taiwan, the annual incidence of pyogenic liver abscess has increased steadily from 11.15 cases/100,000 persons in 1996 to 17.59 cases/100,000 persons in 2004, and *K. pneumoniae* was found in 79.9% of culture-positive cases (*5*). We showed that >3,000 new cases of pyogenic liver abscess were found each year during 2005–2008 (*6*). However, the pathogenesis of *K. pneumoniae* liver abscess remains unclear, and the source of endogenous or exogenous infections has been debated.

To determine whether strains recovered from liver aspirate samples originated from gastrointestinal flora of patients, we investigated isolates from liver aspirate, nasal swab, saliva, and fecal samples by using genomic analysis. Using serotyping and molecular typing methods, we systematically investigated the association between isolates from liver aspirates and those from other body sites in patients with *K. pneumoniae* liver abscess. We also investigated *K. pneumoniae* isolates from healthy carriers that were genetically similar to liver abscess isolates to assess whether colonization of virulent *K. pneumoniae* occurs in these persons, which could subsequently lead to development of liver abscess.

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The Study

During January 2009–December 2010, a total of 43 adult patients (mean age 68.2 years) with liver aspirate cultures positive for *K. pneumoniae* in Taipei Veterans General Hospital were consecutively enrolled in the study. All cases of *K. pneumoniae* liver abscess were community acquired. Clinical characteristics of patients are shown in Table 1.

To determine whether *K. pneumoniae* liver abscess originated from the gastrointestinal tract of patients, we concomitantly tested all liver aspirate, saliva, nasal swab, fecal, and blood samples by using bacterial culture before patients were treated with antimicrobial drugs. A total of 125 *K. pneumoniae* isolates from 43 patients were obtained. Information on culture-positive sites is shown in the online Appendix Table (wwwnc.cdc.gov/EID/article/18/8/11-1053-TA1.htm). To compare virulence and genetic relatedness of *K. pneumoniae* from liver abscess patients and healthy carriers, we obtained 1,000 *K. pneumoniae* isolates from fecal samples of asymptomatic adults during routine physical examinations at the Tri-Service General Hospital (Taipei, Taiwan).

All clinical *K. pneumoniae* isolates were serotyped by using countercurrent immunoelectrophoresis with serotype K antiserum. Isolates with serotypes K1 and K2 were confirmed by PCR (*7*). All K1 isolates were screened for CC23 representatives by detection of the *allS* gene by using PCR as described (*7*). Seroepidemiologic study of *K. pneumoniae* isolates from liver abscess patients showed that serotypes K1 and K2 were predominant, accounting for 61% (26/43) and 16% (7/43) of all isolates, respectively.

All K1 isolates had the *allS* gene, which is consistent with results of a study that showed that the virulent K1 clone of CC23 is associated pyogenic liver abscess (*7*). Although there was no difference in clinical characteristics between K1/K2 and non-K1/K2 patients, complications of distal septic metastasis (9%) and death (mortality rate 9%) were found for the K1/K2 group (Table 1).

A total of 17 randomly selected pairs of representative *K. pneumoniae* isolates (from liver aspirate, saliva, and fecal samples), including serotypes K1 (13 isolates), K2 (3 isolates), and non-K1/K2 (1 isolate), from patients with liver abscess were subjected to pulsed-field gel

Table 1. Clinical characteristics of 43 patients with *Klebsiella pneumoniae* liver abscess, Taiwan, January 2009–December 2010*

Characteristic	No. (%) patients		p value
	Serotype K1/K2, n = 33	Non–K1/K2,†(n = 10	
Sex			0.89
M	19 (57.6)	6 (60.0)	
F	14 (42.4)	4 (40.0)	
Symptom/sign			
Fever	33 (100.0)	10 (100.0)	
Chills	20 (60.6)	4 (40.0)	0.25
RUQ pain/tenderness in abdomen	31 (94.0)	9 (90.0)	0.67
Nausea/vomiting	10 (30.3)	3 (30.0)	0.99
Leukocytosis, $>10^3$ cells/μL	32 (97.0)	10 (100.0)	1.00
Complication with distal metastasis	3 (9.1)	0	0.572
Endophthalmitis	1 (3.0)	0	
Meningitis	1 (3.0)	0	
Lung abscess	1 (3.0)	0	
Underlying disease			
Diabetes mellitus	20 (60.7)	5 (50.0)	0.55
Alcoholism	2 (6.0)	1 (10.0)	0.59
Malignancy	4 (12.1)	3 (30.0)	0.13
CVA	2 (6.0)	1(10.0)	0.59
Biliary tract diseases	8 (24.2)	5 (50.0)	0.12
Liver cirrhosis	2 (6.0)	0	1.00
COPD	1 (3.0)	0	1.00
Chronic renal insufficiency	7 (21.2)	2 (20.0)	0.93
HBsAg positive	2 (6.0)	0	1.00
Deaths	4 (12.1)	0	0.593

*Mean ages of patients were 67.3 for those infected with serotype K1/K2 and 68.6 y for those infected with non-K1/K2. RUQ, right upper quadrant; CVA, cerebrovascular accident; COPD, chronic obstructive pulmonary disease; HBsAg, hepatitis B surface antigen.
†Patients with serotypes K15 (1), K19 (3), K31 (1), K46 (1), K54 (2) and nontypeable (2).

electrophoresis (PFGE) analysis with *Xba*I. All liver aspirate isolates had a PFGE profile identical or closely related to those of fecal or saliva samples from the same patient (Figure 1). Isolates from different patients that belonged to serotypes K1 and K2 were distinguishable from each another, indicating epidemiologic unrelatedness of these strains.

Among 17 patients, PFGE matching of liver aspirate *K. pneumoniae* isolates to isolates from fecal samples of 1,000 healthy adults was performed by using computer program analysis. PFGE showed that 7 groups of serotype K1 *K. pneumoniae* isolated from fecal samples from 10 patients and 7 healthy carriers had identical and >90% similarity in PFGE patterns (Figure 2). No serotype K2 or non-K1/K2 isolates could be matched with other isolates from healthy carriers.

The *rmpA* and aerobactin virulence genes were detected in all K1/K2 isolates (*8*). In vitro and in vivo assays to assess virulence were performed by using neutrophil phagocytosis and serum resistance assays as described (*9*). PCR showed that *rmpA* and aerobactin virulence genes were present in all 17 matched isolates from liver aspirate specimens and healthy carriers. Virulence assessment demonstrated that groups 1–6 (except patient 7) were resistant to phagocytosis and showed evidence of serum resistance (Table 2). In mouse lethality assays, various 50% lethal dose (LD_{50}) were observed among 7 groups of isolates from liver aspirates. Similar LD_{50} values were observed among all 7 groups of isolates, indicating no difference in virulence between isolates from patients and those from healthy carriers, and that the healthy adults carried the virulent strains in their intestines

Conclusions

Molecular typing of the *K. pneumoniae* strains from different patients showed different patterns, indicating epidemiologic unrelatedness of these strains, a finding that excludes a common origin of *K. pneumoniae* and

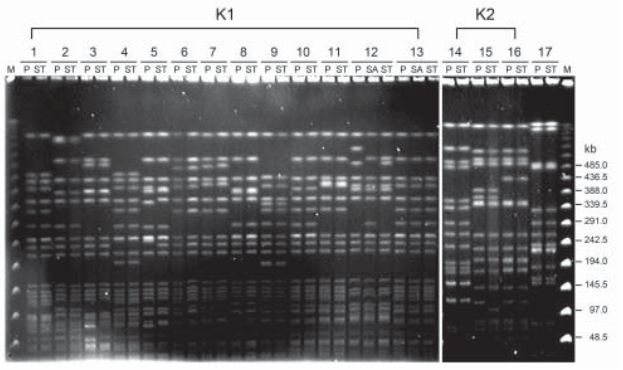

Figure 1. Pulsed-field gel electrophoresis of randomly selected isolates of *Klebsiella pneumoniae* from 17 patients with liver abscess, Taiwan, January 2009–December 2010. DNA fragments were subjected to electrophoresis after digestion with *Xba*I. Lanes 1–13, serotype K1 isolates; lanes 14–16, serotype K2 isolates; lane 17, serotype non-K1/K2 isolates. M, molecular mass marker; P, liver aspirate; ST, stool; SA, saliva.

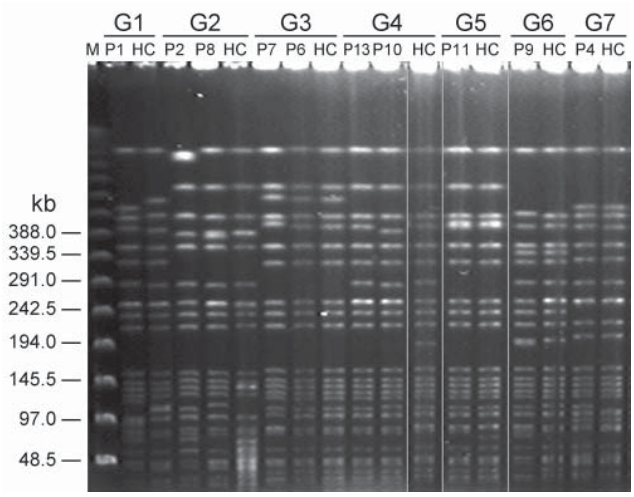

Figure 2. Pulsed-field gel electrophoresis of *Klebsiella pneumoniae* isolates from fecal samples of 7 patient groups with liver abscess and healthy carriers, Taiwan, January 2009–December 2010. G, patient group; M, molecular mass marker; P, patient; HC, healthy carrier.

transmission between patients. All PFGE profiles for liver aspirate isolates were identical or clonally related to those for isolates from fecal samples, suggesting that these infections originated from patient flora.

A previous study showed that serotype K1 and K2 isolates with aerobactin and *rampA* genes were more virulent (*10*). We found 3 isolates from patient 7, patient 4, and healthy carrier 7 that had aerobactin and *rmpA* genes and were less virulent than other matched groups. This finding is compatible with results of our previous investigation (*8*). Thus, development of *K. pneumoniae* liver abscesses probably results from a combination of virulence determinants rather than a single factor (*8*). Furthermore, we found that 6 groups of serotype K1 *K. pneumoniae* strains isolated from liver abscess patients (not patient 7) and 6 from healthy carriers had identical PFGE profiles with the same virulence-associated genes and similar LD_{50} values. This finding indicated that healthy adults had virulent strains in their gastrointestinal tracts.

Table 2. Virulence of serotype K1 *Klebsiella pneumoniae* isolates and clonal relationship from patients with liver abscess and healthy carriers, January 2009–December 2010, Taiwan*

Group no.†	Source	Serum resistance	Phagocytosis, %‡	Mice LD_{50}, CFU
1	P1	R	30.9	$<2.6 \times 10^2$
	HC-1	R	31.0	2.3×10^3
2	P2	R	24.2	$<2.9 \times 10^2$
	P8	R	18.3	$<1.5 \times 10^2$
	HC-2	R	8.8	3.5×10^2
3	P7	S	86.9	$>1.7 \times 10^5$
	P6	R	29.7	2.2×10^2
	HC-3	R	35.6	3.0×10^2
4	P13	R	37.6	3.5×10^2
	P10	R	30.7	$<3.8 \times 10^2$
	HC-4	R	18.8	$<3.0 \times 10^2$
5	P11	R	20.4	2.3×10^3
	HC-5	R	13.5	$<2.4 \times 10^2$
6	P9	R	17.8	3.4×10^3
	HC-6	R	31.7	1.7×10^3
7	P4	S	35.6	1.9×10^4
	HC-7	S	33.1	5.2×10^3

*All groups were positive for the *rmpA* and aerobactin genes. LD_{50}, 50% lethal dose; P, patient; R, resistant: HC, health carrier; S, sensitive.
†Isolates with an identical or clonally related pulsed-field gel electrophoresis profile from liver aspirate and feces of healthy carriers.
‡Percentage of *K. pneumoniae* phagocytosed by neutrophils after 15 min.

Although *K. pneumoniae* liver abscess might be induced by inhalation of *K. pneumoniae* and a bacteremic phase, we showed that none of 49 patients with community-acquired pneumonia and bacteremia caused by *K. pneumoniae* showed development of *K. pneumoniae* liver abscess concomitantly (*11*). A study from Japan reported familial spread of a virulent K1 clone causing *K. pneumoniae* liver abscess (*12*). In this study, 1 family member without *K. pneumoniae* liver abscess had the same clone in her feces (*12*). Thus, the route of entry is probably from the gastrointestinal tract. An animal study demonstrated that *K. pneumoniae* strains with genetic regulatory networks for translocation have the ability to cross the intestinal barrier and cause liver abscess (*13*).

Carrier rates of *K. pneumoniae* have differed considerably among studies. The rate of detection in fecal samples from healthy persons ranged from 19.4% to 38% in studies in Europe (*14*). In a recent investigation, we reported a fecal carriage rate of *K. pneumoniae* in healthy adults of 75% and high prevalence (23%, 17/76) of serotype K1/K2 isolates among typeable strains in Taiwan (*15*). The high carriage of virulent *K. pneumoniae* strains in feces is probably the reason why there are so many cases of *K. pneumoniae* liver abscess in Taiwan.

Our study demonstrated that intestinal colonization of virulent type *K. pneumoniae* is highly associated with pyogenic liver abscess. Early detection of colonization by *K. pneumoniae*, especially serotypes K1 and K2 in patients with diabetes, might help with making treatment decisions for infected patients.

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Dr Fung is an attending physician at the Taipei Veterans General Hospital. His research interests included pathogenesis of *K. pneumoniae* infections and antimicrobial drug resistance.

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Third-Generation Cephalosporin–Resistant *Vibrio cholerae*, India

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Vibrio cholerae resistance to third-generation cephalosporins is rarely reported. We detected a strain that was negative for extended-spectrum β-lactamase and positive for the AmpC disk test, modified Hodge test, and EDTA disk synergy test and harbored the *blaDHA-1* and *blaNDM-1* genes. The antimicrobial drug susceptibility profile of *V. cholerae* should be monitored.

Vibrio cholerae has developed enormous capabilities to combat antimicrobial drug effect. It possesses efflux pumps that act on multiple classes of antimicrobial drugs and elaborates enzymes that can hydrolyze complex antimicrobial drugs. It also can share antimicrobial resistance genes through integrons and conjugative plasmids that enable easy transfer of antimicrobial drug resistance genes and thus contribute to spread of antimicrobial resistance (*1*).

Even though fluid replacement plays a major role in treating cholera during outbreaks, antimicrobial drugs are crucial for controlling the disease and its spread. Antimicrobial therapy reduces shedding of the *Vibrio cholerae* bacillus in feces from ≥5 days to 1–2 days, thereby reducing the volume of diarrheic stool and the duration of illness, hastening recovery, and decreasing the chances of disease spread. In the absence of effective antimicrobial therapy, infected persons shed the bacillus for ≥5 days. Reported resistance to most commonly used antimicrobial drugs, i.e., tetracycline and ciprofloxacin (*1*), has limited options for therapy. Such drug-resistant *V. cholerae* strains threaten public health (*2*). Resistance to third-generation cephalosporins has rarely been reported (*1*). Our goal was to determine the mechanism(s) of resistance to third-generation cephalosporin by phenotypic and genotypic methods.

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The Study

A 2-year-old child was admitted to the department of pediatrics at the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India, with a provisional diagnosis of cholera. Before the child received antimicrobial therapy, a fecal specimen was submitted to the Department of Microbiology at JIPMER. The child was treated successfully with intravenous fluid supplements and ciprofloxacin.

Meanwhile, a strain of *V. cholerae* O1 El Tor Ogawa was isolated from the fecal specimen. This strain was biochemically identified (*3*) and confirmed by agglutination with specific antiserum (BD Difco, Becton Dickinson, Sparks, MD, USA). Antimicrobial drug susceptibility testing was conducted by Kirby–Bauer method in accordance with the Clinical and Laboratory Standards Institute (*4*) against ampicillin (10 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), furoxone (300 μg), cotrimoxazole (25 μg), and tetracycline (30 μg). The MIC for ceftriaxone was determined by the agar dilution method and Etest for the El Tor Ogawa strain. For the agar dilution method, we doubled dilutions of ceftriaxone sodium (Himedia, Mumbai, India) from 0.5 μg/mL to 128 μg/mL, which included the recommended break points (*5*). American Type Culture Collection *Escherichia coli* 25922 was spread on each plate as growth control. The Etest was performed according to the manufacturers' instructions (AB bioMérieux, Solna, Sweden). We performed the combination disk test using cefotaxime and ceftazidime, alone and in combination with clavulanic acid, to detect extended-spectrum β-lactamase (ESBL) in accordance with Clinical and Laboratory Standards Institute guidelines (*4*). The AmpC disk test was performed as described (*6*). The modified Hodge test and imipenem-EDTA disk synergy test for the phenotypic detection of carbapenemase were performed as described (*4*,*7*). We extracted DNA from the strain by using the boiling method. PCR was performed to detect ESBL genes, i.e., *blaCTX* (*8*), *blaSHV* (*8*), and *blaTEM* (*9*); a multiplex PCR was used to detect the AmpC group of genes, i.e., *blaMOX*, *blaCIT*, *blaDHA*, and *blaACC* (*10*). A multiplex PCR to detect carbapenemase genes, i.e., *blaKPC*, *blaIMP*, *blaVIM*, and *blaNDM*, was performed (*11*). Sequencing was performed to identify AmpC β-lactamase gene and the carbapenemase gene. Sequencing of these genes was conducted by Macrogen Inc. (Seoul, South Korea). We used the BLASTN program (www.ncbi.nlm.nih.gov/BLAST) for database searching.

The strain was resistant to ampicillin, ceftriaxone, cotrimoxazole, and furoxone and sensitive only to ciprofloxacin and tetracycline. The MIC for ceftriaxone was 16 μg/mL. The phenotypic tests for ESBL detection were negative. PCRs for detection of the ESBL genes were negative. Because the strain was negative for the

phenotypic and the genotypic tests for ESBL production, in our attempt to determine the reason for the increased MIC, we further tested for the AmpC type of β-lactamase production. The strain, an AmpC β-lactamase producer, was positive for the *blaDHA* gene (405 bp) by multiplex PCR. On sequencing, the *blaDHA* gene detected had 99% identity with *Klebsiella pneumoniae* β-lactamase *blaDHA-1* gene (GenBank accession no. AY635140.1). Because carbapenems are considered the treatment of choice for AmpC-producing organisms, we tested the strain for the production of carbapenemase and found it to be a carbapenemase producer by a positive modified Hodge test and by the EDTA disk synergy test. The multiplex PCR for detection of carbapenemase genes yielded an amplicon of 660 bp, which was confirmed by sequencing to be an *blaNDM-1* gene and had 100% identity with *E. coli* strain HK-01 plasmid pNDM-HK (GenBank accession no. HQ451074.1).

To demonstrate the presence of a plasmid bearing the *blaNDM-1* gene, we isolated a plasmid using the alkaline lysis method (*12*) from the *V. cholerae* strain. This plasmid was subjected to the multiplex PCR to detect the carbapenemase genes mentioned earlier (*11*), which yielded an amplicon of 660 bp matching that of the *blaNDM-1* gene amplicon. We transferred this plasmid by a method described by Lee et al. (*13*) and used *E. coli* J53 as the recipient, which was isolated on meropenem (4 mg/L) containing Luria-Bertani agar and MacConkey agar, meropenem (4 mg/L), and sodium azide (100 mg/L) (*14*). We also subjected the *V. cholerae* plasmid to the multiplex PCR to detect the AmpC gene, as mentioned earlier, but none of the genes were amplified. We failed to transfer the AmpC gene (*blaDHA-1*) by using the plasmid transfer experiment. Such failures of transfer of *blaDHA-1* have been mentioned by Yan et al. (*15*).

Of the many AmpC genes known are 2 types of *blaDHA* genes: *blaDHA-1* and *blaDHA-2*; both are inducible plasmid-mediated genes. Because the strain reported here was already resistant to a third-generation cephalosporin, i.e., ceftriaxone, we did not perform the inducible AmpC test and proceeded to perform the AmpC disk test. Plasmid-mediated inducible β-lactamases are extremely rare and are most often constitutive (*15*). The AmpC disk test does not differentiate between chromosomal and plasmid-mediated AmpC β-lactamases. We do not have complete information about the chromosomal AmpC genes in *V. cholerae*.

Carbapenems are the treatment of choice for AmpC-producing strains (*6*) and are hydrolyzed by carbapenemases, which have many families, including the metallo-β-lactamases (*7*). The most recently described $bla_{\text{NDM-1}}$ (New Delhi metallo-β-lactamase) is plasmid-mediated metallo-β-lactamase and has been isolated from the environment and from hospitals (*14*).

Conclusions

We isolated a clinical strain of *V. cholerae* producing an AmpC β-lactamase and a carbapenemase. Our findings, although perhaps not an issue for treatment of cholera, have other implications. The critical role of all bacteriology laboratories needs to be emphasized for determining not only resistance patterns but also the mechanisms of resistance. Health care–associated networks need to be strengthened to ensure justified and appropriate use of antimicrobial agents that will result in safe drinking water and improved sanitation; these can have remarkable effect in reducing the spread of many communicable diseases, such as cholera, and can go a long way in controlling the growing menace of antimicrobial drug resistance. In light of the above findings, the antimicrobial drug profile of organisms, such as *V. cholerae*, needs to be under constant surveillance in the community.

Dr Mandal is an assistant professor in the Department of Microbiology at JIPMER, Puducherry, India, and is in charge of the stool and urine section and the molecular biology division in the department. Her research interests include diarrheal diseases, urinary tract infections, and antimicrobial drug resistance.

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Seroprevalence and Cross-reactivity of Human Polyomavirus 9

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Many humans have antibodies against simian lymphotropic polyomavirus (LPyV), but its DNA has not been found in humans. Identification of human polyomavirus 9 (HPyV9) led us to compare the seroprevalence and cross-reactivity of LPyV and HpyV9. Results could indicate that humans who have antibodies against LPyV are infected by HPyV9.

To date, 9 human polyomaviruses (HPyVs) have been identified: BK polyomavirus, JC polyomavirus, Karolinska Institute polyomavirus, Washington University polyomavirus, human polyomavirus 6 and 7, Trichodysplasia spinulosa-associated polyomavirus, Merkel cell polyomavirus (MCPyV), and human polyomavirus 9 (HPyV9), which was identified in 2010 in human blood and skin samples (*1*,*2*). Serologic studies have shown that most adults have been exposed to HPyVs, and cross-reactivity studies have shown antigenic similarities between simian virus 40 and BK polyomavirus and, to a lesser extent, between simian virus 40 and JC polyomavirus (*3*–*5*). Interpretation of phylogenetic analysis of viral protein 1 (VP1) sequences predicts that cross-reactivity might also occur between Trichodysplasia spinulosa-associated polyomavirus and Bornean orangutan PyV, between MCPyV and chimpanzee polyomaviruses, and between HPyV9 and simian lymphotropic polyomavirus (LPyV).

Serologic survey results have shown that 15.0%–30.0% of humans have antibodies against LPyV, suggesting that the human population has been exposed to an antigenically related PyV (*4*–*6*). However, because LPyV DNA sequences have not been reported among humans (*7*,*8*), LPyV has thus far been considered to be a virus with a narrow host range, limited to nonhuman primates. The 2010 identification of HPyV9 (*1*,*2*), a virus phylogenetically related to LPyV (84.0% of identity), led us to investigate the seroprevalence of this newly discovered polyomavirus and to evaluate the existence of cross-reactivity that might explain the LPyV seroprevalence in humans.

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The Study

We investigated the seroprevalence of HPyV9, MCPyV, and LPyV in children 1–14 years of age and adults18–85 years of age in the Ferrara region of Italy during December 2010–September 2011, and attempted to determine whether cross-reactivity between LPyV and HPyV9 might explain the latter's seroprevalence reported among humans. Serum samples from 139 children (63 boys, 76 girls) and 186 adults (82 men, 104 women) were analyzed for HPyV9, LPyV, and MCPyV antibodies. The serum samples from healthy adult blood donors aged 18–65 years were obtained from the University Hospital of Ferrara Blood Center, and samples from children and from adults 66–85 years of age were obtained from the Clinical Analysis Laboratory, University Hospital of Ferrara, by using a protocol approved by the local county ethics committee at University of Ferrara. Consent from participants was not requested for polyomavirus testing; the identity of the sources was removed from the samples, and they were analyzed anonymously. To detect antibodies against HPyV9, we set up a virus-like particle (VLP)–based ELISA similar to the assay we developed for MCPyV (*9*). We obtained the HPyV9 VP1 coding sequence by total synthesis with a codon usage adapted for expression in *Spodoptera frugiperda* cells (Genscript, Piscataway, NJ, USA) (GenBank accession no. HQ696595). We used the Bac-to-Bac Baculovirus Expression System (Invitrogen, Fisher Scientific, Illkirch, France) to generate recombinant baculoviruses. HiFive cells maintained in Grace medium (Invitrogen) were infected with the different recombinant baculoviruses for the production of the different VLPs. VLPs were purified, and the presence of VLPs was determined by electron microscopy (*9*) (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/11-1625-Techapp.pdf).

ELISAs were performed (*9*) in wells coated with 100 ng of VLPs. We determined concentration of HPyV9, LPyV, and MCPyV VLPs by using the Qubit Protein Assay Kit (Invitrogen). We used human serum samples diluted 1:100 and peroxidase-conjugated goat anti-human IgG (Clinisciences, Nanterre, France) diluted 1:20,000 to detect binding of human IgG. The cutoff values for all assays were set at 0.2, as determined previously for MCPyV and LPyV (*9*).

In all, 81 (24.9%) of the 325 investigated study participants were seropositive for HPyV9, 82 (25.2%) were

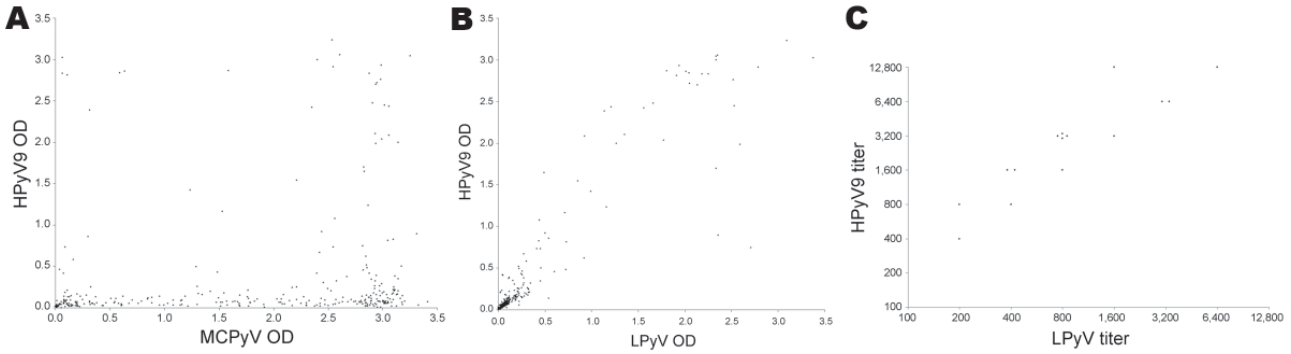

Figure 1. Cross-reactivity between human polyomavirus 9 (HPyV9), simian lymphotropic polyomavirus (LPyV), and Merkel cell polyomavirus (MCPyV) virus–like particles (VLPs). Correlation between A) seroreactivity of 325 serum samples from children and adults in Italy against HPyV9 VLPs and B) MCPyV VLPs and HPyV9 VLPs was analyzed by ELISA. Each point represents 1 serum sample. Correlation coefficients (Spearman ρ) were determined by using XLStat software (Addinsoft, Paris, France). Correlation coefficient was 0.842 in panel B and 0.139 in panel A. C) Titers of 15 serum samples from adults against HPyV9 VLPs and LPyV VLPs. Each point represents 1 serum sample. OD, optical density.

positive for LPyV, and 252 (77.5%) were seropositive for MCPyV. Seventy-six HPyV9-positive participants were also LPyV positive, whereas the ELISA optical density was found to be close to the cutoff value for the remaining 11 discordant cases. We performed proportional analysis between groups, correlation analysis between HPyV9 and LPyV reactivity and between HPyV9 and MCPyV reactivity by using the χ^2 test and the nonparametric Spearman test, respectively, using XLStat software (Addinsoft, Paris, France). The antibody reactivity of the 325 samples analyzed showed no correlation between HPyV9 and MCPyV ELISA results ($\rho = 0.14$, $p = 0.0001$; Figure 1, panel A) but a strong correlation between HPyV9 and LPyV ELISA results ($\rho = 0.84$, $p = 0.001$; Figure 1, panel B). In addition, antibody titers against HPyV9 and LPyV were determined for a subset of 15 human samples. As shown in Figure 1, panel C, the antibody titer against HPyV9 was always higher than the titer against LPyV (mean geometric antibody titer 2,660 against HPyV9 and 877 against LPyV).

To assess the degree of antigenic cross-reactivity between HPyV9 and LPyV, we performed competition assays by preincubation (1 h at 37°C) of the 15 anti-HPyV9–positive serum samples (diluted 1:100) by using 2 μg of HPyV9, LPyV, and MCPyV VLPs. In HPyV9 ELISA, the reactivity declined dramatically to undetectable levels after preincubation by HPyV9 VLPs but not by LPyV and MCPyV VLPs (Table). Similarly, the serum reactivity in LPyV ELISA declined to undetectable levels (or to low levels in 3 cases) after preincubation with both HPyV9 and LPyV but not with MCPyV VLPs. Dose-ranging competition experiments were performed on serum samples from 2 persons (online Technical Appendix Figure 2), confirming the specificity of the inhibition. These results

Table. Cross-competition between HPyV9, LPyV, and MCPyV VP1 in serum samples reactive against HPyV9 and LPyV VP1 virus-like particles*

Serum	HPyV9 reactivity (OD) after preincubation with				LPyV reactivity (OD) after preincubation with			
sample no.	Control	HPyV9	LPyV	MCPyV	Control	HPyV9	LPyV	MCPyV
H1	0.988	0.054	0.737	0.943	0.848	0.049	0.036	0.841
H2	1.083	0.128	0.737	1.018	1.007	0.128	0.104	0.995
H3	1.154	0.100	0.921	1.128	1.080	0.073	0.061	1.159
H4	2.147	0.164	1.308	1.869	1.680	0.126	0.100	1.629
H5	2.256	0.195	2.023	2.152	2.525	0.349	0.153	2.671
H6	1.610	0.127	0.846	1.544	2.068	0.099	0.095	2.179
H7	1.223	0.088	0.577	1.456	1.816	0.069	0.062	1.828
H8	1.136	0.017	0.629	0.935	0.599	0.015	0.162	0.545
H9	0.732	0.055	0.437	0.648	0.441	0.119	0.075	0.448
H10	1.216	0.036	0.683	1.057	0.771	0.045	0.032	0.659
H11	1.489	0.070	1.114	1.303	0.801	0.203	0.070	0.522
H12	1.406	0.016	0.776	1.265	0.885	0.041	0.032	0.858
H13	1.682	0.042	0.944	1.199	0.853	0.168	0.065	0.547
H14	2.424	0.061	1.566	2.145	1.536	0.064	0.058	1.516
H15	2.843	0.067	2.109	2.611	2.339	0.075	0.318	2.205

*HPyV9, human polyomavirus 9; LPyV, simian lymphotropic polyomavirus; MCPyV, Merkel cell polyomavirus; VP1, viral protein 1; OD, optical density. Fifteen serum samples from blood donors reactive against HPyV9 and LPyV VP1 virus-like particles were preincubated with HPyV9, LPyV, or MCPyV VLPs.

indicate that the HPyV9 reactivity detected in human serum samples was caused by HPyV9 infection and not by infection with the closely related LPyV. The findings also indicate that detection of LPyV antibodies in human serum samples should be attributed to cross-reacting HPyV9 antibodies.

HPyV9 infection was confirmed in 27.5% of adults18–50 years of age and in 42.4% of adults 51–85 years of age (Figure 2). MCPyV infection was confirmed in 82.5% and 83.3%, respectively, of the same population groups. Among children 1–7 years of age, few HPyV9-positive subjects (10.1%) were detected compared with MCPyV-positive subjects (49.3%). HPyV9 and MCPyV antibodies were detected in 18.6% and 74.3% of children 7–14 years of age, respectively. No difference in HPyV9 seroprevalence was observed among children according to sex (14.3% vs. 14.5%, p = 0.98). Among adults, HPyV9 seroprevalence was higher among men than women (47.6% vs. 21.1%, p = 0.007). No difference in MCPyV antibodies according to sex was observed among children (69.8% vs. 55.3%, p = 0.39) or adults (86.6% vs. 91.3%, p = 0.80).

Conclusions

The HPyV9 antibodies detected in humans did not cross-react with MCPyV VLPs but cross-reacted strongly with LPyV VLPs in agreement with the recently published findings by Trusch et al., who used VP1 capsomers (*10*). Moreover, inhibition analysis suggested that LPyV does not infect humans or is limited in scope and does not circulate widely among humans.

Our finding that 61 (32.8%) of 186 adults were seropositive for HPyV9 supports the view that this virus is commonly circulating and infects humans throughout life.

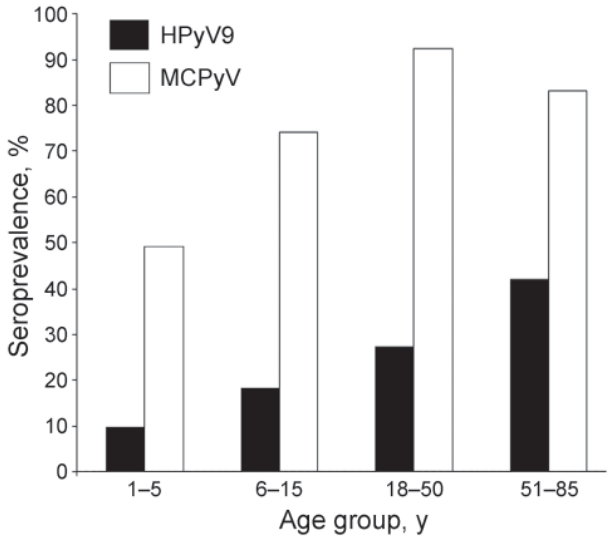

Figure 2. Human polyomavirus 9 (HPyV9) and Merkel cell polyomavirus (MCPyV) seroprevalence in children and adults in Italy.

However, the HPyV9 seroprevalence was low compared with that observed for other human polyomaviruses (*3*,*5*,*11*–*14*), with the exception of that for human polyomavirus 7 (*13*).

Primary exposure for other HPyVs often occurs in early childhood, as found for MCPyV in this study and others (*5*,*12*). The detection of antibodies in 10.1% of children1–7 years of age and the increasing seroprevalence related to increasing age among adults suggests that infection by HPyV9 occurs at all ages. It is not surprising that HPyV9 seroprevalence is lower than the seroprevalence observed for other polyomaviruses because HPyV9 DNA has been detected in only a few persons (*1*,*2*), compared with the high frequency reported for other skin polyomaviruses (*13*,*15*). In conclusion, HPyV9 circulates widely in humans but not as commonly as other polyomaviruses. Further studies are needed to identify the clinical role of this polyomavirus.

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Lack of Evidence for Schmallenberg Virus Infection in Highly Exposed Persons, Germany, 2012

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Schmallenberg virus, a novel orthobunyavirus, is spreading among ruminants, especially sheep, throughout Europe. To determine the risk for human infection, we conducted a survey among shepherds to assess possible exposure and symptoms. We also performed serologic and molecular assays. No evidence of transmission to humans was detected.

In November 2011, a new virus of the genus *Orthobunyavirus* was isolated from diseased cattle in Germany and was provisionally called Schmallenberg virus (SBV) (*1*). It has caused disease in ruminants, i.e., sheep, cattle, and goats. Acute clinical signs such as fever and diarrhea; severe congenital malformation, such as arthrogryposis and hydroencephaly; and a high proportion of stillbirths have been reported among infected animals (*2*). Transplacental transmission leads to fetal infection. The virus is vector borne and has been isolated from biting midges (*Culicoides* spp.) (*3*–*5*). Genomic analyses showed a close phylogenetic relationship to epizootic viruses of the Simbu serogroup, for which zoonotic transmission has not been shown (*1*). However, SBV also bears new genetic and animal-related clinical and epidemiologic properties. Iquitos and Oropouche viruses of this serogroup are also transmitted by culicoids and cause outbreaks in humans (*6*). La Crosse virus and California encephalitis virus can cause disease in humans and belong to the genus *Orthobunyavirus*. A few vector-borne zoonoses from the same family *Bunyaviridae*, i.e., Rift Valley fever virus and Crimean-Congo hemorrhagic fever virus, also are highly transmissible to humans through handling of infectious animal tissue. However, this mode of transmission has not been described for orthobunyaviruses. Shortly after its recognition, SBV and associated disease were reported from an increasing number of European countries, and further spread is conceivable. The virus currently is isolated mainly from sheep farms (*7*,*8*). In Germany, North Rhine-Westphalia is the area most affected. Viral loads are high in infected animals and their birth products (*2*). Thus, shepherds can be considered as strongly exposed, especially during animal obstetric events.

Because SBV emerged recently, transmission from animals to human cannot be completely excluded. Knowing whether SBV poses a risk to humans is vital. Therefore, we conducted a seroprevalence study among exposed shepherds in the area in Germany most affected (North Rhine-Westphalia) to determine whether zoonotic or vector-borne infections occur in humans.

The Study

At an SBV information meeting, 60 shepherds >18 years of age were recruited for this study. After obtaining written informed consent, we administered a standardized questionnaire. We collected information about age, sex, SBV infection in their livestock, exposure to sick lambs, frequency of insect bites, personal health, and categories of signs of disease after exposure. In addition, a serum sample was taken from each participant.

We developed an indirect fluorescent antibody test (IFAT) for primary testing of human serum. For this test, antihuman fluorescein isothiocyanate–conjugated secondary antibodies against SBV-specific IgM or IgG (antibovine for positive control) were used. For the IFAT, all heat-inactivated serum specimens were tested in dilutions of 1:20 and 1:80 on glass slides with noninfected and SBV-infected Vero cells. An SBV antibody–positive serum sample from an experimentally infected cow was used as a positive control. To check for background signals and possible cross-reactivity, we tested 80 serum samples from healthy blood donors; none were positive. A serum neutralization test (SNT) was developed for confirmation of indeterminate and positive results. Serial dilutions of the test serum (lowest dilution 1:5) were incubated for 1 h at 37°C with an equal volume of cell culture supernatant containing 100 infectious doses of SBV and subsequently mixed with Vero cells. To detect SBV-specific RNA, we performed a 1-step real-time reverse transcription quantitative PCR (RT-qPCR) on serum, as described (*1*). The ethics committee of the University Medicine Charité Berlin approved our study.

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Table 1. Self-reported exposure and symptoms of persons exposed to novel SBV, Germany, 2012*

Exposure category†	No. shepherds exposed/total no. (%)	No. shepherds with symptoms/total no. exposed (%)‡
Sheep husbandry in SBV-epizootic area	60/60 (100)	9/60 (15)
Laboratory-confirmed SBV infection in livestock	36/60 (60)	5/36 (14)
Contact with birth products or with lambs that had characteristic signs of SBV disease	48/60 (80)	8/48 (17)
Contact with adult sheep that had characteristic signs of SBV disease	28/51 (55)	5/28 (18)
Frequent insect bites in SBV-epizootic area§	22/56 (39)	5/22 (23)

*SBV, Schmallenberg virus.
†Multiple responses possible.
‡Self-reported signs and symptoms of fever, headache, skin rash, myalgia/arthralgia, respiratory problems, or photophobia since SBV infection appeared in the study area or after handling diseased animals and resulting from unknown cause in each exposure category.
§Self-reported as ‘"very often" or "often."

All 60 participants (75% male; median age 48 years [interquartile range (IQR) 41–56 years]) reported sheep husbandry in the SBV-epizootic area (Table 1). Altogether, 48 (80%) participants had contact with lambs that had characteristic malformations or with the respective birth products (median 10 [IQR 4–20] sick lambs). In livestock from 36 (60%) participants, SBV was laboratory confirmed. Characteristic signs among adult animals had first been noted in September 2011. Median time from first signs in animals to blood withdrawal was 45 days (IQR 39–66 days). A total of 55 (98%) of 56 participants self-reported insect bites during late summer to autumn; among these, 22 (39%) indicated frequent insect bites. Nine (15%) shepherds reported having had signs and symptoms since the disease had appeared in the study area or after handling diseased animals (Table 1): myalgia and arthralgia (7 shepherds), headache (4), fever (4), skin rash (2), and respiratory problems (2). No shepherds reported hospitalization. Of the 36 shepherds whose livestock had laboratory-confirmed SBV infection, 5 (14%) reported signs and symptoms: myalgia and arthralgia (4 shepherds), headache (2), fever (2), skin rash (2), and respiratory problems (2).

No SBV-specific antibodies were detected in any serum specimens (Table 2) . Eight specimens showed indeterminate fluorescent signals in the IFAT at a 1:20 serum dilution for IgG (n = 1) or IgM (n = 7) but were not reactive at 1:80 (Figure). These 8 samples were retested by SNT (serum dilution 1:10) and showed no virus inhibition at any serum dilution during 7 days of incubation. Two (25%) of these 8 shepherds reported symptoms. For the bovine control serum, the titer of the SNT was 320. RT-qPCR was negative in all serum samples.

Table 2. Results of diagnostic tests for SBV in serum samples from exposed shepherds, Germany, 2012*

Test system and dilution	Test results, no. (%) Positive/total	Indeterminate/total
IFAT IgG 80	0/60	0/60
IFAT IgM 80	0/60	0/60
IFAT IgG 20	0/60	1/60 (2)
IFAT IgM 20	0/60	7/60 (11)
SNT titer 10†	0/8	0/8
RT-qPCR	0/60	0/60

*SBV, Schmallenberg virus; IFAT, indirect fluorescent antibody test; SNT, serum neutralization test; RT-qPCR, quantitative reverse transcription PCR.
†Performed in only 8 serum samples with IFAT indeterminate results; 2/8 (25%) reported symptoms as outlined in Table 1.

Conclusions

We investigated the risk for human infection after possible high exposure to an emerging vector-borne epizootic disease through contact with infected animals and tissues or through insect bites. No evidence of SBV infection among the shepherds was found by molecular and serologic tests, even though most of the shepherds had received substantial exposure through repeated direct contact with sheep with laboratory-confirmed SBV-infection and with birth products known to contain high virus loads in the SBV-epizootic area. Reported symptoms were compatible with illnesses commonly experienced during the winter (i.e., influenza-like illness caused by human respiratory viruses) without considerable differences between the exposure categories. The likelihood of virus detection by RT-qPCR is certainly limited because SBV viremia in livestock lasts only a few days (*4*,*8*). Viremia could be of short duration in humans as well. However, after the end of the viremic phase, detection of specific antibodies can be expected. The period between exposure and sampling was sufficiently long to for antibodies to have developed after infection. Furthermore, a large proportion of the participants indicated having been frequently bitten by insects in the epizootic area. Midge bites are difficult to recall, and therefore this exposure could not be assessed precisely. Recollection of insect bites might not be equivalent to exposure to the vector species. Although SBV has been isolated from certain midge species, entomologic knowledge about the ability of different midge species to transmit SBV, i.e., vector competence and host feeding behavior, is still scarce. Nevertheless, on the basis of results from our study and the phylogenetic relationship of SBV, we conclude that the novel virus is unlikely to pose a threat to humans by transmission from infected livestock or from midges.

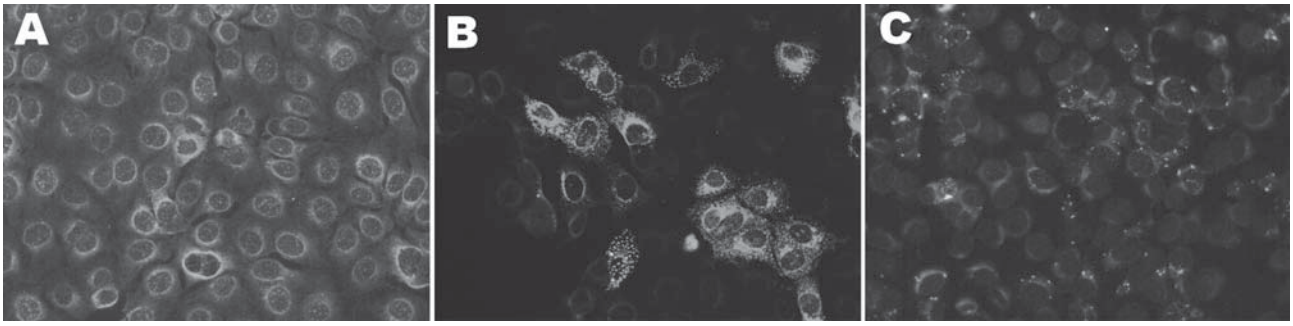

Figure. Fluorescent light microscopy images of serum samples tested for antibodies to Schmallenberg virus by indirect fluorescent antibody test on infected Vero cells mixed with noninfected Vero cells. A) Nonreactive negative serum; B) positive serum reactive with infected cells only; C) indeterminate serum with faint nonspecific reactivity. A color version of this figure is available online (wwwnc.cdc.gov/EID/article18/8/12-0533-F1.htm).

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Capsular Switching in Invasive *Neisseria meningitidis*, Brazil[1]

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During the 1990s, an epidemic of B:4 *Neisseria meningitidis* infections affected Brazil. Subsequent increase in C:4 disease suggested B→C capsular switching. This study identified B→C switches within the sequence type 32 complex. Substantial disease related to capsular switching emphasizes the need for surveillance of circulating meningococcal strains to optimize disease control.

The species *Neisseria meningitidis* includes successful commensal strains and devastating human pathogens (*1*–*3*). Invasive strains produce a polysaccharide capsule, which is essential for virulence (*3*) and is a target for most licensed vaccines. Characteristic genomic fluidity (e.g., facilitated by transformation, slipped-strand mispairing, or 2-component regulatory pathways) enables *N. meningitidis* to alter its antigenic profile (*1*). Capsular switching, which occurs through transformation and horizontal gene transfer, enables *N. meningitidis* to escape from host defenses against the original serogroup. This event is common in the absence of vaccination and has implications for meningococcal vaccines that do not cover all serogroups (*4*–*6*).

Brazil experienced a prolonged epidemic of B:4 *N. meningitidis* infections during 1988–1999. In 1990 and 1994, mass vaccination was performed by using a vaccine consisting of a serogroup B outer membrane vesicle (B:4:P1.15) and a serogroup C polysaccharide. During 1993–1994, a total of 4 C:4 *N. meningitidis* isolates were identified in samples from patients in Rio de Janeiro State (RJ). The presence of class3-PorB in these strains suggested the possibility of B→C capsular switching (*7*). Since 2000, the number of cases of serogroup C disease has steadily increased in RJ (*8*), reaching 90% of laboratory-confirmed cases in 2009. Concomitantly, the proportion of C:4 isolates increased from 2% (1988–1999) to 25% (2000–2009). To determine whether C:4 strains arose as a result of B:4 capsular switching, we performed molecular characterization of these isolates.

The Study

The study was performed at Oswaldo Cruz Institute, RJ, Brazil. The case definition for invasive meningococcal disease was isolation of *N. meningitidis* from a normally sterile body fluid from an RJ resident. Patient characteristics were obtained from the epidemiologic records of the RJ Department of Health during 1988–2009 and analyzed with EpiInfo (version 3.5.3; Centers for Disease Control and Prevention, Atlanta, GA, USA). This study was approved by the Ethical Committee of the Evandro Chagas Research Institute of the Oswaldo Cruz Foundation (protocol 0070.0.009.000–10).

Meningococcal isolates were serogrouped by agglutination and serotyped and serosubtyped by dot blot. The C:4 phenotype was used as a marker of isolates that could have resulted from B:4 capsular switching. Of 41 C:4 isolates recovered from RJ patients during the study period, 35 were viable for further characterization. We also randomly selected 35 B:4 isolates (≈10%) from a list of B:4 isolates available in the laboratory collection from the same period by using SPSS-15 for Windows (SPSS Inc., Chicago, IL, USA).

Multilocus sequence typing (MLST) (*9*) was performed in combination with outer membrane protein (OMP) gene sequencing (*10*) of *porA* variable regions (VRs) 1 and 2, *porB*, and *fetA* VR. Serogroup-specific PCR (*8*) and MLST were repeated for isolates with a suspected capsular switch by using the same template DNA. The assignment to allele, sequence type (ST), clonal complex (CC), *porA, porB,* and *fetA* was performed by querying the Neisseria Sequence Typing Home Page (http://pubmlst.org/neisseria).

STs were considered part of the same clonal complex if they shared at least 4 alleles of the 7 MLST loci with the designated central genotype. OMP gene sequence results were expressed as *porB*:P1, *porA*-VR1, *porA*-VR2, and F.*fetA*-VR (*10*). Meningococcal capsular switching was presumed to have occurred when an ST within a serogroup not usually associated with that ST and more frequently associated with another serogroup was found. MLST and OMP genotypes were used to define capsular switching in specific clones.

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The genotyping profiles of 35 C:4 isolates (1993–2009) were the following: C:4.7:P1.7.1 (54%), C:4.7:P1.19.15 (21%), C:4.7:nt (11%), C:4.7:P1.9 (5%), and single genotypes (9%): C:4.7:P1.3,. C:4.7:P1.5, and C:4.7: P1.16. Profiles of the 35 B:4 isolates collected during1988–2009 were: B:4.7:P1.19.15 (48%), B:4.7:P1.7.1 (26%), B:4.7:nt (17%), and single genotypes (9%) B:4.7:P1.7.13, B:4.21:P1.15, and B:4.23:P1.19. A comparison of patients infected by C:4 (n = 30) versus B:4 (n=179) strains showed that the former were more likely to be 5–14 years of age (Table 1). Sex distribution, case-fatality rates, and clinical features were similar for the 2 groups.

Two case clusters of C:4.7:P1.7.1 meningococcal disease occurred in RJ. The first cluster occurred during 2006 at 1 workplace and involved 4 young women. All survived; 1 was left with neurologic deficits. The second cluster occurred in 2009 in a neighborhood and involved 6 children 2–12 years of age and 1 adult. Three patients died.

MLST results showed that of the 35 B:4 isolates, 30 (86%) belonged to ST32CC and most were ST33 and ST639. There were 2 predominant OMP profiles: 3–1: P1.19.15: F5–1 (47%) and 3–79: P1:7–1.1: F5–1(33%), which are consistent with Brazil B epidemic clones. The other STs were single locus variants of ST32 or ST33. Of the 5 remaining isolates, 3 belonged to ST41/44CC, 1 to ST35CC, and 1 to ST3766 (no defined CC).

For C:4 isolates, 29 (83%) of the 35 belonged to the ST32CC. These isolates were detected first in 1993 and became common from 2000 onward. The ST32CC was represented by 6 STs; STs 639 and 33 (79%) predominated. The 2 most common OMP profiles were 3–1: P1.19.15: F5–1 and 3–79: P1:7–1,1: F5–1 (79%). Other STs detected in the ST32CC were ST34 (1 isolate) and 3 new STs (5 isolates): ST7692, ST7696, and ST7709. Of the 6 remaining isolates, 1 belonged to ST41/44CC (ST41) and 5 did not belong to a defined CC: ST7690 (3 isolates), ST7712 (1 isolate), and ST7691 (1 isolate).

OMP genotyping data demonstrated capsular switching in 4 specific meningococcal clones (Table 2). The switch to the ST639 (32CC), 3–79:P1.7–1.1:F5–1 clone resulted in a substantial number of cases and was the cause of the 2009 cluster. A highly related clone (3–79:P1.7–1.1:F5–1:ST-7696), which is a single-locus variant by MLST of the 2009 outbreak clone, was responsible for the 2006 cluster.

Conclusions

This study demonstrated that after a prolonged epidemic of serogroup B ST32 complex *N. meningitidis* in Brazil, isolates genetically indistinguishable, but expressing the serogroup C capsule, emerged and persisted as a cause of invasive disease during 2000–2009. A plausible explanation for this clonal emergence is the maintenance of virulence after capsular switch and a lack of immunity in a portion of the population in RJ. One of the clones associated with the serogroup B epidemic, B:3–1:P1.19.15:F5–1:ST-33(32CC), against which mass vaccination was directed, resulted in only 2 serogroup C cases.

Capsular switching represents a mechanism by which meningococci escape protective immunity directed at specific capsular polysaccharides (*4*). The emergence and expansion of the serogroup W135 Hajj clone in the setting of vaccination against serogroups A and C illustrate this phenomenon (*11*). However, capsular switching does not always lead to clonal emergence. For example, the serogroup C clone that resulted from capsular switching within the serogroup B epidemic in Oregon has caused relatively few cases (*5*).

We report a substantial number of cases of meningococcal disease and case clusters during 2000–2009 that were associated with ST32/ET-5 serogroup C clones that had emerged in Brazil during 1993–1998. These strains might retain the attributes of the lineages of the ST32 complex B clones, including a propensity to cause outbreaks. Additionally, some capsular variants may have a selective advantage and might, at least in part, replace original strains circulating in the population (*12*). In studies of *N. meningitidis* ST11CC strains bearing serogroups C or W135 in Brazil (*13*,*14*) and serogroups C and B in

Table 1. Characteristics of patients with confirmed serogroup B:4 and serogroup C:4 meningococcal disease, Rio de Janeiro, Brazil, 2000–2009

Characteristic	No. (%) serogroup B:4, n = 179	No. (%) serogroup C:4, n = 30	p value
Female sex	93 (52.0)	20(66.7)	0.14
Age group, y			0.046*
<1	27 (15.1)	1 (3.3)	
1–4	50 (27.9)	7 (23.3)	
5–14	56 (31.3)	15(50.0)	
15–24	24 (13.4)	4 (13.4)	
25–64	22 (12.3)	3 (10.0)	
Deceased	30 (16.8)	7(23.3)	0.38
Clinical features			0.11†
Septicemia without meningitis	22 (12.3)	7(23.3)	
Meningitis + septicemia	93 (51.9)	12(40.0)	
Meningitis only	64 (35.8)	11(36.7)	

*p value for comparison of 5–14 y versus all other age groups combined.
†p value for comparison of septicemia without meningitis versus other clinical syndromes combined.

Table 2. Serogroup B→C capsular switching among *Neisseria meningitidis* isolates belonging to 4 ST32 clonal complex populations with identical OMP genotypes, Rio de Janeiro, Brazil, 1988–2009*

ST and OMP genotype profile	No. isolates	Year(s) of isolation
ST33, 3–1:P1.19,15:F5–1		
Serogroup B:4	13	1988–2009
Serogroup C:4	2	2004–2005
ST34, 3–1:P1.19,15:F5–1		
Serogroup B.4	3	2008–2009
Serogroup C.4	1	2005
ST639, 3–79:P1.7–1,1:F5–1		
Serogroup B:4	10	1989–1997
Serogroup C:4	13	1998–2009
ST639, 3–294:P1.7–1,1:F5–1		
Serogroup B:4	1	1994
Serogroup C:4	1	2008

*ST, sequence type; OMP, outer membrane protein.

Spain (*15*), a pattern of preserved hyperinvasiveness in the emerged W135 and B strains was also found. The ability of *N. meningitidis* to cause substantial disease after capsular switching highlights the need for surveillance of circulating meningococcal strains to delineate optimal disease control policies.

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Avian Influenza and Ban on Overnight Poultry Storage in Live Poultry Markets, Hong Kong

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We analyzed ≈12 years of surveillance data on avian influenza in Hong Kong live poultry markets. A ban on keeping live poultry overnight in these markets reduced virus isolation rates by 84% in chickens (p = 0.006) and 100% (p = 0.01) in minor poultry.

Previous influenza pandemics originated from influenza viruses of birds (*1*). Live poultry markets play a crucial role in maintenance, amplification, and dissemination of avian influenza viruses (*2*,*3*) and are high-risk locations for potential zoonotic transmission of highly pathogenic avian influenza (HPAI) virus (H5N1) to humans (*4*,*5*). From September 1999 through May 2011, fecal dropping samples were collected monthly under the poultry cages in live poultry markets in Hong Kong as part of a systematic longitudinal avian influenza surveillance program. During the 12-year period of surveillance, several interventions were implemented by the Hong Kong government in response to outbreaks of influenza virus (H5N1) in live poultry markets and on poultry farms. In July 2001, a monthly rest day was first implemented; under this system, all poultry in live poultry markets must be sold or slaughtered at the end of the day, poultry stalls must be cleaned and disinfected, and the stalls must be left free of live poultry for 1 day before restocking any live poultry the next day. In February 2002, a ban on sales of live quail was implemented in because an influenza virus (H9N2) lineage commonly isolated from quail possessed the internal genes of the virus that caused the avian influenza (H5N1) outbreak in Hong Kong in 1997 (*6*). In response to further incursions of avian influenza (H5N1) into poultry markets and farms in Hong Kong, a second monthly rest day in live poultry markets was introduced in March 2003, and a complete ban on holding live poultry overnight in live poultry markets was implemented in July 2008.

Previously, we analyzed data from September 1999 through December 2005 and demonstrated that 1 rest day per month significantly reduced isolation rates of influenza virus in minor poultry (i.e., silkie chickens, pigeons, chukars, guinea fowls, and pheasants) but that an additional rest day each month did not significantly reduce the isolation rate further (*7*). In this follow-up study, which includes an additional 6 years of data, we investigated the effect of a ban on keeping live poultry overnight at live poultry markets on isolation rates of influenza A virus (H9N2) from chickens and minor poultry.

The Study

When the live poultry market surveillance program began in September 1999, eight of a total of 80 live poultry markets were selected to represent the 3 major regions of Hong Kong: Hong Kong island, Kowloon, and the New Territories. Since then, the number of markets has declined, and by May 2011, only 5 of the 8 selected live poultry markets continued in operation (of a total of 39 operating live poultry markets). A total of 53,541 samples were collected during these 141 months of consecutive sampling.

We previously published data on the effect of introducing various interventions in live poultry markets, which included the ban on the sales of live quail and the introduction of rest days (*7*). In addition to collecting fecal droppings from the cage floors for virus isolation, we collected data on the total sales of chickens and minor poultry, the proportion of chickens imported as a ratio of the whole, the temperature and relative humidity, and the type of ventilation used, as described (*7*). Laboratory processing of the specimens was conducted as described (*7*). Samples collected in virus transport medium were inoculated into 9–11-day-old embryonated eggs, and allantoic fluid with positive hemagglutination was confirmed and subtyped using standard antiserum.

Because HPAI A virus (H5N1) is rarely detected in live poultry markets in Hong Kong, we used isolation rates of influenza A virus (H9N2) as an indicator of the effect of these interventions on avian influenza virus circulation. The median numbers of samples collected weekly from chickens and minor poultry were 107 (range 3–722) and 23 (range 1–397), respectively (see online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1879-Techapp.pdf, for weekly numbers of samples). The Poisson generalized model (*8*) with influenza virus (H9N2)

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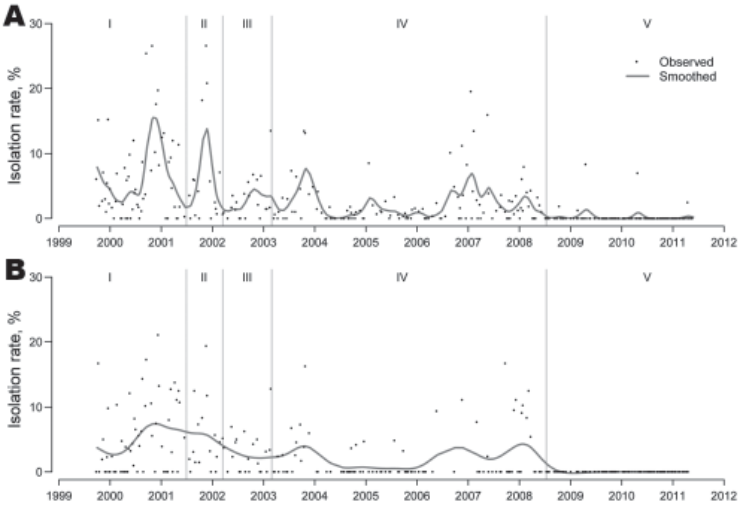

Figure. Weekly influenza virus A (H9N2) isolation rates for chickens (A) and minor poultry (B) in live poultry markets, Hong Kong, September 1999–May 2011. Vertical lines denote periods for different interventions: I, no rest day; II, 1 monthly rest day with quail sold in live poultry markets; III, 1 monthly rest day with no sales of quail in live poultry markets; IV,: 2 monthly rest days; V, ban on keeping live poultry overnight in live poultry markets.

weekly isolation counts as the outcome variable was fitted as described (*7*) and adjusted for proportion of chickens imported; total sales of chickens and minor poultry; ventilation system; weekly average temperature; relative humidity; seasonal variations; sample size; and periods corresponding to the respective interventions: period I (no rest day), II (1 monthly rest day with quail being sold in the live poultry market), III (1 monthly rest day with elimination of live quail from the live poultry market), IV (2 monthly rest days), and V (ban on holding poultry overnight in live poultry market). These variables were considered potentially important confounders related to transmission efficiency of avian influenza virus (*9*), source, type, and volume of poultry.

Weekly virus isolation counts were analyzed from September 22, 1999, through May 31, 2011. A separate model for poultry and minor poultry was fitted, and all analyses were implemented by using R version 2.12.1 software (R Development Core Team, Vienna, Austria).

The Figure shows overall isolation rates by week for chicken and minor poultry from 1999 through 2011; the Table gives the parameter estimates for the final fitted models, which were adjusted for the effect of covariables that could affect the isolation of influenza in the study. For

Table. Poisson generalized linear models for influenza virus (H9N2) isolation rates in live poultry markets, by poultry type, Hong Kong, September 1999–May 2011*

Variable	Chickens		Minor poultry	
	aRR (95% CI)	p value	aRR (95% CI)	p value
Period				
No rest day	1.69 (0.91–3.15)	0.10	2.47 (1.23–4.98)	0.01
1 rest day with quail†	1.25 (0.73–2.15)	0.42	0.99 (0.49–2.01)	0.97
1 rest day without quail†	1.00 (0.60–1.64)	0.97	0.99 (0.53–1.85)	0.97
2 rest days	Reference		Reference	
Ban on keeping live poultry overnight in live poultry markets	0.16 (0.04–0.60)	0.006	‡	0.01‡
Proportion of chickens imported, per 10% increase	0.87 (0.73–1.02)	0.09	1.02 (0.79–1.32)	0.87
Total sales				
Chickens, per 100,000 sold	1.04 (0.98–1.09)	0.19	1.05 (0.98–1.13)	0.15
Minor poultry, per 100,000 sold	2.52 (1.49–4.25)	0.001	3.15 (1.54–6.44)	0.002
Chicken × minor poultry§	0.98 (0.97–1.00)	0.03	0.97 (0.95–0.99)	0.007
Ventilation system				
Natural ventilation	Reference		Reference	
Market economic air treatment system	1.02 (0.79–1.31)	0.89	1.02 (0.78–1.34)	0.87
Air conditioned	0.71 (0.42–1.22)	0.21	0.97 (0.56–1.68)	0.90
Temperature, °C	0.98 (0.99–1.02)	0.96	1.05 (0.96–1.16)	0.29
Relative humidity, %	1.00 (0.99–1.02)	0.63	0.99 (0.97–1.00)	0.10
Seasonality term¶				
α (cosine component)	0.19 (–0.19 to 0.58)	0.33	–0.10 (–0.57 to 0.37)	0.68
β (sine component)	0.30 (–0.11 to 0.70)	0.15	0.47 (–0.06 to 0.99)	0.08

*aRR, adjusted relative risk.
†Indicates before and after ban on sales of live quail.
‡Reliable confidence interval cannot be estimated because of zero isolation of influenza virus (H9N2) from minor poultry after introduction of a ban on keeping live poultry overnight in live poultry markets. p value was calculated using likelihood ratio test.
§Interaction term.
¶The seasonality coefficients α and β contribute to the estimated isolation rate in week t via the terms $\alpha\cos(2\pi t/52) + \beta\sin(2\pi t/52)$.

chickens and minor poultry, compared with the reference category of 2 monthly rest days, the ban on keeping live poultry overnight in live poultry markets was associated with dramatic and significant reduction of influenza virus (H9N2) isolation. The isolation rate of influenza virus (H9N2) among chickens declined 84% (adjusted relative risk 0.16; p = 0.006), and no influenza subtype H9N2 viruses were isolated from minor poultry after the ban on holding poultry overnight in live poultry markets was implemented. Higher volume of minor poultry sales was also significantly associated with higher isolation rate of influenza virus (H9N2).

Conclusions

A previous study that used a stochastic metapopulation model showed that frequent rest days in live poultry markets were effective for reducing transmission of avian influenza (H5N1) (*10*). Our findings show a large additional decline in the influenza virus (H9N2) isolation rate after implementation of a ban on keeping live poultry overnight, which suggests that this intervention has an even greater effect on reducing viral load in live poultry markets than the previous intervention of 1 or 2 rest days per month. While low pathogenic influenza virus (H9N2) was the indicator virus in our study, it is likely that these interventions would have comparable effects on highly pathogenic viruses such as avian influenza (H5N1); this effect has been demonstrated by mathematical modeling (*10*). Studies by others on social network analysis have shown that daily cage cleaning and disinfection of live poultry markets in southern China (*11*), and protective factors including removal of waste in Indonesia (*12*) contributed to a reduction of HPAI (H5N1) in live poultry markets. Taken together, these studies show that eliminating the carryover of live poultry in markets from one day to the next, in the form of rest days or a total ban, is highly effective for reducing viral amplification and persistence in live poultry markets and consequently minimizes zoonotic risk.

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Drug-Resistant Tuberculosis Transmission and Resistance Amplification within Families

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Drug-resistant tuberculosis is caused by transmission of resistant strains of *Mycobacterium tuberculosis* and by acquisition of resistance through inadequate treatment. We investigated the clinical and molecular features of the disease in 2 families after drug-resistant tuberculosis was identified in 2 children. The findings demonstrate the potential for resistance to be transmitted and amplified within families.

The devastating effects of extensively drug-resistant tuberculosis (XDR TB) gained international attention after the 2006 outbreak in Tugela Ferry, South Africa. The evolution of the epidemic is the result of transmission of resistant strains and strain acquisition of resistance through inadequate treatment (*1*). Multidrug-resistant (MDR) TB is disease caused by *Mycobacterium tuberculosis* that is resistant to isoniazid and rifampin, and XDR TB is disease caused by *M. tuberculosis* that is additionally resistant to a fluoroquinolone and an injectable second-line anti-TB drug. Because children usually have transmitted resistance (*2*), they can be seen as the end of a sequence of transmission events. We describe investigations of 2 families after the identification of children with drug-resistant TB in terms of clinical features and molecular characteristics of the isolates.

The Study

This investigation was conducted in a suburban community of Cape Town, South Africa, where TB incidence was 978/100,000 population in 2009 (Health Systems Trust). Since 1994, microbiological samples from all patients treated for TB in this area have been sent to the research laboratory at Tygerberg Hospital, Stellenbosch University. From 2008 through 2010, two children from this community received a diagnosis of MDR TB.

Information was obtained from several sources to document the sequence of events that culminated in the development of MDR TB in each child. A home visit was made, and the family was interviewed after written informed consent was obtained. Family members were included if they either lived with or spent substantial amount of time with the child (*3*). Information on TB diagnosis, treatment, and outcome was obtained at interview. If a family member was identified as having had TB, family contacts of that person were included. Searches for case notes for those included were made at the local clinic, the academic hospitals, and the regional TB hospital responsible for drug-resistant TB management. Also, the local clinic TB register was consulted. The investigation was approved by the Stellenbosch University Ethics Committee.

Sputum samples from the 2 families were identified, and isolates were genotyped by spoligotyping (*4*) and IS*6110* DNA fingerprinting (*5*). Strains were identified according to distinct IS*6110* banding patterns by using Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium) or characteristic spoligotype pattern (*6*). Mutations conferring resistance to isoniazid, rifampin, ethambutol, pyrazinamide, ofloxacin, and amikacin were determined by DNA sequencing of the *inhA* promoter, *katG*, *rpoB*, *embB*, *pncA*, *gyrA*, and *rrs* genes, respectively (*7*).

A 19-month-old girl (A3) received a diagnosis of TB in March 2008 after a 6-month course of preventive therapy with isoniazid. She was brought for assessment with a 2-weeek history of cough, respiratory distress, and fever. She had contact with a patient with pre–XDR TB (MDR TB resistant to either a fluoroquinolone or a second-line injectable drug), and therefore the following antimicrobial drugs were administered: capreomycin, ethionamide, ethambutol, *para*-aminosalicylic acid, terizidone, clarithromycin, and high-dose isoniazid. Gastric aspirate samples were sent to the National Health Laboratory Service; *M. tuberculosis* grew in culture and was resistant to rifampin, isoniazid, and ofloxacin and susceptible to amikacin and ethionamide. She received treatment for 18 months from the time of her first negative culture (the first 6 months included the injectable medication) and recovered.

Patient 1's family consisted of 18 persons (Figure 1). The husband of her aunt (A2) had drug-resistant TB. He cared for the girl on a daily basis. He had received treatment initially for drug-susceptible TB; this was changed to MDR TB therapy when resistance to rifampin and isoniazid was determined and then to XDR TB treatment when resistance

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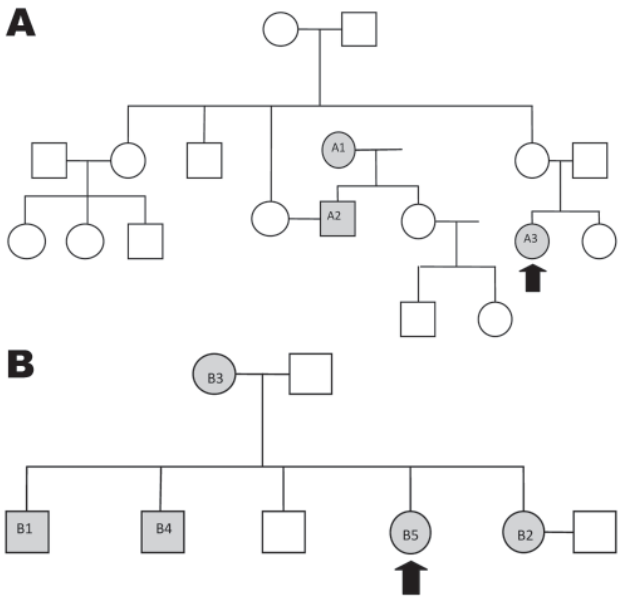

Figure 1. Patients with drug-resistant tuberculosis in families 1 (A) and 2 (B), South Africa, 2008–2010. Gray shading indicates person identified with tuberculosis; arrows indicate child index case-patients; circles indicate female family members; squares indicate male family members.

to second-line drugs was discovered. He subsequently died. His mother (A1) had repeatedly dropped out of treatment, and drug-resistant TB was finally diagnosed in 1998. She refused further treatment and died in 2003. The clinical chronology is shown in Figure 2; molecular details regarding the samples analyzed are shown in the Table.

A 13-year-old girl (B5) was identified in April 2009 as a contact of multiple family members with XDR TB. She was asymptomatic, but a chest radiograph showed abnormalities. A regimen was begun of capreomycin, ethionamide, pyrazinamide, terizidone, *para*-aminosalicylic acid, co-amoxicillin/clavulanic acid, clarithromycin, linezolid, and high-dose isoniazid. *M. tuberculosis,* cultured from a sputum sample, was resistant to isoniazid, rifampin, ethambutol, ofloxacin, and amikacin. Capreomycin was given for 6 months, and she received treatment for 18 months in total. The condition was cured.

Patient 2's family is depicted in Figure 1. The eldest brother (B1) had been in prison, and TB developed soon after his release in 1998. First-line treatment was begun, but he died soon afterward. TB then developed in his sister (B2), mother (B3), and brother (B4). All were given first-line therapy, which was changed, when resistance profiles became available, to the regimen for MDR TB and, for the brother, to the regimen for XDR TB. All 3 patients died. A chronology is shown in Figure 2; molecular details regarding the samples are provided in the Table.

Conclusions

In family 1, the uncle's mother (A1) had pre–XDR TB and probably transmitted it to her son (A2). He likely transmitted it to his niece (A3). Strains for all 3 were identical. In family 2, whether the oldest brother (B1) had drug-resistant TB is unknown. His sister (B2) had pre–XDR TB; then, in sequence, XDR TB developed in her mother (B3), brother (B4), and sister (B5), caused by a strain identical to hers. This investigation, therefore, demonstrates the potential for resistance to be transmitted and amplified within families.

Other than the 2 index case-patients (A3 and B5), all were initially given first-line therapy and received treatment until drug susceptibility test (DST) results became available, often despite a drug-resistant contact being known. Local policy is to diagnose TB solely from sputum smear in new patients who have no risk factors for drug resistance. Retreatment patients and those at risk for resistance have strains tested for drug susceptibility to rifampin and isoniazid. If MDR TB is diagnosed, DST to second-line drugs is then performed. Giving inadequate regimens not only leads to more advanced disease until effective treatment is initiated but also risks amplifying resistance (*8*,*9*). For a patient with TB symptoms who is in contact with a drug-resistant TB patient, it is essential to obtain microbiological samples and then start treating the disease according to the DST results for the source case. If a less-resistant organism is grown, treatment can be changed.

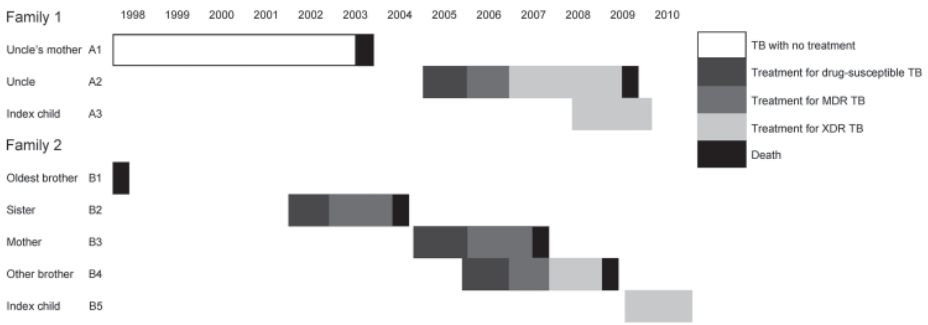

Figure 2. Chronology of tuberculosis treatment and outcomes for 2 families with drug-resistant tuberculosis (TB), South Africa, 2008–2010. MDR TB, multidrug-resistant TB; XDR TB, extensively drug-resistant TB. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/11-1650-F2.htm).

Table. Gene sequencing, IS*6110* DNA fingerprinting, and genotype results for isolates from members of 2 families with drug-resistant tuberculosis, South Africa, 2008–2010*

Family and family member	ID no.	Gene†							IS*6110* cluster no.
		rpoB	*inhA*	*katG*	*embB*	*gyrA*	*pncA*	*rrs* 1401	
		R	H	H	E	F	Z	A	
Family 1									
Uncle's mother	A1	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	WT	213
Uncle	A2	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	WT	213
Index child	A3	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	WT	§
Family 2									
Oldest brother‡	B1								
Sister	B2	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	WT	213
Mother	B3	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	1401, ACG→GCG	213
Other brother	B4	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90, GCG→GTG	160, ACA→GCA; 100, ACC→ATC	1401, ACG→GCG	213
Index child	B5	531, TCG→TTG	WT	315, AGC→ACC	306, ATG→ATA	90 GCG→GTG	160, ACA→GCA; 100, ACC→ATC	1401, ACG→GCG	§

*All isolates were *Mycobacteria tuberculosis* Beijing genotype. The earliest sample available for each patient is shown; in all instances in which >1 sample was available for a patient, all samples demonstrated identical gene sequence and strain type results. ID, identification; R, rifampin; H, isoniazid; E, ethambutol; F, fluoroquinolones; Z, pyrazinamide; A, aminoglycosides, WT, wild type.
†Numbers indicate specific mutations, which are shown.
‡Tuberculosis developed and patient died before systematic sample collection and storage. No culture or drug susceptibility testing was requested for sample.
§Only spoligotyping performed because isolates repeatedly lost viability on culture.

In the context of multiple possible TB sources, deciding on treatment is challenging. Consideration must be given to the infectiousness of potential sources as well as the intensity, frequency, and duration of exposures. Local policy is to carry out household contact tracing for drug-resistant TB patients. Although this tracing occurs infrequently, we demonstrate the value of careful investigation of contacts to identify those who may have subclinical disease that could be treated at an early stage. Given the social interactions, chronology of illness, and the results of mycobacterial cultures, the transmission sequence in these cases likely occurred as described. However, in both clusters, the strain identified was the predominant local strain and is a potential confounder to the transmission lines suggested.

With the rollout of rapid, genotypic diagnostic tests (*10*), which ultimately should be extended to all persons suspected of having TB, more drug-resistant TB may be diagnosed correctly and earlier. If this leads to prompt, appropriate treatment, further transmission and amplification of resistance could be reduced. For XDR TB treatment, drug options that are not only new to the patient but also new to the community must be available. The use of linezolid and other novel drugs will become crucial in the management of an evolving drug-resistant TB epidemic.

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Chloroquine-Resistant Malaria in Travelers Returning from Haiti after 2010 Earthquake

Myriam Gharbi, Dylan R. Pillai, Rachel Lau, Véronique Hubert, Krishna Khairnar, Alexandre Existe, Eric Kendjo, Sabina Dahlström, Philippe J. Guérin, Jacques Le Bras, and members of the French National Reference Center for Imported Malaria Study[1]

We investigated chloroquine sensitivity to *Plasmodium falciparum* in travelers returning to France and Canada from Haiti during a 23-year period. Two of 19 isolates obtained after the 2010 earthquake showed mixed *pfcrt* 76K+T genotype and high 50% inhibitory concentration. Physicians treating malaria acquired in Haiti should be aware of possible chloroquine resistance.

In Haiti (2011 population ≈9.7 million), malaria is endemic. Approximately 30,000 malaria infections are confirmed annually among ≈200,000 estimated malaria cases, mainly *Plasmodium falciparum* infections (*1*). On January 12, 2010, a 7.0 magnitude earthquake struck Haiti near Port-au-Prince, leaving much of the population homeless.

The main malaria vector in Haiti, *Anopheles albimanus* mosquitoes, which mostly bite outdoors during November–January, placed evacuees at high risk for infection (*2*,*3*). Severe flooding after hurricane Tomas in November 2010 probably compounded the problem by facilitating parasite reservoirs and mosquito breeding (*4*). Some studies suggest that these events might have increased malaria transmission in Haiti. Two observational surveys, 1 performed by a mobile medical team during March–April 2010 (*5*) and 1 during November 2010–February 2011 in a primary care clinic in Leogane (*6*), reported a high proportion of malaria infection among persons with fever (20.3% and 46.9%, respectively) compared with reports from a population-based survey in 2006 (14.2%) (*2*). The US National Malaria Surveillance System reported a 3-fold increase in malaria among travelers returning from Haiti in 2010 (170 cases) compared with 2009 (58 cases) (*7*).

Chloroquine associated with primaquine since 2009, is the recommended first-line treatment for uncomplicated malaria. In vitro and molecular surveillance data collected during the past 2 decades suggest continued *P. falciparum* sensitivity to chloroquine (*3*,*8*,*9*). However, a 2006–2007 study in Artibonite Valley, Haiti, showed the chloroquine resistance–associated *Pfcrt*76T genotype in ≈6% (5/79) of *P. falciparum* isolates, although clinical data were lacking (*10*). Subsequently, the Haitian Ministry of Health acknowledged that routine chloroquine efficacy surveillance should be reinforced (*11*). We investigated the chloroquine sensitivity of *P. falciparum* parasites isolated from travelers recently returned from Haiti to Canada and France by using genotypic and phenotypic methods.

The Study

We collected retrospective data from the National Malaria Reference Centre (Paris, France) and Public Health Ontario (Toronto, ON, Canada) during 1988–2010 and 2007–2010, respectively. *P. falciparum* infection was considered probably acquired in Haiti if biologically confirmed by thin and thick blood smears from persons who had recently traveled to Haiti in the 2 months before infection was diagnosed. Basic demographic and epidemiologic data, clinical and parasitologic information, treatment, and history of travel and *P. falciparum* infection were collected systematically. Forty of 80 participating hospitals in the sentinel network in France also documented resistance to antimalarial drugs. Pretreatment isolates were collected to determine chloroquine susceptibility by molecular analysis of the *Pfcrt*76 locus and by comparing the ratio of in vitro chloroquine response of the clinical isolate with a chloroquine-sensitive reference clone.

Seventy-nine imported *P. falciparum* infections were recorded: 49 before the earthquake (all in France) and 30 after the earthquake (3 in Canada and 27 in France). The

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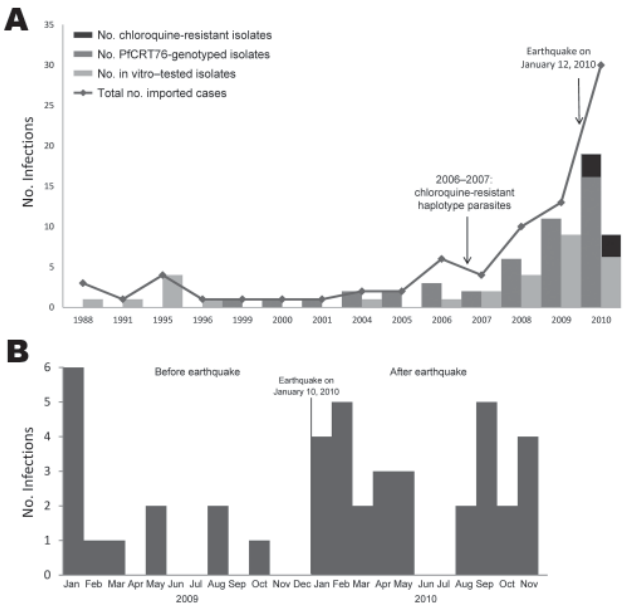

Figure 1. Surveillance during 23 years for antimalarial drug resistance in travelers returning to France and Canada from Haiti after the January 10, 2010, earthquake. A) Imported malaria cases from Haiti reported in France (1988–2010) and Canada (2007–2010). B) Total number of *Plasmodium falciparum* infections, by month, 2009 and 2010.

number of confirmed malaria cases imported from Haiti doubled during 2009–2010 (Figure 1). Approximately half of the travelers were in Haiti 2–4 weeks before the earthquake and >1 month after the earthquake. The main purpose of travel, visiting friends and relatives, decreased from 59% before to 44% after the earthquake. More than 75% of travelers did not take prophylactic medication. The proportion of severe malaria increased from 3% to 11% after January 2010 (Table 1).

Before the earthquake, all 29 isolates had the wild-type *Pfcrt*K76 allele according to analysis by PCR–restriction fragment-length polymorphism. The mean 50% inhibitory concentration (IC_{50}) of chloroquine for the 24 isolates tested ex vivo by the ^{3}H-hypoxanthine uptake inhibition method was 27 nM (95% CI 23–31). These results are consistent with those of an unpublished study conducted in Haiti during 2007 to monitor chloroquine resistance (Jean-François Vely, unpub. data). In that study, Haiti's National Malaria Program, in collaboration with the National Malaria Reference Centre in France, found the chloroquine-sensitive genotype in 146 *P. falciparum*–positive samples in 6 departments (Artibonite, Centre, Grand'Anse, Nord, Nord-Ouest, Ouest) (Figure 2) (*12*). After the earthquake, 2 (11%) of 19 isolates analyzed by pyrosequencing and PCR–restriction fragment-length polymorphism showed a mixed *Pfcrt*76K+T genotype. The ratios of K to T genotypes before and after in vitro adaptation were 0.75:0.25 and 0.23:0.77, respectively, for patient 1, and 0.58:0.42 and 0.25:0.75, respectively, for patient 2. The *Pfcrt*72–76 haplotype was CVMNK before adaptation and CVIET after adaptation for both patients by sequencing. Resistance was confirmed by in vitro methods after culture adaptation. Both isolates had high chloroquine IC_{50} (506 nM and 708 nM, respectively) and high chloroquine IC_{50} isolate:*Pf*3D7 (chloroquine susceptible clone) ratio (20 and 27, respectively) (Table 2).

Patient 1, a 58-year-old woman, was in Haiti during October 2009–January 2010; she returned after the earthquake to Canada, where she sought care for malaise, fever, diarrhea, and vomiting. She reported no previous malaria and no other travel during the previous 2 years. For patient 2, a 16-year-old girl, malaria was diagnosed in Canada on February 25, 2010, after 3 days of fever. She had traveled to Haiti in the past 2 months before malaria was diagnosed and did not report any other recent travel.

Conclusions

The number of *P. falciparum*–infected travelers returning from Haiti has increased since January 2010,

Table 1. Characteristics of travelers returning from Haiti to France, 1988–2010, and Canada, 2007–2010

Characteristic	Before earthquake, n = 49*	After earthquake, n = 30*
Median age, y (range)	44 (0.7–69)	36 (2–77)
Sex		
M	32 (68)	19 (63)
F	15 (32)	11 (37)
Country of residence		
France	49 (100)	27 (90)
Canada	0	3 (10)
Plasmodium falciparum infection	47 (100)	27 (100)
Chemoprophylaxis		
No	37 (76)	23 (77)
Yes	1 (2)	2 (7)
Unknown	11 (22)	5 (17)
Duration of stay		
≤2 wk	5 (17)	3 (14)
2–4 wk	14 (47)	6 (29)
1–3 mo	7 (23)	7 (33)
>3 mo	4 (13)	5 (24)
Purpose of travel		
Tourism	4 (12)	3 (13)
Visit friends and family	20 (59)	10 (44)
Business	5 (15)	3 (13)
Military	1 (3)	0
Residents or expatriates ≥6 mo	1 (3)	5 (22)
Other	3 (9)	2 (9)
Severe malaria		
Yes	1 (3)	3 (11)
No	28 (97)	24 (89)
Median parasitemia (range)†	0.47 (0.001–12.000)	0.57 (0.04–14.00)

*Values are no. (%) except as indicated. Numbers may not add to totals because of missing information.
†Percentage of infected erythrocytes per mL blood.

Figure 2. Departments of Haiti.

probably because of the higher number of aid workers and visitors and increased *P. falciparum* malaria transmission. Data suggest that the earthquake and ensuing hurricane and floods created the necessary conditions—inadequate shelters, population movement, and still water—to increase the incidence of malaria and possibly spread the recently identified chloroquine-resistant strains of *P. falciparum* (*10*). In France and Canada, laboratory surveillance for malaria found that 2 travelers from Haiti carried chloroquine-resistant strains. In vitro culture might have selected resistant strains not observed initially by ex vivo methods. After carefully interviewing these patients about their travels, we found no evidence to cause doubt that they had acquired malaria in Haiti. Alternatively, the resistant strains could have come to Haiti after the earthquake through human activity, as occurred in the cholera outbreak (*13*).

Table 2. Molecular genotypes and in vitro susceptibility for *Plasmodium falciparum* isolates from patients returning to France and Canada from Haiti

Characteristics	Before earthquake, n = 49	After earthquake, n = 30
In vitro analysis	n = 24	n = 10
IC_{50} for chloroquine, nmol/L (mean 95% CI)	27 (23–31)	35 (12–105)*
No. isolates resistant†		
Yes	0	2
No	24	8
Molecular marker analysis, no. (%) isolates	n = 29	n = 19
PfCRT K76	29	17 (89.5)
PfCRT K76+76T	0	2 (10.5)
PfCRT 76T	0	0

*The 2 resistant isolates are included with an IC_{50} of 506 nmol/L and 708 nmol/L. IC_{50}, 50% inhibitory concentration.
†A threshold of IC_{50} = 100 nmol/L is applied to determine resistant isolates (consensus between the laboratories in France and Canada) and in vitro susceptibility (IC_{50}) for the isolates from patients returning from Haiti.

The origin of the chloroquine-resistant strains identified in Haiti is uncertain. The *Pfcrt* CVIET haplotype is common in Southeast Asia and sub-Saharan Africa and was found in the 2006–2007 study in Haiti (*10*).

Regardless of origin, containing the spread of chloroquine-resistant parasites is crucial. Malaria elimination is a goal in Haiti, and it has been strengthened after recent events, but the effects of malaria and many other factors affect the achievability of this goal (*14*). Control measures, possibly mirroring those used to contain artemisinin resistance in Southeast Asia, should be concentrated in Haiti to prevent resistance spreading to the rest of Hispaniola (*15*). However, lack of consensus on the use of molecular and in vitro data for policy change will hamper decision making. Neither the chloroquine-resistant *Pfcrt*76T genotype nor the elevated chloroquine IC_{50} perfectly predicts treatment failure because of confounding factors like acquired immunity.

Our study has several limitations. Returning travelers are not a representative sample of the Haitian population, and the sample of isolates was limited. The origin of the resistant strains is not defined. Also, the precise location of infection is not reported. Nevertheless, travelers are useful sentinels of emerging resistance in areas where little information is available, providing surveillance data in real time with standardized methods. This nonimmune population also facilitates detection of resistant isolates.

Our data highlight the need to implement a therapeutic efficacy surveillance study for assessing in vivo chloroquine sensitivity, which is essential for providing information for rational control strategies and guiding prophylaxis recommendations in Haiti. In addition, physicians treating malaria acquired in Haiti should be aware of the possibility of chloroquine-resistant infections. Patients with persistent fever despite treatment and infected travelers reporting adherence to chloroquine prophylaxis should be treated with alternate antimalarial drug therapy.

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New Variants of Porcine Epidemic Diarrhea Virus, China, 2011

Wentao Li, Heng Li, Yunbo Liu, Yongfei Pan, Feng Deng, Yanhua Song, Xibiao Tang, and Qigai He

In 2011, porcine epidemic diarrhea virus (PEDV) infection rates rose substantially in vaccinated swine herds. To determine the distribution profile of PEDV outbreak strains, we sequenced the full-length spike gene from samples from 9 farms where animals exhibited severe diarrhea and mortality rates were high. Three new PEDV variants were identified.

A member of the family *Coronaviridae*, genus *alphacoronavirus*, porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded positive-sense RNA virus (*1*). PEDV is the major causative agent of porcine epidemic diarrhea, which is characterized by severe enteritis, vomiting, watery diarrhea, and weight loss. PEDV infections have a substantial detrimental effect on the swine industry because the mortality rates are high, especially in sucking piglets (*1*). The major structural gene of the 28-kb PEDV genome encodes the multifunctional virulence factor, spike (S), which is responsible for viral receptor binding, induction of neutralizing antibodies, and host cell fusion. The S gene sequences are a distinguishing feature of PEDV strains, which affect virulence and evolution (*2*–*4*).

The first confirmed PED case in the People's Republic of China was reported in 1973. Almost 2 decades later, an oil emulsion, inactivated vaccine was developed and has since been in wide use throughout the swine industry in China. Until 2010, the prevalence of PEDV infection was relatively low with only sporadic outbreaks; however, starting in late 2010, a remarkable increase in PED outbreaks occurred in the pig-producing provinces. The affected pigs exhibited watery diarrhea (Figure 1, panels A, B), dehydration with milk curd vomitus (Figure 1, panel C), and thin-walled intestines (Figure 1, panel D) with severe villus atrophy and congestion (Figure 1, panels E, F). The disease progressed to death within a few days. Pigs of all ages were affected and exhibited diarrhea and loss of appetite with different degrees of severity, which were determined to be age dependent; 100% of suckling piglets became ill. Pigs >2 weeks of age experienced mild diarrhea and anorexia, which completely resolved within a few days (*5*). Morbidity and mortality rates were lower for vaccinated herds than for nonvaccinated herds, which suggests the emergence of a new PEDV field strain(s) for which the current vaccine, based on the CV777 strain, was partially protective. To identify the PEDV strain(s) responsible for the recent outbreak in China, we sequenced the full-length S gene of isolates obtained from diarrhea samples collected from pigs at 9 affected pig farms.

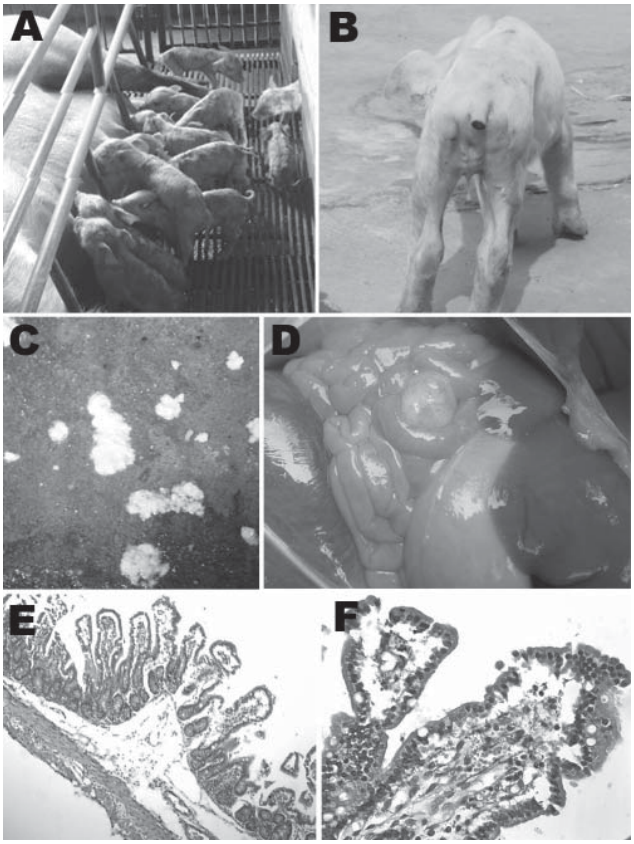

Figure 1. Clinical features of pigs infected with porcine epidemic diarrhea virus from pig farms in the People's Republic of China, 2011. A) Litter of pigs infected with this virus, showing watery diarrhea and emaciated bodies. B) A representative emaciated piglet with water-like feces. C) Vomitus from a representative sucking piglet. D) Thin-walled intestinal structure with water-like content. E) Congestion in the small intestinal wall and intestinal villi; desquamated epithelial cells from the intestinal villus (original magnification ×100). F) Congestion in the lamina propria of intestinal mucosa, and degeneration, necrosis, and desquamation of epithelial cells of the intestinal villi (original magnification ×400). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/11-1343-F1.htm)

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Table 1. Primers used in study of PEDV, China, 2011*

Primer name	Nucleotide sequence, 5′ → 3′	Primer location†
PEDVS1F	GGTAAGTTGCTAGTGCGTAA	20,570–20,589
PEDVS1R	CAGGGTCATCACAATAAAGAA	22,010–22,030
PEDVS2F	TTTCTGGACCGTAGCATC	21,939–21,956
PEDVS2R	TCCTGAAGTGGGACATAG	22,917–22,935
PEDVS3F	GAGTTGCCTGGTTTCTTC	22,816–22,833
PEDVS3R	TATAATTGCGCCTCAAAG	24,979–24,996

*PEDV, porcine epidemic diarrhea virus; F, forward; R, reverse.
†Numbers correspond to the nucleotide positions within the CV777 genome.

The Study

From January 2011 through October 2011, a total of 455 samples (fecal, intestine, and milk) were collected from 57 farms in 12 provinces of China. All samples were evaluated by reverse transcription PCR (RT-PCR), by using previously described primers (*6*). Forty-five (78.95%) of the farms had at least 1 PEDV-positive sample. A total of 278 (61.11%) samples were PEDV positive, including 253 (of 402; 62.94%) fecal samples, 20 (of 31; 64.52%) intestine samples, and 5 (of 22; 22.73%) milk samples. The representative detection of PEDV in fecal samples of PED-affected farms is shown in Technical Appendix Figure 1 (wwwnc.cdc.gov/EID/article/18/8/12-0002-FA1.htm).

Nine diarrhea samples were collected from pigs at 9 farms (where animals had severe diarrhea and mortality rate was high) for sequencing analysis of the full-length S gene (Technical Appendix Table 1; wwwnc.cdc.gov/EID/article/18/8/12-0002-TA1.htm). RT-PCR gene-specific primers were designed on the basis of the sequence of PEDV-CV777 strain (GenBank accession no. AF353511.1) (Table 1) and used to amplify 3 overlapping cDNA fragments spanning the entire S gene. The amplicons were sequenced in both directions (GenScript Co., Nanjing, PRC).

The 9 PEDV S gene sequences were aligned with the sequences of 24 previously published PEDV S genes (Table 2) by using the ClustalX (version 1.82), Bioedit (version 7.0.9.0) and MegAlign version 5.0 (DNAStar Inc., Madison, WI, USA) software packages (*14*). The full-length S gene sequences of the 9 isolates from our study showed overall high conservation with the reference strains, up to 94.9%–99.6% homology (Technical Appendix Table 2; wwwnc.cdc.gov/EID/article/18/8/12-0002-TA2.htm). By phylogenetic analysis, 4 of the field isolates (CH2, CH5, CH6, CH7) clustered with the previously described strain JS-2004–2 from China. Three field isolates (CH1, CH8, CHGD-01) formed a unique cluster with the sequence-confirmed variant strain CH-FJND-3, which had been isolated from China in 2011 (*7*). CH1 and CH8 were isolated from 2 farms, where all sucking piglets had died from diarrhea, even though all of the sows had been

Table 2. Isolates and reference strains used in study of porcine epidemic diarrhea virus outbreak, China, 2011

Virus strain	Country and year of isolation	GenBank accession no.	Reference
CH1	China 2011	JQ239429	This study
CH2	China 2011	JQ239430	This study
CH3	China 2011	JQ239431	This study
CH4	China 2011	JQ239432	This study
CH5	China 2011	JQ239433	This study
CH6	China 2011	JQ239434	This study
CH7	China 2011	JQ239435	This study
CH8	China 2011	JQ239436	This study
CHGD-01	China 2011	JN980698	This study
CH-FJND-1	China 2011	JN543367.1	Unpublished
CH-FJND-2	China 2011	JN315706.1	Unpublished
CH-FJND-3	China 2011	JN381492.1	(*7*)
JS-2004–2	China 2004	AY653204	Unpublished
LJB/03	China 2006	DQ985739	Unpublished
LZC	China 2006	EF185992	Unpublished
DX	China 2007	EU031893	Unpublished
CHS	China 1986	JN547228.1	(*8*)
Chinju99	South Korea 1999	AY167585	(*9*)
Spk1	South Korea 2002	AF400215	(*10*)
Parent DR13	South Korea 2006	DQ862099	(*11*)
Attenuated DR13	South Korea, 2006	DQ462404.2	(*11*)
KNU-0801	South Korea, 2008	GU180142	(*2*)
KNU-0802	South Korea, 2008	GU180143	(*2*)
KNU-0901	South Korea, 2009	GU180144	(*2*)
KNU-0902	South Korea, 2009	GU180145	(2)
KNU-0903	South Korea, 2009	GU180146	(*2*)
KNU-0904	South Korea, 2009	GU180147	(2)
KNU-0905	South Korea, 2009	GU180148	(2)
Br1/87	Great Britain, 1993	Z25483	(*12*)
MK	Japan, 1996	AB548624.1	(*3*)
NK	Japan	AB548623.1	(*3*)
Kawahira	Japan	AB548622.1	(*3*)
CV777	Belgium, 1988	AF353511	(*13*)

vaccinated with the PEDV-CV777 strain–based inactivated vaccine. The isolated variant strains, CHGD-01 and CH1, were tested in experimental infection studies and found to cause illness in 100% of sucking piglets (data not shown).

The phylogenetic analysis of the S gene nucleotide sequences revealed 3 major clusters (Figure 2). Clade 1 comprised 6 strains from our study (CH2, CH3, CH4, CH5, CH6, CH7), the vaccine strain CV777 from China, the attenuated strain DR13 from South Korea, and 2 strains (CHFJND-1, CHFJND-2) that had been isolated in China in 2011. Clade 2 consisted of 4 variant strains (CH1, CH8, CHFJND-3, CHGD-01) that were identified from China in 2011. Clade 3 was composed of 9 isolates from South Korea and 2 strains from Japan (NK and Kawahira). The deduced amino acids of the 4 variant strains in clade 2 had 93% homology to CV777. Furthermore, the 4 variant strains from China (CH1, CH8, CHGD-01, CH-FJND-3) and 9 PEDV isolates from South Korea shared a 5-aa insertion (at positions 56–60 of the S protein) with CV777. One amino acid insertion at position 141 was shared among all variant strains and 6 isolates from South Korea (Technical Appendix Figure 2; wwwnc.cdc.gov/EID/article/18/8/12-0002-FA2.htm). In the S genes, 132 point mutations were found that accounted for genetic diversity among the isolates.

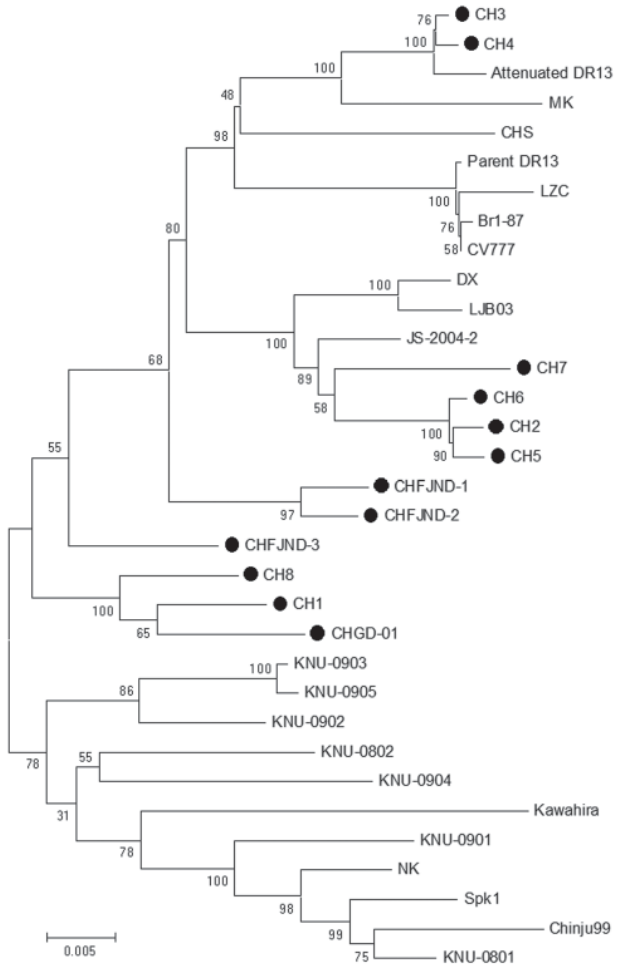

Figure 2. Phylogenetic trees of porcine epidemic diarrhea virus (PEDV) strains generated by the neighbor-joining method with nucleotide sequences of the full-length spike genes. Bootstrapping with 1,000 replicates was performed to determine the percentage reliability for each internal node. Horizontal branch lengths are proportional to genetic distances between PEDV strains. Black circles indicate PEDV field isolates from the 2011 outbreak in China. Scale bar indicates nucleotide substitutions per site.

The recent 4 isolates from China (CH2, CH5, CH6, CH7) were closely related to the previously identified isolates from China (JS-2004–2, LJB03, DX) and another 4 variant strains. Three of the new isolates (CH1, CH8, CHGD-01) were highly pathogenic in piglets. All strains were obtained from farms that used the CV777-based inactivated vaccine but had 100% prevalence of diarrhea in pigs (Technical Appendix Table 1). Another 2 field isolates (CH3, CH4) from 2 farms with pigs with severe diarrhea shared the highest sequence identity with attenuated strain DR13 from South Korea (99.2% and 99.1%, respectively), which has been in routine use as an oral vaccine against PEDV in South Korea since 2004 (*15*). The appearance of strains in China similar to those from South Korea and their role in the recent PEDV outbreak should be further investigated.

Conclusions

RT-PCR amplification and sequencing analysis of the full-length PEDV spike genes were used to investigate isolates from diarrhea samples from local pig farms with severe diarrhea in piglets. Both classical and variant strains were detected, implying a diverse distribution profile for PEDV on pig farms in China. The sequence insertions and mutations found in the variant strains may have imparted a stronger pathogenicity to the new PEDV variants that influenced the effectiveness of the CV777-based vaccine, ultimately causing the 2011 outbreak of severe diarrhea on China's pig farms. Future studies should investigate the biologic role of these particular insertions and mutations. Furthermore, our study of the full-length S gene revealed a more comprehensive distribution profile that reflects the current PEDV status in pig farms in China, including the presence of a strain similar to strain DR13, isolated in South Korea. Collectively, these data indicate the urgent need to develop novel variant strain–based vaccines to treat the current outbreak in China.

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Severe Human Granulocytic Anaplasmosis Transmitted by Blood Transfusion

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A 36-year-old woman acquired severe human granulocytic anaplasmosis after blood transfusion following a cesarean section. Although intensive treatment with mechanical ventilation was needed, the patient had an excellent recovery. Disease caused by *Anaplasma phagocytophilum* infection was confirmed in 1 blood donor and in the transfusion recipient.

Human granulocytic anaplasmosis (HGA), an emerging tickborne zoonosis caused by *Anaplasma phagocytophilum*, has been recognized in the United States since 1994 and in Europe since 1996 (*1*,*2*). Most patients acquire *A. phagocytophilum* infection by tick bite, although individual cases of nosocomial, perinatal, and transfusion-associated transmission have been reported (*3*–*5*). We report a case of severe HGA acquired from blood transfusion.

The Case-Patient

On August 26, 2010, a 36-year-old woman, 29 weeks pregnant without underlying chronic illness, was admitted to the University Medical Center Ljubljana with preeclampsia and restriction of intrauterine growth. Because her previous pregnancy ended in spontaneous abortion, the patient was monitored closely in an inpatient setting. On September 15, an elective cesarean section was performed. Later that day, hemorrhagic shock developed. Surgical revision of the source of the blooding was performed, and she received 6 units of packed erythrocytes and 2 units of fresh frozen plasma, originating from 6 donors. Ten days later, on September 25, the patient became febrile, which was associated with an elevated C-reactive protein level and mild abnormalities in liver enzyme levels, but with no signs of localized infection (Table 1). Antimicrobial drug therapy with amoxicillin/clavulanic acid was initiated, but the regimen was changed after 3 days to gentamicin and metronidazole because the high fever did not abate. At that time, a chest radiograph revealed mild interstitial edema, and a vaginal ultrasound showed no abnormalities.

The patient's condition deteriorated further, and on September 27 she was transferred to an intensive care unit. Tachypnea (30–40 breaths/min) without hypoxia, tachycardia (120 beats/min), elevated temperature (37.8°C), and hypotension (90/60 mm Hg) were recorded at admission. Antimicrobial drug therapy was changed to imipenem, azithromycin, and vancomycin. Computed tomography scan of the chest showed consolidation in the lower right lobe. Blood cultures and other relevant microbiological tests remained negative for infectious agents. Antiphospholipid syndrome was suspected, and treatment with corticosteroids, immunoglobulins, and heparin was initiated. However, corresponding tests did not confirm the diagnosis. Drug therapy was changed to piperacillin/tazobactam, daptomycin, and azithromycin.

The fever continued, laboratory test results worsened (Table 1), and acute respiratory distress syndrome (ARDS) developed. Bone marrow examination, performed because of persistent thrombocytopenia, showed reactive changes. Because of the febrile illness associated with laboratory indicators of inflammation, presence of thrombocytopenia, and elevation of transaminases, as well as the ineffectiveness of treatment, a working diagnosis of HGA was posed, and doxycycline was added to the treatment regimen on October 1.

The diagnosis was confirmed by demonstration of morulae on examination of whole blood smears by microscopy (Figure), by a positive PCR for DNA coding 16S rRNA of *A. phagocytophilum* in whole blood, and later by seroconversion to *Anaplasma* antigens (Table 2). Morulae and *A. phagocytophilum* DNA were also detected in bone marrow biopsy samples (*6*,*7*). In addition, all samples positive by PCR were tested for the *groESL* operon of *A. phagocytophilum,* and reliability of products was confirmed by direct sequencing. On the second day of doxycycline treatment, respiratory distress progressed further and artificial ventilation was necessary. However, the next day the patient experienced dramatic improvement; on the fourth day after initiation of doxycycline, the breathing tube was removed, and her later clinical course was uneventful. She was discharged at the end of a 14-day treatment course of doxycycline, and at follow-up visits she reported no difficulties.

Because the patient denied having been bitten by ticks, had not left her house for several weeks before admission to the hospital on August 26 because of a complicated pregnancy, was continuously hospitalized for 30 days

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Table 1. Blood test results for a patient with severe human granulocytic anaplasmosis, Slovenia, 2010*

Date, 2010	CRP, mg/L	PCT, µg/L	Leukocytes, 10^9 cells/L	Band cells, %	Erc, 10^{12} cells/L	Hb, g/L	Pt, 10^9/L	LDH, µkat/L	AF, µkat/L	AST, µkat/L	ALT, µkat/L	GGT, µkat/L
Sep												
13	<3	ND	8.2	ND	4.23	113	230	2.67	1.8	0.46	0.53	0.19
16	45	ND	11.1	ND	4.57	127	142	3.74	1.5	0.78	0.53	0.23
25	97	ND	9.2	ND	4.76	128	258	ND	2.54	1.39	1.65	ND
27	95	0.75	6.2	ND	3.91	106	80	6.1	3.56	1.39	1.12	2.67
28	120	0.73	9.1	48	3.67	104	39	8.28	5.3	1.9	1.17	3.07
29	167	0.98	10	23	3.82	106	21	9.23	6.11	2.74	1.33	2.98
30	121	1.02	9.4	15	3.79	106	11	13.6	5.75	3.69	1.36	2.95
Oct												
1	88	0.83	6	10	3.92	111	21	18.12	5.07	4.06	1.29	3.28
2	60	1	12	10	3.94	104	50	23.2	4.3	4.86	1.48	3.8
5	34	0.23	13.9	3	3.2	91	52	16.03	3.33	1.8	1.4	4.06
6	15	0.19	14.3	2	3.38	97	141	10.48	2.72	1.2	1.52	3.21
7	4	ND	12	2	3.52	101	210	7.41	2.49	0.97	1.99	3.11
8	ND	ND	11.5	0	3.54	104	275	6.03	2.23	0.73	1.83	2.77
10	<3	ND	8.5	0	3.96	107	401	5.65	2.11	0.62	1.5	2.62

*CRP, C-reactive protein (reference <5 mg/L); PCT, procalcitonin (reference <0.5 µg/L); leukocytes, leukocyte count (reference 4–10 × 10^9 cells/L); Erc, erythrocyte count (reference 4.2–5.4 x 10^{12} cells/L); Hb, hemoglobin (reference120–160 g/L); Pt, platelets (reference 140–340 × 10^9/L); LDH, lactate dehydrogenase (reference <4.12 µkat/L); AF, alkaline phosphatase (referrence <1.74 µkat/L); AST, aspartate transaminase (reference <0.52 µkat/L); ALT, alanine transaminase (reference <0.56 µkat/L); GGT, γ-glutamyl transferase (reference <0.63 µkat/L); ND, not determined.

before the onset of fever on September 25, and received transfusions during her hospital stay, transfusion-associated transmission of HGA was suspected and searched for. Blood taken from the patient for pretransfusion cross-matching on September 15 tested negative by PCR and by immunofluorescence assay for antibodies against *A. phagocytophilum*. Stored plasma samples from all 6 blood donors, frozen on the day of donation (2 donated blood on August 10, 4 on September 7, 2010), were tested for antibodies against *A. phagocytophilum* and the presence of corresponding DNA. The results were negative for all but 1 donor. This 42-year-old man, a regular blood donor who lived in a region where sporadic HGA cases had been established (*8*), reported being an outdoor person who received several tick bites every year (the most recent in July 2010). He donated blood twice in 2010, on May 12 and September 7; blood obtained at the latter visit was transfused as packed erythrocytes to the patient reported here. At the end of August, a self-limited illness had developed in the donor with fever (39°C), myalgia, and arthralgia (Table 2).

Conclusions

HGA is an acute febrile illness that causes headache, myalgia, malaise, elevated levels of C-reactive protein and serum transaminases, leukopenia, and thrombocytopenia; the disease seems to have milder manifestations in Europe than in the United States (*8*,*9*). The fatality rate is <1% (*9*), although a literature search did not reveal any report of a fatal case in Europe. The patient fulfilled the criteria for proven HGA (*10*). She had an acute febrile illness with thrombocytopenia, *A. phagocytophilum* infection demonstrated by the presence of corresponding DNA in plasma and bone marrow in conjunction with seroconversion, and spectacular improvement after treatment with doxycycline was instituted. The course of her illness was severe and encompassed pneumonia, ARDS, and the need for treatment in the intensive care unit, including mechanical ventilation. Although cough has been reported in 19% of patients with confirmed HGA cases in the United States, pneumonia or ARDS has been documented in only 1% (9). In Europe, pneumonia was recorded for just a few cases, and no data on respiratory failure exist (*11*).

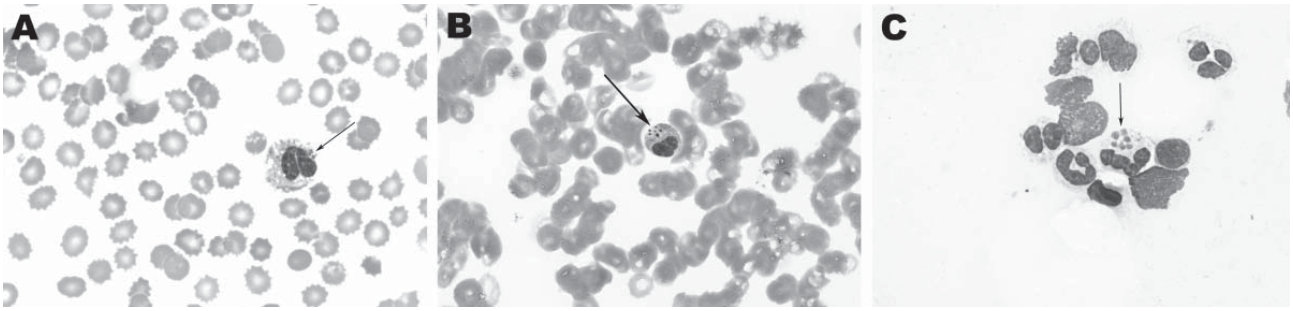

Figure. Histopathology slides from 36-year-old woman with human granulocytic anaplasmosis, Slovenia, 2010. Peripheral blood smear (A, B); bone marrow smear (C). Modified Giemsa staining, original magnification ×1,000. Morulae (clusters of *Anaplasma phagocytophilum* in granulocytic leukocytes) are indicated by arrows. In Europe, morulae have been reported in only 1 patient (*6*), but they are a relatively common observation in the United States, associated predominately with severe cases of human granulocytic anaplasmosis (*7*).

Table 2. Results of PCR and IFA for IgG against *Anaplasma. phagocytophilum* for index patient and blood donor, Slovenia, 2010*

	Patient			Donor		
Date, 2010	PCR	IFA	Remarks	PCR	IFA	Remarks
May 12†				–	–	118 d before index donation
Aug 28–29				ND	ND	Febrile illness
Sep						
7†				+	>1,024	Index donation
15	–	–	Transfusion			
25	ND	ND	Onset of febrile illness			
Oct						
1	+‡	–	Treatment with doxycycline			
4	+	128	Oct 1–14			
6	+	512				
9	+	ND				
10	–	ND				
12	–	512				
19	ND	512				
20				–	>1,024	43 d after index donation
Nov 23	ND	>1,024	No reports of problems			

*Results for IFA are shown in titers. IFA, immunofluorescence assay; ND, not done; –, negative; +, positive.
†Retrospective testing of stored blood specimen.
‡DNA detected in stored bone marrow–biopsy specimen.

In our patient, pregnancy, cesarean section, blood loss after the operation, an additional surgical procedure, corticosteroid treatment, and an interval of 6 days before correct diagnosis and treatment could have contributed to the severity of her illness. It is also possible that infection acquired through transfusion results in a more severe illness than infection after the bite of an infected tick. However, only a few reports of presumed transmission of *A. phagocytophilum* from sources other than ticks have been published (*3*–*5*,*12*). In a previous single report of transmission by blood transfusion (*5*), the evidence that HGA was acquired through the transfusion was convincing, but the report could not prove that the patient was free of *A. phagocytophilum* infection beforehand.

For the patient reported here, findings exclude tick transmission and convincingly favor transfusion-associated transmission of *A. phagocytophilum*. The latter was confirmed by the presence of *A. phagocytophilum* DNA in stored plasma specimens of 1 of the 6 blood donors. This donor, who had negative test results for the bacteria 4 months earlier, reported having had an acute self-limited febrile illness 2–3 weeks before blood donation. This infection probably resulted in severe acute HGA in the patient reported here.

A. phagocytophilum remains viable under refrigeration conditions at 4°C for up to 18 days, enabling potential transmission of infection by blood transfusion (*13*). This case of transfusion-associated HGA in Europe is practical evidence of such transmission and corroborates findings from the United States (*5*) that transfusion-associated febrile illness with thrombocytopenia could be caused by *Anaplasma* infection. Because transfusion-associated HGA appears to be very rare, routine screening of blood donors for the presence of *A. phagocytophilum* genome is not likely to be cost-effective. Nevertheless, when febrile illness associated with leukopenia or thrombocytopenia develops in a patient after transfusion, testing for infection with *A. phagocytophilum* may be beneficial.

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Hepatitis E Virus in Pork Food Chain, United Kingdom, 2009–2010

Alessandra Berto, Francesca Martelli, Sylvia Grierson, and Malcolm Banks

We investigated contamination by hepatitis E virus (HEV) in the pork production chain in the United Kingdom. We detected HEV in pig liver samples in a slaughterhouse, in surface samples from a processing plant, and in pork sausages and surface samples at point of sale. Our findings provide evidence for possible foodborne transmission of HEV during pork production.

During the past 10 years, hepatitis E virus (HEV) infection acquired in industrialized regions worldwide in the apparent absence of contaminated drinking water for fecal–oral transmission has been reported (*1*). In Japan, foodborne transmission of HEV through ingestion of contaminated Sika deer, wild boar, and pig meat has been demonstrated (*2*) and, in France, after ingestion of pig liver sausage (*3*). Detection of HEV in pig liver sold in retail locations has been reported from Japan, the United States, and the Netherlands in 1.9%, 14.0%, and 6.5%, respectively (*4*,*5*). PCR indicated that 1 (1.3%) of 76 pig livers collected at retail outlets in southwestern England was positive for HEV (*6*).

The European Commission Framework Program 7 project, Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains, aimed to gather data on virus contamination of food and environmental sources for quantitative viral risk assessment and development of virus-specific guidance for food supply chain operators. The UK Animal Health and Veterinary Laboratories Agency investigated the pork food chain for HEV from slaughterhouse to point of sale. We investigated fecal contamination of pork and work surfaces during this study.

The Study

During September 2009–October 2010, we collected samples from slaughtered pigs, human hands, and the environment at perceived critical points for virus contamination in a pig slaughterhouse, a processing plant, and 3 points of retail sale. Food safety fact-finding visits were made to the premises during which, through direct observations of conditions and practices, more points were identified where contamination with viruses possibly could occur and from where ad hoc surface samples were collected by using sterile gauze swabs and placed in in phosphate-buffered saline plus antimicrobial drugs.

We tested all samples collected by using real-time reverse transcription PCR (rRT-PCR) (*7*) for HEV. In addition, samples were tested for porcine adenovirus (PAdV) and human adenovirus by PCR as indicators of porcine and human fecal contamination, respectively. Nucleic acid extraction and rRT-PCR were performed according to standardized Integrated Monitoring and Control of Foodborne Viruses in European Food Supply Chains protocols. An extraction control virus, murine norovirus (MNoV), was placed in all samples before the lysis step of the extraction (*8*) to demonstrate the extraction of amplifiable nucleic acid. We performed all rRT-PCRs with an internal amplification control (*8*).

At the slaughterhouse, 40 carcasses were selected. Ten carcasses were randomly selected from each of 4 groups of pigs slaughtered that day; each group corresponded to a different farm of origin. From each carcass, the visceral pack was removed and 2–3 g of liver and 8–10 g of feces were collected. Also, 10 swab samples were collected from the handlers and environment (Table 1).

At the processing plant, 10, 18, and 14 carcasses from 3 slaughterhouses, representing 12 farms of origin, were randomly selected. The set of 14 carcasses came from the slaughterhouse we had visited, but for logistical reasons the carcasses were not the same as those that we sampled at the slaughterhouse. We collected 5 g of muscle from the ventral abdomen of each carcass. The extraction control MNoV was not detected in 2 of the samples, which we therefore excluded from the study, leaving 40 samples. Also, 10 surface swab samples were collected (Table 1).

At points of retail sale, 75 sausages were collected in 11 batches from 3 locations representing 2 types of retail outlet (2 UK supermarket chains and 1 butcher). Sausages were collected on different days to ensure that they were from different batches of pigs. MNoV was not detected in 12 of the samples, leaving 63 sausages for investigation. Eight surface swab samples were collected at point of sale (Table 1).

HEV RNA was detected at each of 3 sites in the pork food supply chain (Table 2). At the slaughterhouse, 5 (13%) of 40 fecal samples, 1 (3%) of 40 livers, and 1 (25%) of 4 swabs of workers' hands were HEV positive. At the processing plant, each of 40 pig muscle samples was negative for HEV, and a surface swab from a metal point used to hook the carcasses was HEV positive. At points of sale, 6 (10%) of 63 sausages and 2 (25%) of 8 surface samples (knife and slicer swabs) were HEV positive. Five of the 6 positive sausages were in 1 of the 11 batches

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Table 1. Surfaces sampled to investigate hepatitis E virus in the pork food chain, United Kingdom, 2009–2010*

Slaughterhouse	Processing/cutting plant	Point of sale
Surface swab		
Bar under operator inspecting livers	Bench on which meat is sold	Chopping board
Floor under carcasses in clean area	Box in which cuts are collected	Slicer
Floor under which livers are hung	Door handle	Cold-room door handle
Boxes in which livers are collected before freezing and sale	Hook	Sausage maker
Knife used immediately after scraping	General purpose knife	General purpose knife
Knife used on livers	Point	NT
	Saw	Toilet
	Scale	Sink
Swab, handlers' skin		
Hand 1	Hand 1	Hand 1
Hand 2	Hand 2	Hand 2
Hand 3	NT	NT
Hand 4	NT	NT

*NT, sample not taken.

collected. Control results showed no evidence of cross-contamination in the laboratory.

The indicator of pig fecal contamination, PAdV, was detected at 2 of 3 sites (Table 2). Of 40 fecal samples, 39 (98%) from the slaughterhouse were PAdV positive , as were 6 (15%) of 40 livers and 4 (40%) of 10 swab samples (knife swab immediately after evisceration, 2 hand swabs, and floor swab from area under which pigs were hung). At the processing plant, PAdV was not detected in any of the 40 pig muscle samples or the 10 swab samples tested. At points of sale, PAdV was not detected in any of the 63 sausages tested, but 1 (13%) of the 8 swab samples from the door handle of the cold room was PAdV positive. This finding could have resulted from transfer from a contaminated pig carcass, but the in-test controls and method of sampling exclude this contamination as a source of the HEV in the sausage meat. Human adenovirus was not detected in any samples from any of the locations.

Conclusions

In industrialized regions, although the incidence of clinical hepatitis E in humans is low, the seroprevalence of antibodies against HEV is relatively high (*9*), indicating a high proportion of subclinical disease and/or underdiagnosis (*10*). A small proportion of this exposure to HEV is likely to result from travel to regions to which the virus is endemic or migration from such regions (*11*). However, a substantial level of exposure to HEV seems to have an indigenous source.

Pork products are eaten in several industrialized regions, including the United Kingdom (*2*,*12*,*13*). A recent cluster of illnesses in southern France was associated with ingestion of *figatelli*, a pig liver sausage mainly eaten raw (*3*). Foodborne illness has been associated with ingestion of pig liver and pig muscle tissue (*14*,*15*). Our study showed that in the United Kingdom, pork products with high-volume nationwide consumption might be contaminated with HEV. We chose sausages as the type of point-of-sale pork product investigated for HEV because they are widely eaten throughout the United Kingdom (>212,746 tons in Great Britain during February 2011–February 2012, BPEX, Agriculture and Horticulture Development Board, UK), unlike pig liver, and a 10% HEV detection rate in pork sausages at point of sale could be a cause for concern.

In terms of the potential for virus transmission, the surface swabs provided evidence that PAdV and HEV contamination occurs in the slaughterhouse and at point of sale. From the processing plant, HEV was detected on 1 surface swab. The 98% positive rate recorded for pig feces with the PAdV indicator validates this approach to detecting fecal contamination of porcine origin. No evidence of human fecal contamination was detected at any point in the chain, indicating that personal hygiene standards were high and that the HEV detected was unlikely to have come from human contamination of the samples.

Because the numbers of samples tested for viral contamination in this study were relatively small, these results should be taken as indicators only. For greater

Table 2. Prevalence of PAdV, HEV, and HAdV in the pork food chain, 2009–2010*

Point in chain	Sample type	No. PAdV positive/ no. collected (%)	No. HEV positive/ no. collected (%)	No. HAdV positive/ no. collected
Slaughterhouse	Feces	39/40 (98)	5/40 (13)	NT
	Liver	6/40 (15)	1/40 (3)	NT
	Surface swab	4/10 (40)	1/10 (10)	0/10
Processing plant	Muscle	0/40	0/40	NT
	Surface swab	0/10	1/10 (10)	0/10
Point of sale	Sausage	0/63	6/63 (10)	NT
	Surface swab	1/8 (13)	2/8 (25)	0/8

*PAdV, porcine adenovirus; HEV, hepatitis E virus; HAdV, human adenovirus; NT, sample not taken.

confidence in the results, we recommend a larger study. In addition, we will assess the viability and genotype of HEV by further work of in vitro culture and nucleotide sequencing, respectively.

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Autochthonous Infections with Hepatitis E Virus Genotype 4, France

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During January–March 2011, diagnoses of hepatitis E virus (HEV) infection increased in Marseille University hospitals in southeastern France. HEV genotype 4, which is described almost exclusively in Asia, was recovered from 2 persons who ate uncooked pork liver sausage. Genetic sequences were 96.7% identical to those recently described in swine in Europe.

In industrialized countries, most cases of hepatitis E virus (HEV) infection in humans are autochthonous (*1*). Pigs are a major reservoir of HEV, and transmission of virus to humans who ate raw or undercooked pork has been reported (*1*–*3*). In France, >300 cases of HEV infection are reported annually (*3*); most infections are autochthonous and occur in southern France, where the prevalence of anti-HEV IgG is higher than in northern France (*3*–*5*). Almost all cases of autochthonous HEV infection reported in Europe have involved genotype 3 strains (*1*–*6*).

Beginning February 21, 2011, a biosurveillance program (EPIMIC) (*7*) detected an increase in the number of HEV infections diagnosed at Marseille University hospitals in southeastern France. During February 21–March 28, the weekly number of serum samples that were tested and found positive for HEV was above the elected critical threshold (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/11-1827-Techapp.pdf).

The Cases

In Marseille during January–March 2011, a total of 11 cases of HEV infection were confirmed by anti-HEV IgM testing and detection of HEV RNA in serum samples. EIAgen assays (Adaltis Italia, Casalecchio di Reno, Italy) were used to detect anti-HEV IgM and IgG. Additional anti-HEV IgM testing was performed by using the Assure HEV IgM Rapid Test (MP Biomedicals, Illkirch, France) and the *recom*Line HEV IgG/IgM test (Mikrogen Diagnostik, Neuried, Germany). HEV RNA was detected by using a real-time reverse transcription PCR targeting open reading frame (ORF) 2 (*2*).

The mean age of the case-patients was 57 years (±11 years). Of the 11 case-patients, 10 were male and 3 were kidney transplant recipients (Tables 1, 2). HEV infection was clinically asymptomatic in all transplant recipients; the infection was diagnosed after routine posttransplant laboratory tests showed elevated levels of liver enzymes. Longitudinal testing indicated chronic HEV infection in 1 case-patient (no. 5), and an 80-year-old case-patient died 9 weeks after disease onset.

HEV 5′-ORF2 RNA was recovered from the serum of 8 case-patients. Phylogenetic analysis (*2*) showed that 4 patients each had HEV genotype 3c or 3f (online Technical Appendix Figure 2). Sequences from 2 unrelated case-patients (nos. 7, 8) showed 99.8% identity; for other pairwise comparisons, maximal identity was 93.4% (mean 83.6%).

We could not recover HEV 5′-ORF2 RNA from the serum of 3 case-patients (nos. 1, 2, 6). For case-patient 6, this could be explained by a low viral load. When targeting ORF1 and 3′-ORF2, we recovered HEV genotype 4 (HEV-4) from serum samples from case-patients 1 and 2 (Figure; online Technical Appendix Figure 3) (*5*,*10*). Molecular testing results for these samples were checked in duplicate and by testing different serum samples from the same case-patients. HEV-4 RNA from the 2 case-patients showed 100% identity for ORF1 and 99.8% identity for ORF2. The next closest match (96.7% identity) was the first HEV-4 RNA in swine reported in Europe (GenBank accession no. HQ857384) (Figure) (*8*). The next 2 closest matches (91.0%–91.3% identity) were recovered from swine in China (GenBank accession nos. EU676172 and DQ279091) (Figure). However, the sequences showed only 86.0% identity with a genotype 4f HEV RNA recovered from a patient in Germany (*9*).

None of the 11 case-patients had traveled abroad during the 2–9 weeks before hepatitis onset. Of the 11 case-patients, 6 (55%), including the 2 with HEV-4, ate uncooked pig liver sausage (*figatelli* [2]) (Table 2). The 2 patients with HEV-4 were not related. Because 4 contacts of the 2 HEV-4–infected case-patients also ate some of the figatelli eaten by the case-patients (Tables 1, 2), we investigated whether these contacts had markers for HEV infection. Test results for anti-HEV IgM were positive for the wife of case-patient 1 and a friend of case-patient 2; the HEV PCR result was barely positive for contact 1a, who had an elevated level of alanine aminotransferase (165 IU/L; reference <45 IU/L). These 2 persons were clinically asymptomatic.

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Table 1. Epidemiologic and virological findings for case-patients and contacts with HEV infection, Marseille, France, January–March 2011*

Case-patients and contacts, by ID no.	Day obtained, serum sample (retrospectively tested sample)	Anti-HEV IgG EIA result†	Anti-HEV IgM result, by testing method			HEV RNA in serum (C_t)	HEV genotype
			EIA†	Rapid test‡	Immunoblot		
1	Feb18	10.3	14.2	Positive	Positive	Positive (29)	4
1a§	*Mar 2*	*11.4*	*0.2*	*Negative*	*Not tested*	*Positive (38)*	NA
1a§	*Mar 16*	*>10.0*	*2.0*	*Not tested*	*Borderline*	*Negative*	NA
1b¶	*Mar 2*	*Negative*	*Negative*	*Negative*	*Negative*	*Negative*	NA
2	Mar 21 (Feb 7)	11.2	15.6	Positive	Positive	Positive (28)	4
2a#	*Mar 21*	*6.3*	*15.6*	*Positive*	*Positive*	*Negative*	NA
2b**	*Mar 21*	*0.1*	*Negative*	*Negative*	*Negative*	*Negative*	NA
3	Jan 4	10.7	16.6	Positive	Not tested	Positive (27)	3f
4	Feb 3 (Jan 6)	5.2	6.3	Weakly reactive††	Positive	Positive (26)	3c
5‡‡	Feb 3	0.1	1.6	Weakly reactive	Negative	Positive (31)	3f
6	Mar 1	11	14	Positive	Positive	Positive (38)	NA
7	Mar 2	10.6	15.1	Positive	Negative	Positive (27)	3c
8‡‡	Mar 9	11.1	14.9	Positive	Not tested	Positive (28)	3c
9	Mar 18	10.5	16.4	Positive	Not tested	Positive (28)	3f
10‡‡	Mar 21	0.3	15.6	Positive	Not tested	Positive (24)	3c
11	Mar 23	1.2	13.8	Positive	Positive	Positive (27)	3f

**Italics* indicate results for contacts of case-patients. HEV, hepatitis E virus; ID, identification; EIA, enzyme immunoassay; C_t, cycle threshold; NA, not applicable.
†EIA score represents test/cutoff optical density ratios.
‡An immunochromatographic rapid test.
§Contact (wife) of case-patient no. 1.
¶Contact (friend) of case-patient no. 1.
#Contact (friend) of case-patient no. 2.
**Contact (sister) of case-patient no. 2.
††Tested on a serum sample collected on March 3, 2011.
‡‡Kidney transplant recipient.

Conclusions

We report a concurrent rise in testing for and diagnoses of HEV infections in Marseille during January–March 2011. This rise may reflect increased clinician awareness of HEV or an increased incidence of HEV infection that may have resulted from the use of improved tools for HEV diagnosis.

In industrialized countries, HEV has increasingly been recognized as a possible cause of hepatitis in diverse clinical settings, including in solid-organ transplant recipients. In our study, 3 (27%) of the case-patients were solid-organ transplant recipients. This number suggests a high incidence of HEV infection among transplant recipients in the geographic area we studied, and it is consistent with the incidence (≈3.2/100 person-years) of HEV infection among organ transplant recipients in Toulouse in southwestern France (*4*). Liver

Table 2. Epidemiologic and clinical findings for case-patients and contacts with HEV infection, Marseille, France, January–March 2011*

Case-patients and contacts, by ID no.	Age, y/sex	Clinical signs	ALT, IU/L†	Consumed uncooked pig liver sausage
1	62/M	Asthenia, jaundice, nausea	2,124	Yes
1a‡	*57/F*	*None*	*165*	*Yes*
1b§	*60/M*	*None*	NA	*Yes*
2	49/M	Asthenia, jaundice	138	Yes
2a¶	*46/F*	*None*	*40*	*Yes*
2b#	*51/F*	*None*	*40*	*Yes*
3	45/F	Jaundice	1,696	Yes
4	80/M	Asthenia, jaundice	1,885	No
5**	63/M	None	340	No
6	68/M	Asthenia, fever, jaundice	1,135	Yes
7	54/M	Asthenia, jaundice	88	No
8**	58/M	None	21	No
9	38/M	Asthenia, fever, dark urine	1,554	Yes
10**	52/M	None	71	Yes
11	58/M	Asthenia, jaundice	2,500	No

**Italics* indicate features for contacts of case-patients. HEV, hepatitis E virus; ID, identification; ALT, alanine aminotransferase; NA, not available.
†Upper limit of normal, 40 IU/L.
‡Contact (wife) of case-patient no.1.
§Contact (friend) of case-patient no.1.
¶Contact (friend) of case-patient no.2.
#Contact (sister) of case-patient no.2.
**Kidney transplant recipient.

biochemical testing is routinely performed after organ transplantation; such testing enabled the detection of subclinical HEV infection in the 3 transplant recipients reported here. Subclinical HEV infection can also occur in immunocompetent persons (*1*,*11*,*12*); thus, we question whether the incidence of HEV infection among transplant recipients in southern France might reflect that in the general population. Moreover, 52% of adult blood donors sampled in southwestern France in 2003–2004 were retrospectively shown to be HEV seropositive by using a newly available assay (*4*), and HEV was retrospectively shown to be an underdiagnosed cause of liver injury in the United States and United Kingdom (*13*).

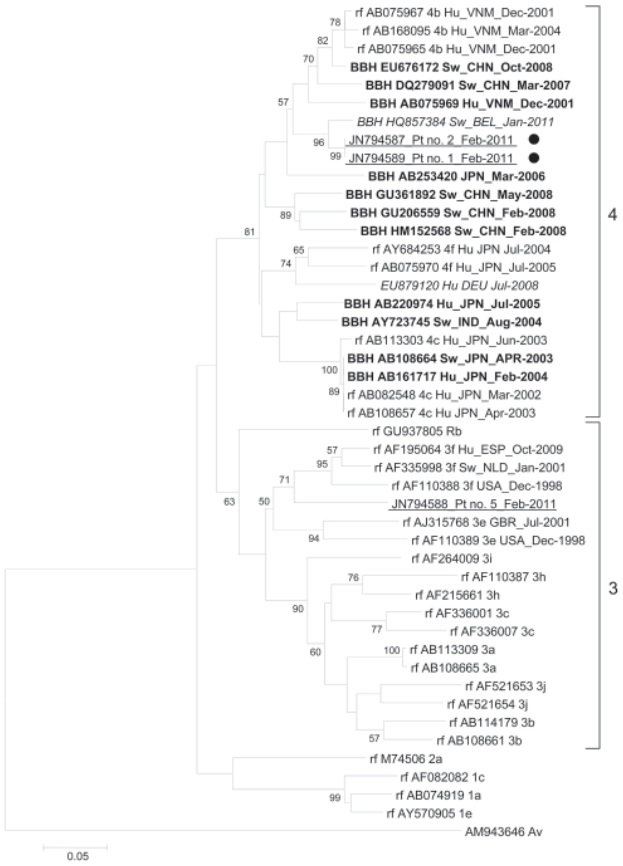

Figure. Phylogenetic tree based on partial (186 nt) sequence of the 5′ end of open reading frame 1 of the hepatitis E virus (HEV) genome (nt 133–318; GenBank accession no. AB291961). **Boldface** indicates sequence with the highest BLAST scores (http://blast.ncbi.nlm.nih.gov/Blast.cgi); *italics* indicates sequences obtained from a swine in Belgium with HEV genotype 4 (*8*) and a human in Germany with autochthonous HEV infection (*9*); underlining indicates sequences obtained from humans in Marseille, France, during January–March 2011; black dots indicate patients who ate uncooked pig liver sausage. Reference sequences (rf) with known genotypes and subtypes are indicated (*6*). Numbers on right indicate genotype. For sequences from this study, nucleotide alignments were performed by using ClustalX version 2.0 (www.clustal.org/download/current). The tree was constructed by using MEGA4 (www.megasoftware.net) and the neighbor-joining method as described (*2*). Branches were obtained from 1,000 resamplings of the data; those with bootstrap values >50% are labeled on the tree. The avian HEV sequence AM943646 was used as an outgroup. Scale bar indicates nucleotide substitutions per site. HEV sequences are labeled with GenBank accession number, host, country where isolated, and collection or submission date. Hu, human; VNM, Vietnam; BBH, best BLAST hit; Sw, swine; CHN, China; BEL, Belgium; Pt, patient; JPN, Japan; DEU, Germany; IND, India; Rb, rabbit; ESP, Spain; NLD, the Netherlands; USA, United States; GBR, United Kingdom; Av, avian.

A considerable proportion of HEV infections in southern France may be linked to the consumption of uncooked figatelli, which has been identified as a source of zoonotic foodborne HEV transmission (*2*,*4*). In our study, 55% of the HEV-positive patients reported eating uncooked figatelli, and in another study in France, 2 HEV-positive patients reported eating uncooked figatelli (*11*). These cases indicate insufficient prevention of HEV transmission to humans through uncooked figatelli consumption (*2*).

The major finding in our study was the recovery of HEV-4 sequences from 2 of 6 case-patients who ate uncooked figatelli purchased in southern France. These sequences were closely related to the sequence for the first-reported HEV-4 RNA in swine in Europe (*8*), which was recovered in Belgium and classified as genotype 4b. HEV-4 is indigenous to Asia, where it has been recovered from pigs and humans (*1*,*6*,*14*). For the 2 HEV-4 RNA sequences obtained in our study, the best matches among sequences from Asia were from HEV strains from swine in China. Since its first report in China in 1999, HEV-4 has been increasingly described as endemic in pigs and involved in most sporadic cases of hepatitis E (*1*,*14*). Several HEV subtypes have been described in China, including subtype 4b (*1*,*6*,*14*). In Europe, autochthonous infection with a genotype 4f HEV in a person in Germany was reported in 2008 (*9*), and a case of HEV-4 infection acquired in India was reported for a person in England in 2010 (*15*). Studies in China have shown relatedness between HEV-4 sequences recovered from humans and swine in the same geographic area, which suggests zoonotic transmission (*1*). Moreover, sequences for HEV-4 RNA recovered from a father and son in Japan who ate barbecued pig meat were almost identical, and sequences were identical for HEV-4 RNA from packaged pig liver sold in a grocery store and from a patient in whom HEV infection developed after the person ate grilled pig liver (*1*,*2*).

Together with previous findings, our results bring up the question as to whether HEV-4 was introduced into domestic pigs or whether pig meat of Asian origin was introduced into France. Moreover, it prompts a study of whether HEV-4 strains circulate and spread in Europe as an effect of globalization of HEV zoonosis.

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Putative Novel Genotype of Avian Hepatitis E Virus, Hungary, 2010

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To explore the genetic diversity of avian hepatitis E virus strains, we characterized the near-complete genome of a strain detected in 2010 in Hungary, uncovering moderate genome sequence similarity with reference strains. Public health implications related to consumption of eggs or meat contaminated by avian hepatitis E virus, or to poultry handling, require thorough investigation.

Avian hepatitis E virus (aHEV) is a causative agent of big liver and spleen disease (BLSD) in chickens (*1*), which manifests itself, as its name indicates, by hepatosplenomegaly. The etiologic association has been demonstrated worldwide, including Australia, North America, Asia, and Europe (*2*,*3*). In Hungary, BLSD was observed during the 1980s, but association of the disease with hepatosplenomegaly was not made in Hungary until 2008 (*4*,*5*). Ivanics et al. (*5*) reported outbreaks of aHEV infection and increased deaths of chickens caused by hepatitis and splenitis and reduced egg production in chicken flocks in Hungary. Tissue samples from chickens collected during 2010 were positive for aHEV by reverse transcription PCR (RT-PCR) (*6*,*7*). Paired samples indicated seroconversion upon inoculation as measured by serologic assay, strongly suggesting an etiologic association between BLSD and aHEV infection in the affected chicken flocks (*5*).

The aHEV is characterized by a small, nonenveloped virion and a 6.6-kb, capped, poly-A tailed single-stranded RNA genome. The genome encodes 3 open reading frames (ORFs). ORF1 is a polyprotein encoding putative functional domains of methyltransferase, papain-like cystein protease, helicase, and RNA polymerase. The capsid protein is encoded by ORF2; ORF3 encodes a multifunctional cytoskeleton-associated protein linked to viral morphogenesis and pathogenesis (*1*).

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aHEV is ≈50% similar at the genomic level to mammalian HEV genotypes; recent proposals classify aHEV into a different taxon, possibly a new genus, within *Hepeviridae*. GenBank now includes 5 full or nearly complete aHEV genome sequences that could be classified into 1 of the 3 recognized aHEV genotypes. These 5 prototype strains with full or nearly complete genome sequences include a genotype 1 strain from Australia, 2 genotype 2 strains from the United States, and 2 genotype 3 strains from Europe and the People's Republic of China (*6*,*8*–*10*). Additional putative genotypes have been identified through analysis of an ≈130-nt fragment of the helicase domain, suggesting that diversity within aHEV is higher than recognized (*11*). Our primary objective was the genetic characterization of an aHEV strain from the 2010 outbreak in Hungary to increase understanding of the origin and evolution of these emerging pathogens.

The Study

Preliminary sequence analysis of the amplicons obtained by the diagnostic HEV RT-PCR from the 2010 BLSD outbreaks in Hungary (strains identified by HUN prefix) showed a moderate genetic relationship with reference aHEV sequences deposited in GenBank (*5*). One strain, HUN-16773–2010, found in a sample of liver tissue, was selected for further analysis. Viral RNA was extracted by using the SV96 total RNA nucleic acid extraction kit (Promega, Madison, WI, USA). Oligonucleotides were designed for our primer walking sequencing strategy on the basis of aHEV genome sequence data (Table). Genomic fragments were amplified by using a OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) as described by the manufacturer. Amplicons were subsequently subjected to direct sequencing (Biomi Ltd., Gödöllő, Hungary).

The final assembly was 6,543 bp (GenBank accession no. JN997392), with negligible amounts of missing information at the 5′ (75 nt) and 3′ ends preceding the poly A tail (36 nt). Nucleotide and deduced amino acid sequences of HUN-16773–2010 aligned well with the other 5 prototype aHEV strains. The nearly complete coding sequence (1,515 aa) with a missing 17-aa region was determined for ORF1. Predicted functional domains (*12*) were identified. The sequence similarities (Megalign; DNASTAR Inc., Madison, WI, USA) of ORF1 in comparison with genotypes 1–3 aHEV strains were 81.0%–83.9% at the nucleotide level and 93.0%–95.4% at the amino acid level.

Unique amino acid changes in the ORF1 of HUN-16773–2010 were found in 16 positions compared with the European prototype aHEV strain (not shown) (*6*). ORF2 encodes the 606-aa capsid protein. Similarities to prototype aHEV strains were 83.9%–87.3% at the nucleotide level and 98.2%–99.3% at the amino acid level. We observed 2

Table. Primers used in the generation of nearly complete genome of the novel Hungarian avian hepatitis E virus strain*

Primer name	Sequence, 5′ → 3′	Location	Orientation
F1	CAG ACT ACC ATT ATC GCC AC	2926–2945	+
R1	AGC TGG AGC TTG ACA ACT TC	3319–3338	–
R1b	AGC TGG AGC TTG ACA ACC TC	3319–3338	–
F2	GGA TGC CTG ACT GGA TGG T	3926–3944	+
F2b	CTG ACT GGA TGG TGG CTC T	3932–3950	+
R2	TTA CCA GGC GAC AGA TTC C	5395–5413	–
F3	ACT CGT GTT AAG GTA ACG GC	5430–5449	+
F3b	GTG GCT GAA GAT GGC GAG AT	5541–5560	+
F3c	GGT GTG GCT GAA GAT GGC GA	5538–5557	+
R3	CCG AGA TGG GAG GAT TTC ATA A	6629–6650†	–
F4	GCA GTT TGC AGA GTC CAA GG	36–55†	+
R4	CTG ATC AAA AGT GCT ACC CT	2909–2928	–
R4b	TGA TTG ACC CGG GGT TCG	2876–2893	–
F5	GGT GTT GAG GTC GGC ATG GT	4432–4451	+
F5b	ATA TCC AGC GCA TGC AGG	4325–4342	+
R5	ATC TAC ATC TGG TAC CGT GC	4828–4847	–
R5b	GAC GTT GTG CCA TCC TGA AG	4965–4984	–
F6	TCC TGT TCT CAC CAG TGT	400–417	+
R6	AGC CCA TAA TTA ACC ATG TC	2302–2321	–
R6b	GAT GTC GAT TTA CCG CTG CC	2413–2432	–
F7	GGT GTG GCA GAG GTT AAT GAT G	897–918	+
F7b	GAT GAT GCC TTC TGC TGC TC	937–956	+
R7	AAG ACA GCA AGG ACC TCC TC	1795–1814	–
R7b	AGG CTG ATG ATC ACG GTT GG	1883–1902	–

*The annealing temperature was set at 53°C for each primer combination. +, sense; –, antisense.
†5′ and 3′ end primers were designed on the basis of sequence AM943646.

unique amino acid substitutions in positions A53S and Q/M473T, the second residue located within antigenic region II (*6*). ORF3 encoded an 87-aa polypeptide. This region also shared high (>92%) nt and (>90%) aa similarities with genotype 1–3 aHEVs.

When analyzing the nearly complete genome sequences, we found that strain HUN-16773–2010 shared moderate similarity to genotypes 1–3 aHEV strains (nt, 81.9%–84.9%). Similar ranges of genome sequence similarity (nt, 81.8%–82.7%) distinguish prototype aHEV strains from each other. Using SimPlot analysis (http://sray.med.som.jhmi.edu/SCRoftware/simplot), we observed this moderate nucleotide similarity of HUN-16773–2010 to the 5 prototype strains along the entire genome (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/8/11-1669-FA1.htm), except the near-central region of ORF1, which showed less similarity among aHEV strains; this region encodes the hinge region flanking the papain-like protease and the X domain. Similarity in the genome was highest in the 5′ end of the genome and the overlapping region of ORF2 and ORF3. We found no evidence for recombination between HUN-16773–2010 and genotypes 1–3 strains (*13*), demonstrating that this strain was not generated through recombination from prototype aHEVs.

Maximum-likelihood and neighbor-joining trees were constructed with the substitution model of Tamura and Nei, including gamma distribution shape (G) parameter (TN93+G) as implemented in MEGA 5 (*14*); 500 bootstrap replicates were used to test the reliability of the tree topology. Phylogenetic analysis of the nearly complete aHEV genome revealed 4 major clusters, where genotypes 1–3 strains formed previously recognized branches (*10*); the aHEV strain, HUN-16773–2010, appeared to form a fourth cluster (Figure, panel A). To determine whether HUN-16773–2010 shared genetic relatedness in the partial helicase gene with recently identified variants of European aHEV strains (*11*), we reanalyzed this region by using a larger sample set. Our data reaffirmed that HUN-16773–2010 forms an independent branch (not shown).

To identify the evolutionary driving forces beyond the heterogeneity of aHEV genomes, we individually ran the alignments of all 3 ORFs on the DataMonkey server by using the random effects likelihood method (www.datamonkey.org). Evidence for strong selection pressure was found at the hinge region of ORF1 involving 17 sites between amino acid positions 556 and 605 (Figure, panel B). However, 905, 384, and 3 sites within ORF1, ORF2, and ORF3, respectively, were under purifying selection across aHEV genotypes.

Conclusions

Various genotypes of aHEV have been proposed to represent clusters of strains typical for particular geographic locations. However, new evidence has shown that some genotypes could be present on different continents (*6*,*10*,*11*). For example, although Australia seemingly remained the only continent with genotype 1 aHEV, different genotypes could be endemic to the Americas and particularly Europe, and these areas could show strain diversification. The origin of the novel aHEV strain in Hungary remains enigmatic;

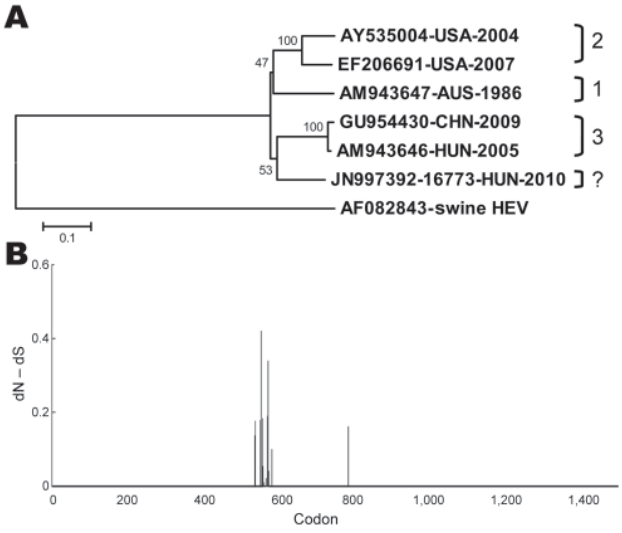

Figure. Genomic structure of HUN-16773-2010 and 5 other avian hepatitis E virus (aHEV) strains. A) Neighbor-joining tree constructed from the nucleotide sequences (*14*) of the nearly complete genome of 6 aHEV strains. Bootstrap values are indicated at branch nodes. A swine HEV served as outgroup in the phylogeny. Genotype assignments are depicted for reference aHEV genomes. Scale bar indicates genetic distance. B) Adaptive evolution of aHEV genomes. Only sites under positive selection in open reading frame 1 are shown with normalized value of dN – dS along the codon positions, as indicated by the random effects likelihood method and by using the default significance level (www.datamonkey.org).

understanding its recent emergence awaits analysis of further isolates within and outside Europe.

Purifying selection was a key mechanism in the evolution of aHEV, suggesting the existence of a strong structural and functional constraint against diversification that would lead to extensive amino acid changes within the 3 ORFs in aHEV strains. In contrast, positive selection was predicted primarily in the hypervariable hinge region flanking the protease and X domains in ORF1. The biological role of this observation is not clear. However, recent findings have demonstrated that this region might be responsible for differences in replication efficiency (*15*). Thus, the positive evolutionary selection in this region could directly affect viral fitness and influence the pathogenesis of aHEV infections.

We provided further evidence for the marked sequence diversity among aHEVs by providing the nearly complete genome sequence of a strain identified during a BLSD outbreak in Hungary. Additional aHEV genomes could facilitate more penetrating insights into their epidemiologic features and evolutionary mechanisms and also could serve as molecular bases for reliable and robust demarcation criteria in future classification proposals.

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Novel Hepatitis E Virus in Ferrets, the Netherlands

To the Editor: Hepatitis E virus (HEV), a member of the family *Hepeviridae* and the genus *Hepevirus*, is transmitted by the fecal–oral route and causes liver inflammation, which leads to mortality rates of ≈20% in pregnant woman (*1*,*2*). Human hepatitis E is a major disease not only in developing countries but also in industrialized countries, and identification of animal strains of HEV in pigs and deer and its zoonotic potential has raised considerable public health concerns (*1*,*3*). Recent reports suggest that other animals such as rats, mongooses, chickens, rabbits, and trout also may harbor HEVs (*1*–*5*). The genomes of these viruses are ≈6.6 kb–7.2 kb and encode 3 open reading frames (ORFs) flanked by a capped 5′ end and a poly A tail at the 3′ end (*1*,*3*). We used random PCR amplification and high-throughput sequencing technology to investigate HEV sequences in ferrets (*Mustela putorius*) from the Netherlands.

In 2010, fecal samples were collected from ferrets in the Netherlands and stored at −80°C. Samples that were negative for ferret coronavirus (*6*) were further characterized for other pathogens. Viral RNA was isolated and viral metagenomic libraries were constructed for 454 pyrosequencing as described (*7*,*8*), and 248,840 sequence reads were generated from 7 fecal samples. Using Blastn and Blastx (www.ncbi.nlm.nih.gov/BLAST), we identified 289 sequence reads in 1 sample that were related to rat HEV and that could be assembled into 6 contigs covering ≈50% of the ferret HEV (FRHEV) genome.

We then developed a set of nested PCR primers on the basis of obtained sequences to detect viral RNA (online Technical Appendix Table 1, www.cdcnc.gov/EID/pdfs/11-1659-Techapp.pdf). Total RNA extracted from 43 ferret fecal samples collected from 19 locations in the Netherlands was used to perform reverse transcription PCR amplification. Using this PCR, we detected viral RNA in 4 (9.3%) fecal samples tested from 4 locations (distance between each sampling location ranged from 25 km to 127 km). All amplicons were confirmed by nucleotide sequencing. We have limited information regarding the clinical disease this virus may cause because these samples were obtained from household pet ferrets that did not show overt clinical signs. In addition, 4/16 animals from a single farm were IgG positive when tested for IgG against HEV by using recombinant human HEV protein (Wantai, Beijing, China).

To characterize the complete genome of this virus, we selected 2 PCR-positive samples (FRHEV4 and FRHEV20), developed different sets of specific primers on the basis of sequence fragments obtained by 454 pyrosequencing, directly sequenced amplicons by Sanger sequencing, and used a rapid amplification of cDNA ends PCR to obtain 5′ and 3′ frame end sequences. Using overlapping fragments we assembled 2 complete FRHEV genome sequences that contained 6,854 nt, including a 13-nt 3′ poly A tail and a 12-nt 5′ end. FRHEV full-genome sequences FRHEV4 and FRHEV20 showed 98.6% sequence identity and were deposited into GenBank under accession nos. JN998606 and JN998607, respectively.

The FRHEV genome contains a complete ORF1 gene that encodes a nonstructural protein of 1,596 aa, an ORF2 gene that encodes a capsid protein of 654 aa, an ORF3 gene that encodes a phosphoprotein of 108 aa, and a 3′ noncoding region of 78 nt. Sequence analyses indicated that the FRHEV genome shared the highest identity (72.3%) with rat HEV. Sequence identity with HEV genotypes 1–4 and rabbit and avian HEVs ranged from 54.5% to 60.5% (online Technical Appendix Table 2). The FRHEV genome organization

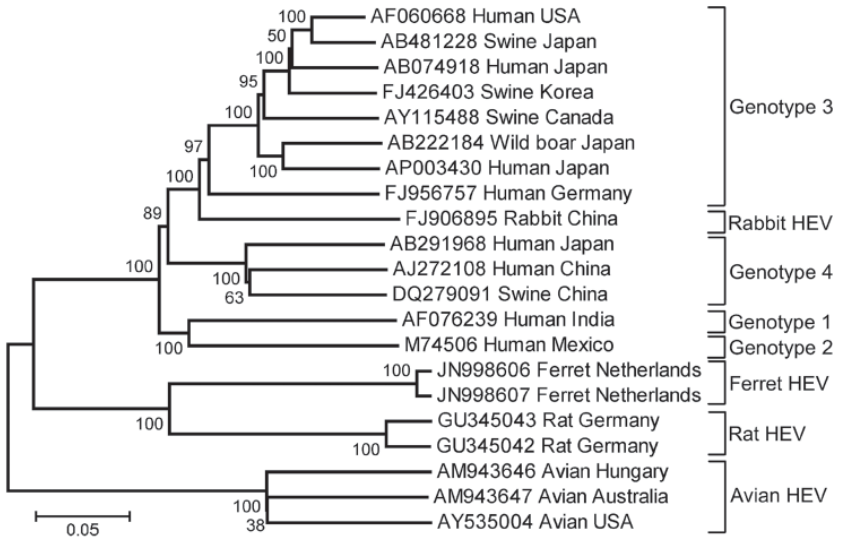

Figure. Phylogenetic tree based on the complete genomic sequences of ferret hepatitis E viruses (HEVs) and human, rabbit, swine, avian, and rat HEV strains. Names of HEV strains follow GenBank accession numbers. Sequence alignment was performed by using ClustalW in the MEGA5.0 software package (www.megasoftware.net), and the trees were constructed by using the neighbor-joining method with p-distance (gap/missing data treatment; complete deletion) and 1,000 bootstrap replicates as in MEGA version 5.0. Scale bar indicates nucleotide substitutions per site.

was found to be slightly different from other HEVs and included a putative ORF (ORF4) of 552 nt that overlapped with ORF1 (online Technical Appendix Figure). A similar pattern of genome organization was observed for both FRHEVs.

Phylogenetic analysis of the complete genomes clearly showed that FRHEV was separated from genotype 1–4 HEVs and clustered with rat HEV (Figure). Similar phylogenetic clustering was observed when nucleotide and deduced amino acid sequences of ORF1, ORF2, and ORF3 were analyzed separately. The phylogenetic distance between rat HEV and FRHEV is larger than the distance between genotype 1 and genotype 2 HEV.

In recent years, an increasing number of sporadic cases of hepatitis E have been reported (*1*,*9*). Several observations suggest that autochthonous cases are caused by zoonotic spread of infection from wild or domestic animals (*1*,*3*,*9*). In addition, IgG anti-HEV seropositivity in the United States has been associated with several factors, including having a pet at home (*10*). Further studies are needed to identify the zoonotic potential of FRHEV.

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Epidemic *Clostridium difficile* Ribotype 027 in Chile

To the Editor: The increased severity of *Clostridium difficile* infection is primarily attributed to the appearance of an epidemic strain characterized as PCR ribotype 027 (*1*). The only report that identified epidemic *C. difficile* ribotype 027 in an American country outside of North America comes from Costa Rica, raising the possibility that strains 027 might also be present in other countries of Latin America (*2*). Several studies between 2001 and 2009 have been conducted in South American countries to detect the incidence of *C. difficile* infection in hospitalized patients, but they did not identify which *C. difficile* strains were causing these infections (*3*).

During an epidemiologic screening of patients with *C. difficile* infection in a university hospital in Chile, we analyzed all stool samples of patients with suspected *C. difficile* infection during a 5-month period (June–November 2011). Two cases of *C. difficile* infection were associated with ribotype 027.

C. difficile was isolated from stool samples according to published protocols (*4*). Briefly, stool samples were spread onto taurocholate-cefoxitin-cycloserine fructose (Merck, Rahway, NJ, USA) agar plates and incubated for 96 hours at 37°C in a Bactron III-2 anaerobic workstation (SHEL LAB, Cornelius, OR, USA.). Plates were examined for the characteristic *p*-cresol odor unique to *C. difficile* culture (*5*). The aminopeptidase test (*6*) was also used to differentiate *C. difficile* strains. Suspected colonies were further analyzed by PCR to amplify *tcdA*, and *tcdB* genes (*7*). The presence of binary toxin gene (*cdtB*) and deletion in the negative regulator of the pathogenecity locus, *tcdC*, were determined by using Cepheid GeneXpert PCR (Cepheid, Sunnyvale, CA, USA). We used *C. difficile* ribotype 027 strain R20291 as a reference strain for comparative purposes. PCR ribotyping was performed as described (*8*). The specific ribotype 027 of each of the clinical isolates was determined by visual analysis and with the GelCompar II v6.5 software (Applied Maths, St-Martens-Latem, Belgium).

Case-patient 1 was a 60-year-old man with a history of coronary disease who required a coronary artery bypass graft because of 3-vessel coronary disease. Forty-eight hours after receiving 3 doses of cefazoline to prevent surgical wound infection, he exhibited severe and diffuse abdominal pain with frequent loose stools (8 bowel movements/day), fever (up to 39°C), and hemodynamic compromise, which required high doses of vasopressors. Stool samples were positive for *C. difficile* by ELISA, and the patient received intravenous metronidazole and oral vancomycin. However, because of the severity of the course of the disease, he underwent an urgent total colectomy with terminal ileostomy. The patient showed progressive improvement, and he was discharged 11 days after surgery. No relapse of *C. difficile* infection was reported in this patient in the next 5 months. Isolation of toxigenic culture and PCR demonstrated that the bacterial pathogen causing the diarrhea was *C. difficile* ribotype 027 (i.e., strain PUC51) (Figure).

Case-patient 2 was a 46-year-old man with a history of ischemic stroke with hemiparesis of the left side who had experienced a urinary tract infection that had been treated with ciprofloxacin 2 months earlier. Four weeks before admission, he had frequent loose stools with no fever and diffuse abdominal pain after meals. On admission, a computed tomographic scan and angiograph of the abdomen showed pancolitis with colonic wall thickening and scant ascites, suggestive of an inflammatory or infectious cause, without vascular compromise. However, ELISA of stool samples was negative for *C. difficile* toxin. Treatment with ceftriaxone reduced his symptoms, and he was discharged. Seven days after discharge, he had intense diffuse abdominal pain, with frequent loose stools and fever up to 38.9°C, and was again admitted to the hospital. A new computed tomographic scan of the abdomen showed no change; however, an ELISA of a new stool sample for *C. difficile* toxin was positive, and the patient was given oral vancomycin. No relapse of *C. difficile* infection was observed within 3 months of observation. Toxigenic culture from stool samples and PCR identified the *C. difficile* isolate as ribotype 027 (i.e., strain PUC47) (Figure).

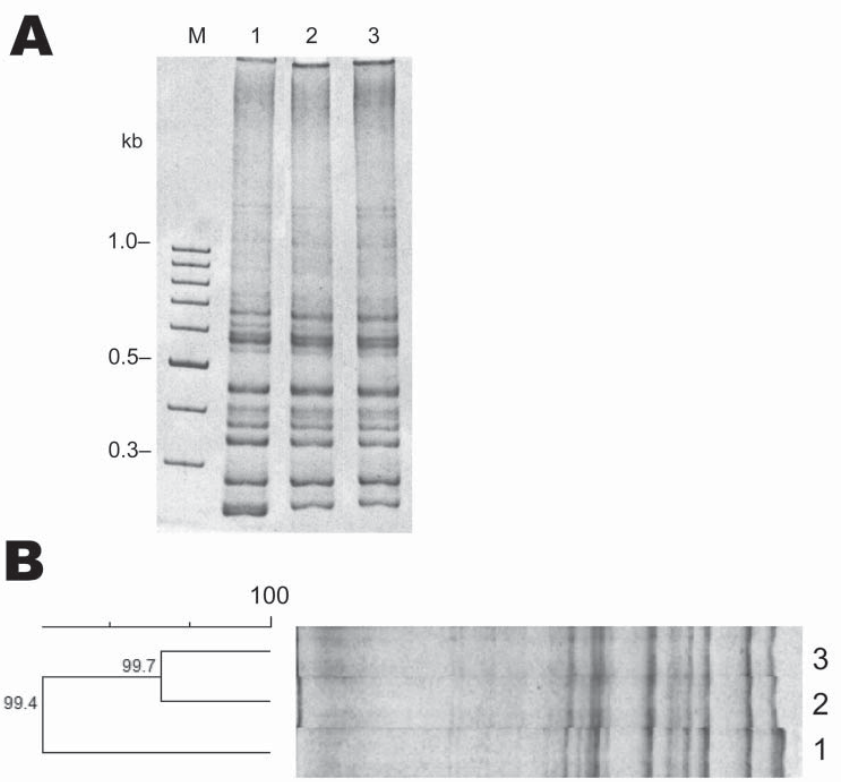

Figure. Results of PCR ribotyping of *Clostridium difficile* 027 strains from Chile. M indicates the 100-bp DNA ladder; lane 2, R20291; lane 2, PUC47; lane 3, PUC51. A) PCR ribotyping of *C. difficile* isolates. PCR results show that that the band pattern of the ribosomal intergenic regions of strains PUC47 and PUC51 are similar to those of the reference (epidemic) strain R20291. B) Cluster analysis of strains PUC47, PUC51, and the epidemic strain 027 R20291 shows >99% similarity and that they belong to the same epidemic clade. Scale bar indicates percent identity.

Molecular typing analysis showed that both case-patients had a monoclonal infection caused by *C. difficile* ribotype 027. Both isolates had *tcdA*, *tcdB,* and *cdtB* and had a deletion in *tcdC* (data not shown).

In summary, the described severe cases of *C. difficile* infection in Chile were caused by epidemic *C. difficile* ribotype 027. One of these case-patients required urgent colectomy. These results demonstrate that epidemic *C. difficile* 027 strains are present in South America, highlighting the need for enhanced screening for this ribotype in other regions of the continent.

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Zoonotic Pathogens among White-Tailed Deer, Northern Mexico, 2004–2009

To the Editor: Intense wildlife management for hunting affects risks associated with zoonotic pathogens (*1*). White-tailed deer (*Odocoileus virginianus*) are increasingly managed by fencing, feeding, watering, and translocation to increase incomes from hunting in northern Mexico (*2*). These deer also play a major role in dissemination and reintroduction of pathogens and vectors from Mexico into the United States (*3*,*4*). White-tailed deer are suitable reservoir hosts for *Mycobacterium bovis* (*1*), and an *M. bovis*-positive white-tailed deer was recently found in Tamaulipas in northeastern Mexico (*2*). Brucellosis is widespread in many animal hosts in Latin America (*5*) and thus of interest in white-tailed deer. Another major zoonosis, sometimes linked to raw deer meat consumption, is hepatitis E, which is caused by genotypes of hepatitis E virus (HEV) (*6*). HEV is increasingly prevalent in red deer (*Cervus elaphus*) (*7*), but its prevalence in white-tailed deer is unknown.

The objective of this study was to determine the prevalence of zoonotic pathogens in white-tailed deer in northern Mexico. This study was conducted under a scientific collecting permit issued by the Mexican Division of Animal and Wildlife Health and on 8 ranches in 3 states in northern Mexico (≈26–28°N, 99–100°W).

Serum samples (n = 347) were collected during 2004–2009 in a cross-sectional survey for antibodies against HEV, *Brucella* spp., and mycobacteria. Deer were opportunistically sampled during live-capture operations as described by Cantú et al. (*8*). Bleeding was performed by using jugular venipuncture and vacuum tubes

without anticoagulant. Samples were allowed to clot and centrifuged to collect serum that was stored at –20°C.

Serum samples were tested for IgG against HEV by ELISA as described (*7*). Serum samples were also tested for antibodies against *Brucella* spp. by using a commercial ELISA (Ingezim Brucella Compac 2.0 Ingenasa, Madrid, Spain), according to the manufacturer's instructions. Detection of antibodies cross-reacting with 2 widely used mycobacterial antigens, bovine purified protein derivative (PPD) and paratuberculosis protoplasmatic antigen 3 (PPA3), was conducted as described (*9*). The sensitivity and specificity of this assay have not been established for white-tailed deer, but it has been used in seroprevalence studies of wild boar and fallow deer (*9*,*10*).

Insufficient volumes of serum samples prevented testing for antibodies against all pathogens (Table). Limited serum volume and lack of other (organ) samples also precluded additional analyses to verify presence of pathogens.

Prevalence was 62.7% (95% CI 54%–70%) for antibodies against HEV, 0.4% (95% CI 0%–2%) for antibodies against *Brucella* spp., 8.9% (95% CI 6%–13%) for antibodies against bovine PPD, and 2.6% (95% CI 1%–5%) for antibodies against PPA3 (Table). Antibody responses to bovine PPD were detected in deer from at 6 of 8 sampling sites; in deer from 3 of these sites, antibodies were also detected against PPA3 antigen. Seroprevalence against bovine PPD was higher than that against PPA3 (χ^2 10.9, df 1, $p<0.01$).

This cross-sectional survey of white-tailed deer in northern Mexico detected antibodies to several pathogens relevant to public and animal health. High prevalence of antibodies against HEV and frequent detection of antibodies against mycobacterial antigens are public health concerns. Prevalence of antibodies against HEV were 3× higher than that reported for red deer in Europe (*7*). This result suggests wide circulation of HEV in the study region and warrants further research, including detection and sequencing of virus RNA. Low *Brucella* spp. antibody prevalence confirms results of a study in this region (*8*).

Antibody responses to bovine PPD were detected in serum samples from deer from most sampling sites, occasionally in the absence of antibodies against PPA3. These results, and a recent report of an *M. bovis*–positive white-tailed deer from this region (*2*), suggest that these deer may be contracting *M. bovis* in northern Mexico. If one considers that white-tailed deer are *M. bovis* reservoirs in other parts of North America and that risk factors such as supplemental feeding are present in northern Mexico, there is a high risk for pathogen transmission to animals and humans (*1*). White-tailed deer are probably exposed to several pathogens that are relevant to animal health in northern Mexico.

Although this cross-sectional survey provided only an indication of pathogen prevalence in the study populations, high antibody prevalence to HEV and mycobacterial antigens requires antigen-targeted surveillance. Risks associated with pathogen translocation by white-tailed deer are also relevant to neighboring states in Mexico and the United States.

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Table. Prevalence of serum antibodies against zoonotic pathogen antigens among white-tailed deer on 8 ranches, northern Mexico, 2004–2009*

Ranch no.	State	Municipality	Sample size	No. positive/no. tested (%)			
				HEV	*Brucella* spp.	Bovine PPD	PPA3
1	Tamaulipas	Guerrero	1	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
2	Tamaulipas	Guerrero	106	41/71 (57.7)	0/102 (0)	9/102 (8.8)	0/102 (0)
3	Tamaulipas	Nuevo Laredo	37	21/27 (77.7)	0	0/37 (0)	0/37 (0)
4	Nuevo León	Unknown	35	0	0	3/35 (8.5)	2/35 (5.7)
5	Coahuila	Guerrero	35	1/1 (100)	0/33 (0)	2/33 (6.0)	1/33 (3.0)
6	Coahuila	Guerrero	50	6/17 (35.2)	0/40 (0)	3/40 (7.5)	0/40 (0)
7	Coahuila	Hidalgo	17	4/6 (66.6)	1/16 (6.2)	3/16 (18.7)	5/16 (31.2)
8	Coahuila	Guerrero	66	16/19 (84.2)	0/39 (0)	7/39 (18.0)	0/39 (0)
NA	Total	NA	347	89/142 (62.7)	1/231 (0.4)	27/303 (8.9)	8/303 (2.6)

*HEV, hepatitis E virus; PPD, purified protein derivative; PPA3, paratuberculosis protoplasmatic antigen 3; NA, not applicable.

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KIs Virus and Blood Donors, France

To the Editor: KIs-V is a new putative virus identified recently in the blood of persons in Japan (*1*). First partial sequence of KIs-V was characterized unexpectedly, when PCR primers were used that were directed primarily to the consensus domain of helicase of positive-stranded RNA viruses. Extensive physicochemical and molecular analysis suggested that KIs-V is an enveloped virus with a circular, double-stranded DNA genome of ≈9,500 bp (prototype isolate: GenBank accession no. AB550431); its genetic diversity is presumed to be extremely low because the 4 complete genomes already characterized in Japan harbor strict identical sequences. The 13 potential genes identified by in silico analysis exhibit an overall low sequence homology to other known viral proteins (*1*). Until now, KIs-V epidemiologic data have been related only to the original study in which the authors analyzed plasma samples from 516 blood donors categorized into 4 groups by alanine aminotransferase (ALT) level (either ≤60 IU/L or >60 IU/L) and the presence or absence of hepatitis E virus (HEV) antibodies. As a result, KIs-V DNA was detectable at elevated prevalence in the high ALT level/HEV antibody–positive group (36%, n = 100); viral loads, checked for a few samples, ranged from 10^6 to 10^8 copies/mL. KIs-V DNA also was identified in HEV antibody–negative samples, with low or high ALT level (≈0.8%, n = 120, and 1%, n = 100, respectively) (*1*).

To gain insights about the potential presence of this virus in the blood of persons in France, we investigated KIs-V DNA in the plasma of 576 healthy blood donors (mean age 40 years; 306 men; men:women 1:1.13). Blood samples were collected in vacuum tubes (Vacutainer, SST, Becton Dickinson, Meylan, France) and centrifuged, and plasma aliquots were stored at −80°C until use. Nucleic acids were extracted from 1-mL plasma volumes (MagNA Pure LC, Roche Diagnostics, Meylan, France) (*2*) and tested for KIs-V DNA by using the same nested PCR system for screening Japanese blood donors (*1*). Briefly, one tenth (5 μL) of extracted nucleic acids were first amplified by using primers 101-C (5′-CAACACCGCA ATCACAAAGT-3′) and N101-B (5′-AACATTGAAACGTCATGT CC-3′) (0.8 μM each) in a 50-μL mix containing deoxynucleotide triphosphates (0.2 mM each) (Roche) and 2 U Taq DNA polymerase (Invitrogen, Cergy Pontoise, France) with its corresponding buffer. One microliter of the amplification mixture was subsequently used in a second-round PCR with primers KS-2 (5′-CTCGTCCTCGTCGTCATC GTA-3′) and N101-D (5′-CA TTTGCTCCCGCTGGAGATG-3′) under the same conditions as above. The amplification conditions for first-

and second-round PCR were 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Expected amplification products were 458 bp (PCR-1) and 304 bp (PCR-2). Using dilutions of a synthetic template corresponding to the target sequence, we estimated the sensitivity of the amplification assay to be <5 copies of target sequence by limiting-dilution assay.

Negative (sterile water) and positive controls (synthetic template dilutions) were added systematically to each amplification run. A PCR control intended to check the quality of the nucleic acids extraction procedure was also performed systematically on 4 randomly selected samples of each batch (n = 32); this control was based on the detection of an extremely prevalent DNA virus (Torque Teno virus and related viruses, family *Anelloviridae*) by using a highly conserved amplification system (*3*).

Among the 576 plasma samples tested, no positive signal was identified for KIs-V DNA after agarose gel electrophoresis of PCR-1 and PCR-2. Amplification controls (negative, positive, anellovirus*e* DNA) confirmed the validity of these results.

Using the PCR detection system adopted by Satoh et al., combined with the extraction of large plasma volumes, we were not able to detect KIs-V DNA in the blood of donors tested, suggesting an uncommon frequency in healthy persons in France. Information related to HEV status or ALT levels were not available here because both parameters are not evaluated for routine blood donor screening in France; HEV seroprevalence studies involving blood donors from northern and southwestern France indicate discrepant results (≈3%–≈52%, IgG), possibly related to serologic assay performances and/or geographic differences (*4*). The precise identity of KIs-V remains uncertain, but according to its extensive initial characterization, complementary studies probably will confirm its viral origin. Molecular characterization of new full-length sequences will be needed to investigate the real genetic diversity of KIs-V and to help design optimized molecular detection systems.

The implication of KIs-V in human health remains under debate. The original publication highlighted the fact that HEV antibody–positive persons in Japan who had moderately elevated ALT levels at a prevalence of KIs-V infection that is non-negligible; such findings could suggest a link between the virus and liver dysfunctions. HEV and KIs-V also could share the same route of contamination, i.e., foods (*5*). Further investigations involving diverse human cohorts need to be undertaken to better understand the natural history of KIs-V in humans.

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Usefulness of School Absenteeism Data for Predicting Influenza Outbreaks, United States

To the Editor: School closure has been proposed as a strategy for slowing transmission of pandemic influenza (*1*). Studies of influenza A(H1N1)pdm 2009 (pH1N1) suggested that early and sustained school closure might effectively reduce communitywide influenza transmission (*2*,*3*). However, empirical evidence identifying the optimal timing of school closures to effectively reduce disease transmission after an outbreak occurs is limited.

That school absenteeism data improve school-based disease surveillance and response has been suggested (*4*–*6*). In 2009, Sasaki et al. demonstrated that the pattern of influenza-associated school absenteeism in the days before an influenza outbreak predicted the outbreak course with high sensitivity and specificity (*7*). However, that study used absenteeism data from Japan, which are generally not applicable to the United States, because most US absenteeism data

collected at the local level do not specify cause. Furthermore, few US jurisdictions collect electronic health data for students.

In New York City (NYC; New York, New York, USA), electronic health data are collected daily on ≈70%–80% of the total nurse visits in the city's public schools, kindergarten through grade 8, and on all-cause school absenteeism. Using these data, we adapted the algorithm developed by Sasaki et al. for use with all-cause absenteeism data from NYC schools and validated our findings by using the daily count of school nurse visits for fever/influenza over the same period (*7*). To reduce variance, we aggregated absenteeism data for September 6, 2005, through June 26, 2009, for 1,206 public schools in NYC at the school day and school district levels. A negative binomial regression model was then fit to these data, adjusting for day of week, whether the preceding day was a holiday, school type (elementary, middle), school day (linear term), and sine and cosine terms to account for seasonality. This modeling approach was used to standardize the outcomes across school districts and to further reduce variance caused by factors unassociated with influenza transmission.

A similar regression model was also fit to the daily school district–level count of school nurse visits for fever/influenza syndrome over the same period. However, for this model, seasonal influenza periods, determined by virus isolate data, were censored before modeling.

Residuals of both models were then used to calculate school district–specific z-scores for each day from September 25, 2006, through June 26, 2009, by dividing the model residual by the school district–specific standard deviation of the outcome. To determine the threshold and pattern in lagged days that best predicts an outbreak of absenteeism and fever/influenza syndrome, we applied the Sasaki et al. algorithm to the absenteeism z-score time series. We calculated receiver operating characteristic (ROC) curves by observing whether z-score thresholds of 1, 1.5, 2, or 2.5 reached either 1, 2, or 3 days in a row, were followed by an influenza outbreak in the same school district in the next 7 days. An influenza outbreak was indicated by a z-score of at least 3 (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/11-1538-Techapp.pdf).

Results revealed a moderately positive in-phase correlation between absenteeism and fever/influenza syndrome by school district during the pH1N1 period (r = 0.264) but a weak correlation over the entire study period (September 6, 2005–June 26, 2009) (r = 0.086). When data were aggregated across the city, the correlation between absenteeism and fever/influenza z-scores during the pH1N1 period and the entire study period increased to 0.304 and 0.210, respectively. When estimating a cross-correlation function to the citywide

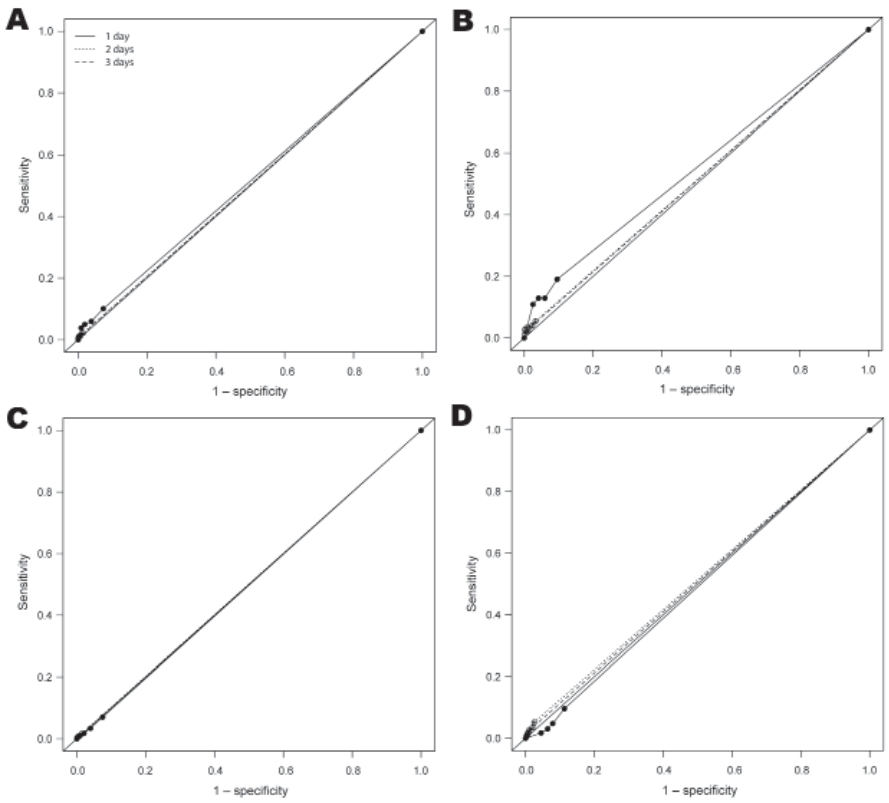

Figure. Receiver operating characteristic (ROC) curves showing A) predictive ability of school absenteeism to detect an outbreak (z-score ≥3) of fever/influenza for the entire study period; B) fever/influenza for the pandemic (H1N1) 2009 period; C) absenteeism for the entire study period; and D) absenteeism for the pandemic (H1N1) 2009 period. ROC curves were based on observations of whether 4 absentee threshold z-score levels—1, 1.5, 2, or 2.5—were reached or exceeded for either 1 day, 2 consecutive days, or 3 consecutive days as a predictor of the school district outbreak status during the next 7 days. In the ROC curves, sensitivity on the y-axis indicates the true-positive rate, and 1– specificity on the x-axis indicates the false-positive rate. The study was conducted September 6, 2005–June 26, 2009, in New York City, New York, USA.

data, the absenteeism time series correlated most strongly with the fever/influenza syndrome time series at a 2-day lag (pH1N1 period, r = 0.550; entire study period, r = 0.213), indicating that changes in absenteeism were most strongly correlated with changes in fever/influenza syndrome visits 2 days earlier.

The ROC curves illustrate the limited ability of absenteeism and fever/influenza visit patterns to predict absenteeism and fever/influenza outbreaks (Figure). The ROC curves also show that absenteeism in the week before an outbreak has little ability to predict an outbreak of either fever/influenza syndrome or absenteeism during the entire study period or during a period of pandemic influenza.

Thus, non–disease-specific absenteeism data alone are of little use for school-based influenza surveillance. Use of all-cause absenteeism data cannot inform influenza mitigation policies, such as school dismissal, at the school or the school district levels. Not surprisingly, the influenza-specific absenteeism data from Japan were better able to predict an influenza outbreak than were our data because our data were not influenza specific. Other factors specific to the school system in Japan might have also played a role.

In the future, it might be beneficial for schools to collect causes of absenteeism, particularly if is it not feasible to electronically collect data on school nurse visits. Creation of school-based early warning systems for pandemic influenza remains a priority. In NYC, efforts to improve emergency department and primary care electronic medical record systems have been successful (*8–10*). Similar efforts to improve electronic health data collection and influenza-related absenteeism data in schools might yet demonstrate the usefulness of school-based surveillance systems.

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Letters

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

Rhodococcus erythropolis Encephalitis in Patient Receiving Rituximab

To the Editor: *Rhodococcus* spp. infections occur predominantly in immunocompromised patients, and most infections are caused by *Rhodococcus equi* (*1*). Seven cases of *R. erythropolis* infections in humans have been described (*2*–*8*). None of these cases included a central nervous system infection (Table). We report a case of *R. erythropolis* meningoencephalitis in a patient with rheumatoid arthritis who was treated with rituximab and methotrexate.

In September 2010, a 44-year-old woman in Amarillo, Texas, USA, with rheumatoid arthritis who was being treated with rituximab and methothrexate was hospitalized with a 5-day history of fever, headache, and confusion. Physical examination showed that the patient was febrile (38.3°C) and drowsy. Results of a computed tomography scan of the brain were normal. Cerebrospinal fluid (CSF) obtained by lumbar puncture showed a leukocyte count of 112 cells/mm^3 (28% neutrophils, 56% lymphocytes) and glucose and total protein concentrations of 64 mg/dL and 81 mg/dL, respectively.

At admission, the patient was given a diagnosis of meningoencephalitis and treated with vancomycin, meropenem, ampicillin, and acyclovir. Magnetic resonance imaging of the brain showed diffuse cortical increased signal on T2-weighted imaging and confluent, near-symmetric T2 signal hyperintensities in the thalami extending into the brain stem that demonstrated mild post contrast enhancement and no evidence of restricted diffusion. This finding was believed to be suggestive of viral encephalitis.

The mental status of the patient deteriorated and she became profoundly weak but had preserved reflexes. She had no movement in her lower extremities and only limited movements in her upper extremities. She was transferred to the intensive care unit for intubation and mechanical ventilation.

Routine CSF bacterial and viral cultures were negative. Serologic results for cryptococcal antigen and West Nile virus in CSF were negative. PCR results for herpes simplex virus, cytomegalovirus, and enteroviruses in CSF were negative.

The patient became afebrile and showed slow but limited improvement. She became more alert and occasionally raised her right index finger on command. After CSF bacterial cultures were reported negative, antimicrobial drugs were discontinued. Five days later, while the patient was still receiving mechanical ventilation, fever relapsed, prompting treatment and tests for a hospital-acquired infection.

A repeat lumbar puncture was conducted 10 days after admission. Opening pressure was 340 mm H_2O. The leukocyte count was 167 cells/mm^3 (2% monocytes, 98% lymphocytes), and glucose and total protein concentrations were 51 mg/dL and 103 mg/dL, respectively. CSF from the repeat lumbar puncture was used for routine bacterial culture but not mycobacterial culture. There were no obvious infection foci and antimicrobial drugs were discontinued.

On the 18th day of hospitalization, the patient became unresponsive and had fixed dilated pupils. Computed tomography scan of the brain showed obstructive hydrocephalus and cerebellar herniation. The patient died 1 day later. During the third week of incubation, gram-positive rods grew in mycobacterial broth medium and were subsequently identified as *R. erythropolis*.

Species of the genus *Rhodococcus* (order Actinomycetales, family *Nocardiaceae*) are aerobic, gram-positive, partially acid-fast, coccoid to rod-shaped bacteria (*9*). They have been isolated from a variety of sources (*6*). *Rhodococcus* spp. are generally considered to have low virulence (*7*). Most documented human infections with *Rhodococcus* spp. have been caused by *R. equi*, and pneumonia is the most commonly described condition (*7*).

R. erythropolis is typically found in soil and has been detected on the surface of the healthy human eye, but there are no reports of its presence at other sites in humans (*7*). *R. erythropolis* colonies are typically rough and orange to red; thus, the name *erythropolis*, which means red city (*8*).

Immunosuppression is a major risk factor in the pathogenesis of *Rhodococcus* spp. infections (*10*). The patient had been treated with rituximab and methotrexate for 2 years. Multiple infectious complications have been

Table. Characteristics of 7 patients infected with *Rhodococcus erythropolis*

Patient no.	Age, y/sex	Cconcurrent condition	Characteristic	Reference
1	63/M		Peritoneal dialysis, catheter-related peritonitis	(*2*)
2	44/F		Exacerbation of bronchiectasis	(*3*)
3	24/M	HIV/AIDS	Disseminated skin infection	(*4*)
4	82/F		Chronic endophthalmitis after lens implantation	(*5*)
5	53/F		Osteomyelitis after first metatarsophalangeal joint arthrodesis	(*6*)
6	6/M	Acute lymphocytic leukemia	Catheter-related bloodstream infection	(*7*)
7	79/M	Esophageal cancer	Catheter-related bloodstream infection	(*8*)

described for each of these drugs, but only 1 case of *Rhodococcus* infection has been reported for a patient receiving methotrexate and none have been described for patients receiving rituximab (*10*).

Because of difficulties in species identification and delays in growth, non–*R. equi* infections might be underdiagnosed (*9*). This finding is complicated by the fact that these gram-positive bacilli may be misidentified as contaminating diphtheroids (*9*). It is unlikely that this organism was a contaminant, given that the fever in the patient relapsed after antimicrobial drugs were discontinued and no other cause was identified. The isolate from the patient was identified by sequencing the first ≈500 bp of the 16S rRNA gene, which is a useful molecular technique for speciation of the genus *Rhodococcus* (*9*). The isolate showed 99.8% identity with *R. erythropolis* type strain DSM 43066.

Antimicrobial drug susceptibility patterns in non–*R. equi* rhodococci have not been studied, and there are no established standards for treating patients with *Rhodococcus* spp. infections. This case, along with previously reported cases, represents emergence of an opportunistic pathogen in a rapidly increasing patient population, namely those with impaired local or systemic immunity.

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Factors Influencing Emergence of Tularemia, Hungary, 1984–2010

To the Editor: *Francisella tularensis*, the etiologic agent of tularemia, is a highly infectious zoonotic agent. *F. tularensis* subsp. *holarctica* (type B) is found throughout the Northern Hemisphere and is the only subspecies found in Europe (*1*). Lagomorphs and rodents probably serve as the primary mammalian reservoir hosts, and hematophagous arthropods, such as ticks, play a role as vectors and hosts (*2*,*3*). Although *F. tularensis* is a potential agent of biological warfare and several emergences and reemergences of tularemia have been reported around the world (*1*,*4*), the epizootiology of the disease is only partially understood. The aim of our study was to analyze factors that influence the emergence of tularemia in Hungary.

The study area (15,475 km^2) included 3 counties in eastern Hungary. The analyzed data represented a period of 25 years, March 1984–February 2010. Annual *F. tularensis*–specific seroprevalence data for the European brown hare (*Lepus europaeus*) population were obtained by slide agglutination testing during the winter (December and January) screening of 2,500–25,000 animals (online Technical Appendix, wwwnc.cdc.gov/EID/article/18/8/11-1826-Techapp.xls). Population density data (animals/km^2) for hares were based on February line transect counts and were obtained from the Hungarian Game Management database (www.vvt.gau.hu/vadgazdalkodasi_statisztikak.htm). Common vole (*Microtus arvalis*) densities (calculated from the number of active burrows/hectare during November) for 1996–2010 were obtained from the Central Agriculture Office, Budapest, Hungary. Vole

density was scaled from 0 (absent) to 10 (peak population). The annual number of tularemia cases in humans (based on clinical history and tube agglutination test results) was obtained from the National Center for Epidemiology, Budapest.

The data were regrouped according to the yearly biologic cycle (March–February) for hares and voles (Figure), and relationships between these yearly data were quantified by using the Spearman rank correlation coefficient (*5*) at county and regional levels. A 2–3 year cycle was characteristic for the analyzed data. A significant positive correlation was found among the number of tularemia cases in humans and the seroprevalence of *F. tularensis* among European brown hares (Spearman $\rho = 0.73$; $p<0.0001$) and the population density of common voles (Spearman $\rho = 0.77$; $p = 0.0081$). A significant negative correlation was found between the population density of hares and the seroprevalence of *F. tularensis* in hares (Spearman $\rho = –0.41$; $p = 0.0365$).

The comprehensive and long-term annual data used in this study provide clues regarding the factors shaping the intraannual epizootiology and emergence or reemergence of tularemia. The European brown hare is moderately sensitive to *F. tularensis* subsp. *holarctica*. The hares produce a heterogeneous response to infection, which means that some die of overwhelming bacteremia and others survive with a protracted course of infection, thereby contributing to the maintenance of tularemia over longer periods and serving as useful sentinels of disease activity. Other studies have concluded that hares, together with infected ticks, may serve as disease reservoirs between epizootics (*2*,*3*,*6*,*7*).

However, we instead hypothesize that the 2–3 year cycling feature of the population dynamics for the common vole (*2*) determines the ecology of *F. tularensis* subsp. *holarctica* in eastern Hungary. The common vole is highly susceptible to *F. tularensis* subsp. *holarctica* (*3*,*8*). When population densities among voles are high, *F. tularensis* disease transmission and spillover to hares may be facilitated by stress-related aggression, cannibalism, and *F. tularensis* contamination of the environment by infectious body discharges (*2*). Enhanced transmission and spillover can expand local outbreaks to epizootic proportions, and infected hares may, in turn, further enhance the spread of disease through bacterial shedding in urine (*6*,*7*).

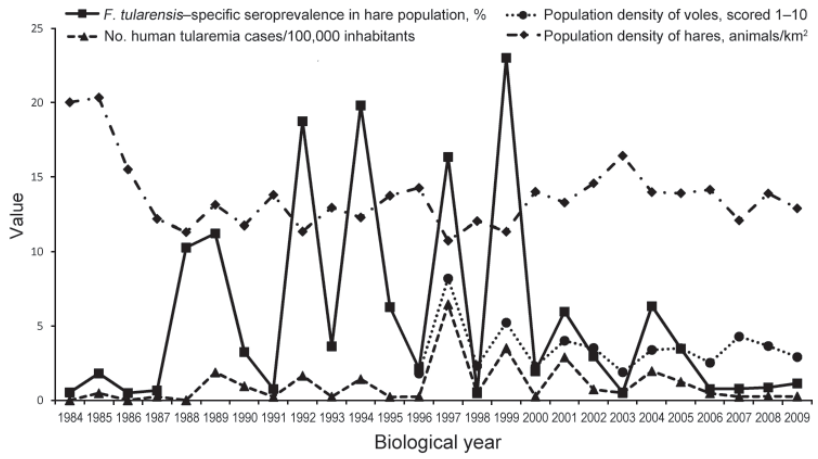

Figure. Correlation between the seroprevalence of *Francisella tularensis* in the European brown hare (*Lepus europaeus*) population, the population density of European brown hares and common voles (*Microtus arvalis*), and the number of tularemia cases in humans eastern Hungary, 1984–2010. Values were determined on the basis of biological years (March–February). Median values from the records of 3 counties were used for analysis.

The disease in hares often results in septicemia and death (*7*), thus decreasing the population density of these animals. Hares and especially voles are also hosts for different stages of several tick species (*2*,*6*), so it can be expected, that higher numbers of infected rodents and lagomorphs result in an increased proportion of infected ticks and, thus, increased transmission of *F. tularensis* subsp. *holarctica*. It can be concluded that a higher number of infection sources in the environment results in elevated numbers of cases in humans, mainly through the handling and skinning of hares, but also through tick bites and, potentially, the inhalation of infectious aerosols originating from, for example, hay or grain.

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Klebsiella pneumoniae Carbapenemase-producing Enterobacteria in Hospital, Singapore

To the Editor: During the past decade, enterobacteria that produce *Klebsiella pneumoniae* carbapenemase (KPC) have become established in the United States and countries in South America and Europe (*1*). In Asia, KPC was reported in the People's Republic of China in 2007 (*2*) and subsequently in South Korea (*3*) and Taiwan (*4*). Public health agencies emphasize screening and strict contact precautions to control multidrug resistant *Enterobacteriaceae* (*5*). Routine testing for mechanisms of resistance facilitates detection of emerging carbapenem-resistant *Enterobacteriaceae*.

In Singapore's 1,000-bed National University Hospital during November 2010–January 2011, we identified New Delhi metallo-β-lactamase 1–producing *Enterobacteriaceae* in 2 clinical specimens but none that produced KPC (I. Venkatachalam et al., unpub. data). We conducted a laboratory screening study to determine the prevalence and nature of carbapenem-resistant *Enterobacteriaceae* in April 2011. Ethics committee approval was waived for this study.

Testing of rectal swab samples is part of an established hospitalwide program for vancomycin-resistant enterococci screening. Using a scoring system to identify patients at high risk for vancomycin-resistant enterococci (*6*), we found that ≈2.5 specimens per 100 admissions were attained each month. During our study, we also tested these samples for carbapenemase-producing *Enterobacteriaceae*.

During April–June 2011, we incubated specimens for 24 h in 10 mL tryptic soy broth containing 1 mg/L imipenem, then streaked 100 μL of the broth onto CHROMagar KPC (CHROMagar, Paris, France). Colonies detected after 24 h incubation at 35°C were identified by using MALDI-TOF MS with a Microflex LT instrument (Bruker Daltonik GmbH, Leipzig, Germany). Imipenem and meropenem MICs for *Enterobacteriacaeae* were confirmed by using Etests (bioMérieux, Marcy l'Etoile, France). Isolates with MIC ≥2 μg/mL underwent analysis with Metallo-β-Lactamase Confirmative Identification Pack (Rosco Diagnostica, Taastrup, Denmark) and Etest MBL (bioMérieux) for metallo-β-lactamase production. Isolates suspected to be producers were genotypically confirmed by PCR.

Of the 201 nonduplicate samples processed, 79 microorganisms exhibited imipenem resistance and were isolated on CHROMagar KPC (Table). Among *Enterobacteriaceae*, carbapenem MIC ≥2 μg/mL was present in 1 *E. aerogenes*, 2 *E. cloacae*, and 4 *K. pneumoniae* isolates. One isolate (*K. pneumoniae*) had a positive combined disk test result with a pattern suggestive of serine carbapenemase production.

We analyzed genomic DNA (DNeasy Blood and Tissue Kit, QIAGEN, Hilden, Germany) from this isolate by using PCR for transmissible carbapenem resistance markers: metallo-β-lactamases (VIM, IMP, and KHM-1), serine carbapenemases (KPC, GES1–5 and 7), and OXA-48. bla_{KPC}-specific primers (for-ward primer 5′-CGTTGACGCCCAATCC-3′; reverse primer 5′-ACCGCTGGCAGCTGG-3′) generated a 390-bp amplicon. Full gene sequencing of bla_{KPC} (forward primer 5′-ATGTCACTGTATCGCCGTCT-3′; reverse primer 5′-CCTAAATGTGACAGTGGTTGG) revealed 100% homology to bla_{KPC-2} (GenBank accession no. FJ628167.2). Further analysis showed that the isolate carried extended-

spectrum β-lactamase (bla_{TEM-1}, bla_{SHV-11}, $bla_{CTX-M-15}$), plasmid-located AmpC (bla_{DHA-1}), and 16S rRNA methylase *armA* genes but was negative for bla_{CMY}, bla_{OXA}, bla_{GES}, metallo-β-lactamases, and plasmid-mediated quinolone (*qnr*) genes. Multilocus sequence typing conducted at Institut Pasteur (Paris, France), identified this isolate as sequence type 11. It was susceptible only to colistin and tigecycline.

Sequence type 11, a single-locus variant of the internationally dominant sequence type 258 clone (*7*), is present in 64.2% of KPC-producing *K. pneumoniae* in China (*8*). In South Korea, sequence type 11 is the most common clone of extended-spectrum β-lactamase–producing *K. pneumoniae* isolates (*3*).

The KPC-producing *K. pneumoniae* originated from a woman in the local community, 89 years of age, who had severe ischemic cardiomyopathy and atrial fibrillation. She was discharged home after a 3-day hospitalization for treatment of stroke in January 2011. During May 2011, she was readmitted after a severe stroke. During week 4, she was transferred to a subacute care hospital but readmitted within 24 hours with a lower respiratory tract infection. A rectal swab sample was collected for routine screening for vancomycin-resistant enterococci. We empirically prescribed a 10-day course of piperacillin-tazobactam. On day 10 of treatment, KPC-producing *K. pneumoniae* was isolated from the rectal specimen. The patient responded to treatment and was discharged to a long-term care facility.

This case demonstrates concerns about a KPC of local community origin because no other KPC-producing *Enterobacteriaceae* were isolated during this inpatient surveillance and the patient had neither received antimicrobial drugs nor traveled in the 6 months before her May admission (*7*). However, she was admitted 3 weeks before sampling; an unidentified hospital source remains a possibility. Of added concern is the potential for dissemination within the facility to which she was discharged.

Resistance to third-generation cephalosporins was reported for 20% of *Escherichia coli*, 32.3% of *K. pneumoniae*, 46.2% of *Acinetobacter* spp., and 7.5% of *Pseudomonas aeruginosa* clinical isolates at 4 major Singapore hospitals during January 2006–December 2008 (*9*). Authors reported positive correlation between meropenem administration and carbapenem resistance development in *Acinetobacter* spp. blood isolates.

When the resistance mechanism to an antimicrobial drug is embedded in highly mobile elements like plasmids, widespread dissemination is possible. Although acute care hospitals are conducive to development of antimicrobial drug resistance, long-term care facilities facilitate spread of these organisms (*10*). Infection control interventions including routine screening for mechanisms of resistance and responsible use of antimicrobial drugs are increasingly critical in hospitals and long-term care facilities; a response plan coordinated between these facilities is needed.

Table. Bacteria isolated on CHROMagar in screening for carbapenemase-producing *Enterobacteriaceae*, Singapore, 2011*

Organism	No. isolated
Enterobacteriaceae	
Klebsiella pneumoniae	11
Enterobacter cloacae	3
Enterobacter aerogenes	2
Proteus mirabilis	2
Escherichia coli	1
Serratia marcescens	1
Non-*Enterobacteriaceae*: gram-negative nonfermenters	
Pseudomonas aeruginosa	20
Acinetobacter baumannii	17
Stenotrophomonas maltophilia	13
Elizabethkingia meningoseptica	3
Wautersiella falsenii	1
Enterococci	
Enterococcus gallinarum	4
Enterococcus faecalis	1

*CHROMagar, Paris, France.

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bla_{NDM-1}–positive *Klebsiella pneumoniae* from Environment, Vietnam

To the Editor: The bla_{NDM-1} gene, which produces the New Delhi metallo-β-lactamase (NDM-1) enzyme, confers resistance to the carbapenem class of antimicrobial drugs and can be transferred among different types of bacteria. NDM-1 was identified in 2008 in Sweden from a patient from India who had been hospitalized in New Delhi (*1*). Since that report, bla_{NDM-1}–positive bacteria have been identified from patients in several countries; most of these patients had a direct link with the Indian subcontinent (*2*). The spread of bla_{NDM-1} among bacterial pathogens is of concern not only because of resistance to carbapenems but also because such pathogens typically are resistant to multiple antimicrobial drug classes, which leaves few treatment choices available (*3*–*5*). In 2011, spread of bla_{NDM-1}–positive bacteria in an environmental setting in New Delhi was reported (*6*).

The possible appearance of bacteria harboring bla_{NDM-1} in Vietnam is of concern because cultural and economic links between Vietnam and India are strongly established, including extensive person-to-person exchanges that could enable easy exchange of pathogens. In addition, Vietnam faces a serious problem of antimicrobial drug resistance because drugs are freely available and used in an indiscriminate fashion. Thus, once bla_{NDM-1}–positive bacteria colonize persons in Vietnam, they would be able to spread easily and pose a serious public health threat.

During September 2011, we collected paired swab samples (1 for PCR, 1 for culture) of seepage water from 20 sites (rivers, lakes, and water pools in streets) within a 10-km radius of central Hanoi, Vietnam. Samples were transported in Transystem (COPAN Italia S.p.A, Brescia, Italy) to preserve bacteria and DNA. The 20 PCR swab specimens were squeezed out into 0.5-mL volumes of sterile water and centrifuged at 3,000 × *g* for 30 seconds; 1 μL of the resulting suspension was then used as PCR template to detect bla_{NDM-1} as described (*7*). Two samples were positive for bla_{NDM-1}; these 2 samples were collected from the same river (Kim Nguu River) but at sites 3 km apart.

To isolate and identify the phenotype and genotype of bla_{NDM-1}–positive bacteria, we repeatedly spread the 20 culture swab specimens onto Muller-Hinton agar (Nissui, Tokyo, Japan) containing 100 mg/L vancomycin (Nakalai, Kyoto, Japan) plus 0.5 mg/L meropenem (LKT Laboratories, St. Paul, MN, USA) until single colonies were obtained. Each colony was then subcultured by plating onto MacConkey agar (Nihon Seiyaku, Tokyo, Japan) containing 0.5 mg/L meropenem to ensure culture purity; colonies were identified by using API 20E strips (bioMérieux, Basingstoke, UK). MICs of these isolates for 13 antimicrobial drugs were calculated by using Etest (bioMérieux), and susceptibility data were interpreted by using Clinical and Laboratory Standards Institute guidelines (www.clsi.org).

We harvested several species of bacteria from the 2 seepage samples positive for bla_{NDM-1}: *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *P. fluorescens/putida*, and *P. luteola*. These isolates were placed onto media containing 0.5 mg/L meropenem, and bacterial DNA was extracted and used for the template for PCR analysis to detect bla_{NDM-1} as described (*7*). bla_{NDM-1} was detected in 3 *K. pneumoniae* isolates from each of the 2 positive samples (6 isolates total); this result was confirmed by sequencing. All 6 isolates were highly resistant to all β-lactam antimicrobial drugs, including carbapenems (Table). To

Table. Resistance to 13 antimicrobial drugs of bla_{NDM-1}–positive *Klebsiella pneumoniae* isolates from the Kim Nguu River, Hanoi, Vietnam*

Antimicrobial drug	MIC, mg/L	
	Site X	Site Y
Piperacillin/tazobactam	64–>256	64–>256
Cefotaxime	48–>256	96–128
Ceftazidime	>256	>256
Ceftriaxone	96–>256	128–>256
Meropenem	8–>32	12–>32
Doripenem	4–>32	8–>32
Imipenem	6–>32	>32
Fosfomycin	3–8	8
Gentamicin	>1,024	>1,024
Tobramycin	384–>1,024	256–384
Ciprofloxacin	0.064–1.5	0.064
Colistin	0.19–2	0.125–0.38
Tgecycline	1.5–3	0.5–1.5

*MICs were interpreted by using Clinical and Laboratory Standards Institute guidelines (www.clsi.org).

detect another β-lactamase, multiplex PCRs were carried out as described (*8*); genetic variants bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, bla_{IMP}, bla_{VIM}, and bla_{KPC} were not detected in any of the isolates other than *K. pneumoniae*. All 6 *K. pneumoniae* isolates were positive for bla_{TEM} and bla_{CTX-M} variants by PCR; these variants were confirmed as bla_{TEM-1} and $bla_{CTX-M-3}$ by sequencing.

Aminoglycosides are often used in the management of severe infectious diseases caused by gram-negative pathogens. 16S rRNA methylases were found to confer high levels of resistance to aminoglycosides such as amikacin, tobramycin, and gentamicin. The 6 *K. pneumoniae* isolates we found were highly resistant to gentamicin (MIC >1,024 mg/L) and tobramycin (MIC 256–>1,024 mg/L) (Table). Therefore, we screened genetic elements of 16S rRNA methylases (*rmtB*, *rmtC*, and *armA*) by PCR and detected *rmtB* in all 6 isolates (*9*). Multilocus sequence typing was applied for these 6 isolates; all were identified as *K. pneumoniae* sequence type 283 (*10*), which had not been reported as harboring bla_{NDM-1}. The azide-resistant *Escherichia coli* strain J53 has been used as recipient for conjugation assay, which had been reported previously (*6*), but we found no transconjugant strain with bla_{NDM-1} on MacConkey agar containing 100 mg/L sodium azide and 0.5 mg/L meropenem.

Our results show that bla_{NDM-1}–positive *K. pneumoniae* sequence type 283 is present in the Kim Nguu River, which flows through the central part of Hanoi at 2 sites. The isolates we obtained were also positive for 2 other β-lactamases, bla_{TEM-1} and $bla_{CTX-M-3}$, were highly resistant to aminoglycosides related to *rmtB*, and showed mild elevation of MIC against ciprofloxacin up to 1.5 mg/L. Wide-scale surveillance of environmental and clinical samples in Vietnam and establishment of a strategy to prevent further spread of bla_{NDM-1} are urgently needed.

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Rickettsia felis in Fleas, Southern Ethiopia, 2010

To the Editor: Fleas (order Siphonaptera) are obligate hematophagous insects. They are laterally flattened, holometabolous, and wingless ectoparasites. More than 2,500 species of flea, belonging to 16 families and 238 genera, have been described. A minority of these genera live in close association with humans (synanthropic), including fleas of these species: *Pulex irritans*, *Ctenocephalides felis*, *Ctenocephalides canis*, *Xenopsylla cheopis*, *Nosopsyllus fasciatus*, *Echidnophaga gallinacea*, and *Tunga penetrans* (*1*). Many fleas are capable of transmitting the following pathogens to their hosts: bacteria (e.g., *Rickettsia typhi, R. felis, Yersinia pestis,* and many *Bartonella* spp.); viruses (e.g., myxoma virus); protozoa (e.g., *Trypanosoma* spp.); or helminths (e.g., *Hymenolepis* spp.) (*2*). *Ctenocephalides* spp. fleas are of special interest as main reservoirs and vectors of *R. felis*, because this agent causes an emerging disease, fleaborne rickettsiosis. The distribution and prevalence of this disease have not been well studied. Symptoms of this disease range from mild to moderate and include fever, cutaneous rash, and sometimes an inoculation eschar (*3*,*4*). *R. felis* can also infect at least 10 other species of arthropods, including *P. irritans* fleas, trombiculid and mesostygmata mites, hard and soft ticks, and booklice (*5*,*6*).

In Africa, the presence of *R. felis* in fleas has been documented in Algeria, Tunisia, Egypt, Ethiopia, Gabon, Côte d'Ivoire, and the Democratic Republic of Congo (*5*). Recent studies conducted in Senegal (*3*) and Kenya (*4*) have shown that as much as 4.4% and 3.7%, respectively, of acute febrile diseases in these regions may be caused by *R. felis* infections. We conducted a study to determine the distribution and prevalence of *R. felis* in fleas in Ethiopia.

In our study, 55 fleas were collected in 2010 in 2 villages in Ethiopia; 25 fleas were collected from Tikemit Eshet (6°51′837″N and 35°51′348″E; altitude2,121 m), and 30 fleas were collected from Mizan Teferi (6°59′640″N and 35°35′507″E; altitude 1,700 m). The specimens were collected by using a plate filled with soapy water with a candle in the middle of the plate. Because fleas are thermotropic, they jumped toward the candle and fell onto the plate, where they rapidly drowned in the soapy water. The fleas were identified by morphologic features and stored in 90% ethanol until DNA extraction.

To confirm the phenotypic identification, we designed primers and probes for quantitative real-time PCR (qPCR) that were specific for 2 species of flea (*P. irritans* and *C. felis*) based on the sequences of mitochondrial cytochrome oxidase gene published in GenBank (Table). All of the identifications made by morphologic appearance were confirmed by qPCR because some specimens were damaged and difficult to identify. We found that most (52/55) of the fleas collected in human dwellings were *P. irritans*, and 3 specimens were *C. felis*. A screening by amplification using primers and probes specific for the 16S–23S internal transcribed spacer of *Bartonella* spp. (*7*) produced no positive results.

We screened rickettsial DNA by using qPCR with a *Rickettsia*-specific, *gltA* gene-based RKND03 system (*8*) and a *bioB*-based qPCR system specific for *R. felis*. We found that the 3 specimens of *C. felis* fleas contained the DNA of *R. felis*; however, 23 (43%) of 53 *P. irritans* specimens also contained DNA of *R. felis*. We amplified and sequenced nearly the entire rickettsial *gltA* gene from 3 *C. felis* and 10 *P. irritans* specimens and found that the sequence was identical to that of *R. felis* (GenBank accession no. NC_007111).

During the field collection of the fleas, the conservation of specimens may be difficult. Degradation of specimens may pose a problem for the ensuing morphologic identification. For fleas, a specific preparation is required that destroys internal organs and produces a chitin complex of the insect. This type of preparation makes it difficult, and sometimes impossible, to use the insect later for molecular studies. The development of qPCR specific for *P. irritans* and *C. felis* fleas facilitated the identification of damaged samples and also precluded the laborious and time-consuming procedure of identification by morphologic features.

We conclude that the reservoirs of *R. felis* in Ethiopia include both *C. felis* and *P. irritans* fleas. In Ethiopia, *P. irritans* fleas have been reported to be prevalent (*9*). *P. irritans* fleas have been shown to be infected by *R. felis* in several locations, notably in the Democratic Republic of the

Table. Sequences of primers and probes used to identify fleas by quantitative real-time PCR, southern Ethiopia, 2010

Species	Forward primer, 3′→5′	Reverse primer, 3′→5′	Fluorescent probe
P. irritans	CGAATACTTTTAGAAAGCCAAAACA	CATTGATGACCAATAGATTTTAGAGTG	TTGCTTTACCGTCTTTACGTTT
C. felis	TCGTTATTTACTTGAAAGACAAAATG	TCATTGATGACCAATTGCTTT	TGCTTTACCTTCTCTTCGACTTTT

**P., Pulex; C., Ctenocephalides.*

Congo and in the United States, and another rickettsia phylogenetically similar to *R. felis* has been detected in *P. irritans* fleas in Hungary (*10*). Reports attributing substantial numbers of acute febrile illnesses to fleaborne rickettsiosis caused by *R. felis* in Senegal and Kenya (*3*,*4*) place fleaborne rickettsiosis among emerging diseases with the potential for adverse public health effects. Furthermore, the identification of the vectors of *R. felis* in Ethiopia reveals the epidemiologic background for the fleaborne spotted fever in this region. We speculate that the elucidation of the full range of possible vectors of *R. felis* may facilitate the development of prevention measures that will help control this disease.

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Identification of Cause of Posttransplant Cachexia by PCR

To the Editor: A man, 56 years of age, was admitted to the hospital for epigastric pain, fever, and fatigue 8 years after a cardiac transplant. His immunosuppressive regimen consisted of cyclosporine A, mycophenolate mofetil, and steroids. Clinical examination revealed a 4-kg weight loss within 3 months without peripheral lymph node enlargement.

Laboratory test results showed moderate anemia, severe lymphopenia, and moderately increased C-reactive protein. Serologic results for HIV, *Brucella* spp., *Coxiella burnetii*, and *Francisella tularensis* were negative. Whole-body computed tomography scanning showed enlarged mediastinal and abdominal lymph nodes. Bone marrow histopathologic results ruled out lymphoma or granuloma but showed a histiocytic infiltrate and intracellular acid-fast bacilli (AFB) with positive Ziehl–Neelsen staining. Sputum, urine, gastric aspirates, and bronchoalveolar lavage specimens revealed no AFB. A mediastinal lymph node biopsy showed few AFB, suggesting *M. tuberculosis* or nontuberculous mycobacteria. Isoniazid, rifampin, ethambutol, and clarithromycin were prescribed for 2 months, followed by rifampin, ethambutol, and clarithromycin. Cultures for mycobacteria remained negative.

Five months after treatment initiation, the patient experienced severe abdominal pain, diarrhea, and continued weight loss. Lymph node biopsy was repeated; results showed intramacrophagic coccobacilli tinted with Ziehl-Neelsen, Gram, and periodic acid–Schiff (PAS) stains. Two diagnoses were considered: malakoplakia and Whipple disease (WD). Screening results from quantitative real-time PCR (qPCR) for *Tropheryma whipplei* were negative for blood, saliva, stools, urine, and lymph nodes.

Although no characteristic Michaelis–Gutmann bodies were seen, the staining characteristics of the intracellular coccobacilli were compatible with *Rhodococcus equi*, a pathogen associated with malakoplakia. Combined treatment with ertapenem, teicoplanin, and amikacin was implemented but failed to induce clinical improvement. Culture of the biopsy specimen failed to grow *R. equi* or mycobacteria, and

the result of 16S rRNA PCR was negative. To investigate the cause of the diarrhea, the patient underwent endoscopy, which showed a thickened duodenal wall. A duodenal biopsy specimen displayed a massive histiocytic infiltrate, with positive PAS and Gram staining but negative Ziehl-Neelsen staining. Cultures remained negative for mycobacteria.

Acting on the hypothesis of WD, we administered doxycycline and hydroxychloroquine for 4 weeks, then discontinued for ineffectiveness. Four weeks after cessation of antimicrobial drug treatment, a third lymph node biopsy was performed, in which the *T. whipplei* PCR result was positive. Antibacterial drug treatment for WD was resumed, but the patient's condition worsened.

Simultaneously, extracted DNA and fresh tissue of all biopsy specimens were sent to the Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, (Marseille, France), a reference laboratory for WD. Immunohistochemical analysis, DNA extraction, and *T. whipplei* qPCR were performed as described (*1*,*2*). Biopsy specimens were subjected to a systematic molecular approach, which included 16S rRNA PCR and several specific PCRs (*3*) (Table).

Histopathologic results of the duodenal biopsy revealed PAS-positive and diastase-resistant macrophages (online Appendix Figure, wwwnc.cdc.gov/EID/article/18/8/12-0309-FA1.htm) with faint immunohistochemical staining. Results of *T. whipplei* PCRs targeting 2 different sequences were negative for the duodenal and lymph node biopsy specimens. These specimens were also negative by PCR for 16S rRNA, *Bartonella* spp., and *F. tularensis*. Conversely, Ziehl–Neelsen staining showed numerous AFB. Results of PCRs were negative for *M. tuberculosis* and *M. avium* but positive for *Mycobacterium* spp.

Sequencing facilitated identification of *Mycobacterium genavense* (99.6% of homology with the isolate with GenBank accession no. HM022216). Combined treatment with amikacin, rifabutin, moxifloxacin, clarithromycin, and ethambutol was implemented. To enhance the chances of eradicating *M. genavense*, mycophenolate mofetil was discontinued and cyclosporine A reduced. The patient's condition was largely unimproved; clinical improvement was observed 9 months after treatment reinitiation. Cardiac allograft function remained unaltered. Optimal duration of therapy is unknown; treatment had been ongoing for nearly 12 months at time of publication. More than the choice of antimycobacterial agents, we believe that it is the reduction in immunosuppression and the duration of therapy that eventually facilitated clinical improvement.

M. genavense is a slow-growing, nontuberculous mycobacterium that infects immunocompromised hosts (*4*). Only 3 cases of *M. genavense* infection in solid-organ transplant recipients have been reported (*5*–*7*,). *M. genavense* has a predilection for the digestive tract, which explains

Table. Approach used to determine the cause of posttransplant cachexia in a patient*

Pathogen	Sequence target	Primers, 5′ → 3′	Probes/identification
Molecular tool to detect and identify *Tropheryma whipplei*			
Real-time PCR			
T. whipplei	Repeated sequence	Twhi2F: TGAGGATGTATCTGTGTATGGGACA Twhi2R: TCCTGTTACAAGCAGTACAAAACAAA	6-FAM-GAGAGATGGGGTGCAGGACAGGG-TAMRA
T. whipplei	Repeated sequence	Twhi3F: TTGTGTATTTGGTATTAGATGAAACAG Twhi3R: CCCTACAATATGAAACAGCCTTTG	6-FAM-GGGATAGAGCAGGAGGTGTCTGTCTGG-TAMRA
Molecular tools to detect and identify *Mycobacterium* spp.			
Step 1: Real-time PCR			
Mycobacterium spp.	ITS	ITSd: GGGTGGGGTGTGGTGTTTGA ITSr: CAAGGCATCCACCATGCGC	6-FAM-TGGATAGTGGTTGCGAGCATC-TAMRA
M. tuberculosis	ITS	ITSd: GGGTGGGGTGTGGTGTTTGA ITSr: CAAGGCATCCACCATGCGC	6-FAM-GCTAGCCGGCAGCGTATCCAT-TAMRA
M. avium	ITS	ITSd: GGGTGGGGTGTGGTGTTTGA ITSr: CAAGGCATCCACCATGCGC	6-FAM-GGCCGGCGTTCATCGAAAT-Mgb
Step 2: Classical PCR			
Mycobacterium spp.	*rpoB*	MycoF: GGCAAGGTCACCCCGAAGGG MycoR: AGCGGCTGCTGGGTGATCATC	Sequencing
Housekeeping gene	β-actin	ActinF: CATGCCATCCTGCATCTGGA ActinR: CCGTGGCCATCTCTTGCTCG	6-FAM-CGGGAAATCGTGCGTGACATTAAG-TAMRA

*ITS, internal transcribed spacer; *rpoB*, RNA polymerase B.

the severity of the gastrointestinal symptoms (*4*). Moreover, it can mimic the endoscopic and histopathological features of WD (*8*).

In this case, the positive PAS-staining, the weak positivity of immunochemical staining for *T. whipplei*, and the false-positive results for 1 PCR temporarily delayed diagnosis. False-positive PCR results have been mainly reported when molecular diagnosis for *T. whipplei* was based on 16S rRNA PCR (*9*). Thus, positivity of a first PCR should be confirmed by using a second PCR with another target (*10*).

Bacteria responsible for lymph node enlargement are rarely isolated by culture. Molecular methods performed on lymph node biopsy specimens are useful diagnostic tools, but the common single molecular approach using 16S rRNA PCR lacks sensitivity, which delayed diagnosis for this patient (*3*). To address this issue, simultaneously to performing 16S rRNA PCR, we followed a strategy of systematic qPCR for lymph node specimens that targeted *Bartonella* spp., *F. tularensis*, *T. whipplei*, and *Mycobacterium* spp. (*3*). This report confirms the power of this systematic molecular approach, which enabled us to identify a rare bacterial agent scarcely reported for transplant patients.

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Murine Typhus in Drug Detoxification Facility, Yunnan Province, China, 2010

To the Editor: An outbreak of murine typhus caused by *Rickettsia typhi* was confirmed among persons attending a 51-acre drug detoxification program 2.5 km from Ruili City in Yunnan Province, People's Republic of China. Ruili City, with an average altitude of 1,381 km, is located in southwestern China near the Myanmar border (Figure). At the time of the outbreak, the detoxification program had 1,264 inpatients and 96 staff members. The facility is divided into sections A (women), B, C, and D. Residents of each section are housed in a 4-story building; each floor contains 9 rooms (2 m^2 per person). During September 4–21, 2010, a total of 76 of the 430 residents of section B were reported with fever of unknown

cause. All patients were men 19–38 years of age who worked in clothing manufacture at the facility and were receiving treatment for drug addiction. Before the outbreak, rats and stray cats were frequently observed in a cafeteria in section B. No persons with similar illness were observed in the other 3 sections.

To investigate the outbreak, we gathered information about demographics; past medical histories; exposures to vectors, such as ticks, mites, fleas, and lice; and symptoms. Patients frequently reported headache, dizziness, diffuse myalgia, high fever (>39°C), and shivers but did not report a rash or eschar. No patients remembered a flea or louse bite, but they frequently reported seeing rats in the area. The Chinese Center for Disease Control and Prevention (China CDC) Institutional Review Board approved the investigation.

Two milliliters of blood was collected from each consenting patient. Separated serum and the remaining blood clots were stored at −70°C and transferred to the Department of Rickettsiology, National Institute of Communicable Disease Control and Prevention, China CDC, for testing. Specimens were tested by indirect immunofluorescence assay (*1*) to detect specific IgM and IgG against 10 common rickettsiae: *Rickettsia prowazekii*, *R. typhi*, *R. heilongjiangensis*, *Orientia tsutsugamushi* types Karp and Kato, *Coxiella burnetii*, *Bartonella henselae*, and *B. quintana*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum*. Antigens were prepared by placing the rickettsial stains in L929 cells and HL60 or and DH82 cells, respectively; collecting the culture when Gimenez stain or Wright staining showed positive results; ultrasonically crushing the culture; and purifying the bacteria by density ultracentrifugation. Positive control serum was prepared by inoculating rabbits with the above standard rickettsiae strains.

We collected 76 serum samples from patients a median of 4 days (range 1–9 days) after illness onset. Thirty-five (40%) were IgM positive for *R. typhi* (titer ≥40, maximum titer 160) and 29 (38%) were IgG positive for *R. typhi* (titer ≥80, maximum titer 320). No samples were positive for the other 8 rickettsial antigens, except for 10 (13%) that had weak reactions for *R. prowazakii* (titer 40). Twelve convalescent-phase serum samples (median interval between acute and convalescent phases 187 days [range 181–192 days]) were IgG positive for *R. typhi* (titer ≥80) and 4 had 4-fold increases in titer; 2 reached titers of 1,280 and 2,560.

DNA was extracted from acute-phase samples by using a QIAGEN DNA extraction kit (Hilden, Germany) and tested by real-time PCR that targeted the *groEL* gene *of R. prowazekii* and *R. typhi* (*2*). Twelve (16%) of the 76 samples were positive. To differentiate between *R. prowazekii* and *R. typhi*, we used a previously developed nested PCR targeting the *groEL* gene *of R. prowazekii* and *R. typhi* (*3*) and found the expected 218-bp fragments in 11 patients. BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that these sequences (200 bp) were 100% homologous with that of *R. typhi* strain Wilmington (GenBank accession no. AF017197).

Initially, patients were treated with antiviral drugs and Chinese herbal medicine for suspected influenza. Subsequently, murine typhus was suspected and doxycycline was administered. All patients recovered fully.

Yunnan Province's subtropical geographic and climate characteristics are advantageous to the vectors of rickettsial diseases, such as murine typhus, scrub typhus, spotted fever, and Q fever (*4*–*6*). Three national murine typhus outbreaks involving >10,000 cases each have been reported since 1949, and each involved Yunnan Province (*7*). In the 1970s, an outbreak of louse-borne typhus occurred in northeastern Yunnan Province (*4*); since then, louse-borne typhus has been rarely reported. Murine typhus was reported from Baoshan City, east of Ruili City, in 2010. However, the currently reported murine typhus

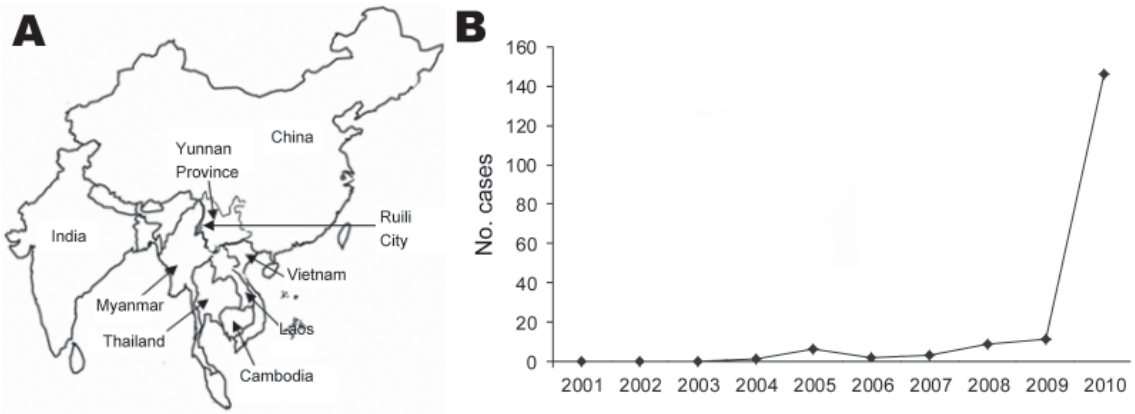

Figure. A) Location of Ruili City, Yunnan Province, People's Republic of China (97°51′–98°02′E, 23°38′–24°14′S; altitude 1,381 m). B) Number of murine typhus cases reported from Ruili City Center for Disease Control and Prevention during 2001–2010.

outbreak in Ruili City near the China–Myanmar border was the largest outbreak in China during the previous decade. None of the 76 patients had rash, a finding similar to that reported in previous outbreaks in Myanmar, Thailand, and other Southeast Asia regions (*8–10*). In addition to the 76 cases reported here, 70 additional sporadic cases of murine typhus were reported to the Ruili CDC in 2010. We conclude that murine typhus should be considered in cases of unexplained fever with nonspecific clinical manifestations in southern Yunnan Province.

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Carpal Tunnel Syndrome with Paracoccidioidomycosis

To the Editor: Paracoccidioidomycosis, a systemic mycosis caused by *Paracoccidioides brasiliensis*, is endemic to rural areas of Latin America (*1*). Persons are infected early in life by inhaling the fungus propagules, which reach the lower airway and cause primary complex (*2*). The most common clinical manifestation of paracoccidioidomycosis, which occurs with the chronic multifocal form, is characterized by pulmonary and extrapulmonary (e.g., skin, central nervous system, osteoarticular system) involvement, which occurs after a prolonged latency period (*2*). Carpal tunnel syndrome (CTS) is seldom associated with pyogenic agents (*3*), *Mycobacterium tuberculosis* (*4*), or fungal agents (*5*). Few reports have described paracoccidioidomycosis in immunosuppressed patients (*6*). We report a rare case of flexor tenosynovitis and severe CTS in the context of reactivated, chronic paracoccidioidomycosis infection.

A 63-year-old white male agricultural worker from São Paulo, Brazil, reported insidious and progressive pain, numbness, and tingling in his right hand and fingers, which began in April 2009. His medical history included symmetric polyarthritis of hands, ankles, and knees, which had been diagnosed elsewhere as seronegative rheumatoid arthritis in 2006. At that point, he also had chronic cough; a computed tomographic (CT) scan of the chest showed small nodules and mild interstitial fibrosis, and sputum specimens were negative for fungi or mycobacteria by microscopy. For treatment, he received prednisone, leflunomide, meloxicam, and methotrexate. Hydroxychloroquine was added in March 2010 because

of worsening polyarthritis. Pain in the right hand also increased, and infiltrations of the right carpal tunnel with methylprednisolone and lidocaine were performed in September and October 2010, with poor response. After that, physical examination showed mild edema and warmth of the flexor surface of the hand and reduced wrist motion. Phalen test and Tinel signs were positive. In February 2011, an outpatient electrophysiologic evaluation showed a severe right focal demyelination of the median nerve at the wrist and mild acute denervation in the abductor pollicis brevis muscle, consistent with CTS.

In August 2011, the patient was admitted to the hospital of the University of Campinas, São Paulo, Brazil, with poor general health, fever, cutaneous nodules on the trunk and limbs, and dyspnea. Purulent material drained from 2 fistulous nodules in the right thumb and forearm (Figure, panel A). A new CT scan of the chest indicated cystic bronchiectasis, bronchial wall thickening, and adjacent areas of consolidation. Microscopic examination of thumb secretion and sputum samples by using 10% potassium hydroxide revealed the characteristic pilot's wheel appearance of *Paracoccidioides brasiliensis*, showing multiple-budding yeast cells with well-defined refringent double walls (*7*) (Figure, panel C). Grocott-Gomori stain of a skin biopsy specimen demonstrated yeast with the same microscopic features (*7*). Serologic tests for *Paracoccidioides* spp. were negative. Blood and thumb secretion cultures were negative for *Mycobacterium* spp. and fungi. Magnetic resonance imaging of the right wrist and forearm showed diffuse inflammatory infiltrates with signs of tenosynovitis and fluid collection involving the flexor compartment and extending to areas corresponding to fistulous skin lesions (online Technical Appendix, wwwnc.cdc.gov/pdfs/18/6/12-0153-Techapp.pdf).

Intravenous co-trimoxazole was prescribed, followed by oral itraconazole. Immunosuppressant drugs were withdrawn. After the patient's general health stabilized, he underwent open carpal tunnel release, flexor tenosynovectomy, and collection of the purulent drainage. When evaluated 5 months after hospital discharge, his right hand symptoms and polyarthritis had almost completely resolved (Figure, panel B). A neurophysiologic examination demonstrated a mild improvement in distal median neuropathy. Results of serologic assessment for rheumatoid factor and antibodies against cyclic-citrullinated peptide were negative. Also, no signs of bone erosions or subcortical cysts were shown on radiograph of wrist and hand joints, which does not support the diagnosis of seronegative rheumatoid arthritis.

Although a direct search for fungi and mycobacterial agents was initially negative, paracoccidioidomycosis should still have been included in the differential diagnosis for this

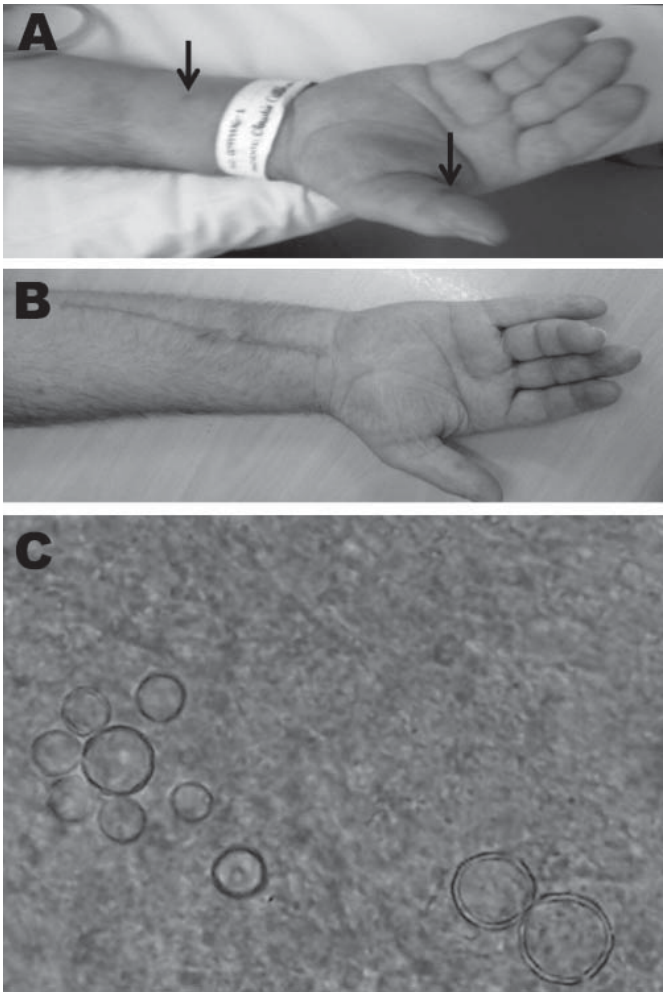

Figure. A) Edema and erythema of the flexor surface of the hand of patient with paracoccidioidomycosis, carpal tunnel syndrome, and flexor tenosynovitis, Brazil. Note a fistulous pustulous nodule in the right thumb and forearm (arrows) and flexor contracture of the fourth finger. B) Flexor surface of the hand and forearm after surgery. C) *Paracoccidioides brasiliensis* was directly identified on the thumb secretion, sputum, and flexor tenosynovectomy specimen by using a 10% potassium hydroxide preparation. This image was obtained from the thumb secretion. Note the characteristic multiple-budding yeast cells (pilot's wheel) with the well-defined refringent double wall. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/8/12-0153-F1.htm).

patient, who exhibited arthritis and pulmonary symptoms and had the risk factors of heavy smoking and living in a paracoccidioidomycosis-endemic region. The initial chest CT scan did not rule out paracoccidioidomycosis (*7*). However, seronegative rheumatoid arthritis was diagnosed and treated. When the patient arrived in our hospital, systemic manifestations, severe pulmonary compromise, and CTS of the right hand were the main features of his condition, and *P. brasiliensis* was detected on direct microscopic observation of sputum and thumb secretions. The central nervous system is a frequent extrapulmonary site of damage by paracoccidioidomycosis (*2*,*8*,*9*), but for paracoccidioidomycosis to cause CTS is unusual. The patient received immunosuppressive drugs during a 5-year period. The immunosuppressive treatment could contribute to reactivation of pulmonary quiescent infection foci and hematogenous fungal spread. Infiltrations of the wrist with corticosteroids could facilitate and enhance local fungal proliferation after hematogenous dissemination. Factors such as inoculum size, pathogenicity and strain virulence, and patient's immune status could explain the development and severity of disease (*2*). Immunocompromised patients, particularly those with cell-mediated immune impairment, are at greatest risk for severe disseminated paracoccidioidomycosis (*6*), as occurred in this patient. Identifying antibodies against *Paracoccidioides* spp. in patient's serum would have helped monitor the host response to treatment (*2*), but he was seronegative, probably because of his immunosuppressed state. Paracoccidioidomycosis serologic testing would be useful early in the disease to help distinguish between seronegative rheumatoid arthritis and reactive arthritis. Paracoccidioidomycosis should always be suspected in *P. brasiliensis*–en-demic areas.

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Fundamental Medical Mycology

Errol Reiss, H. Jean Shadomy, and G. Marshall Lyon, III

Wiley-Blackwell, Hoboken, NJ, USA, 2011
ISBN: 978-0-470-17791-4
Pages: 656; Price: US $99.95

In Fundamental Medical Mycology, Errol Reiss, Jean Shadomy, and Marshall Lyon have produced a valuable new text. Drs Reiss and Shadomy are medical mycologists who have extensive research and educational experience with the increasing spectrum of pathogenic fungi, including diagnosis of infections they cause and host defenses. Dr Lyon is an infectious diseases physician with clinical expertise in the epidemiology and management of opportunistic mycoses. This complementary team has produced a highly readable and comprehensive book, which they intend to be a text for medical and graduate students, a resource for microbiology technologists, and a reference for physicians and researchers.

The book has been carefully organized, and the extensive table of contents enables readers to quickly identify specific areas of interest. The first 3 chapters contain basic information describing fungi, diagnostic methods, and antifungal chemotherapy. The succeeding 19 chapters review specific mycoses, using a similar format that addresses the following topics: etiology, clinical manifestations, ecology of the fungi, epidemiology of the infections, pathogenicity, animal infections, treatment, and laboratory diagnosis. Each chapter provides instructive case histories and ends with references and review questions. The book also includes a helpful glossary.

A book of this nature can be judged by its completeness, accuracy and timeliness of coverage, clarity of the writing, and ease with which information can be accessed. By all of these criteria, Fundamental Medical Mycology merits high marks. The book does a superlative job in addressing recent advances in medical mycology, which include identifying emerging pathogens, new antifungal drugs and strategies for their use; progress in molecular diagnostics; and up-to-date knowledge about host defenses against fungi, especially opportunistic pathogens. For a 1-volume text, this book provides excellent coverage of several critical areas: detailed methods of identifying fungi; descriptions of common and rare mycoses; the nuances of interpreting serologic tests; and ongoing progress in detecting diagnostic fungal antigens, nucleic acids, and signature proteins in clinical specimens.

In addition, the authors provide superb, concise descriptions of the strain diversity of the major pathogenic species and the clinical and epidemiologic relevance of certain phylogenetic clades. Currently unresolved or controversial topics are clearly explained, such as fungal sinusitis and the etiology of *Malassezia* spp. infections. When information is available, each chapter summarizes the mechanisms of pathogenicity and confirmed virulence factors. The authors discuss advances in understanding the innate and adaptive immune responses to fungi at the tissue, cellular, and molecular levels (e.g., the role of Th17 immune responses in candidiasis). Another asset is the frequent but unobtrusive inclusion of key citations to assist anyone seeking additional information. The illustrations include diagrams, clinical photographs, and photomicrographs from a variety of sources as well as original figures. Rather than attempt pictorial consistency throughout, the authors have gleaned images for their relevance to the text.

This book will serve medical and graduate students who will value the book's succinct, lucid coverage of key fungal infections, as well as instructive case vignettes and review questions. Clinical fellows and physicians will appreciate the readable summaries of specific mycoses, diagnostic procedures, common symptoms, and appropriate antifungal drugs. Biomedical scientists and educators in related fields will use this text as a resource for a quick review of specific topics.

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Jules Adler (1865–1952) *Transfusion of a Goat's Blood* (1892) Oil on canvas (129.5 cm × 195.6 cm) (detail) Photo by Alyssa Cwanger, 2006

Heart Fastened to a Dying Animal

Polyxeni Potter

"Medea unsheathed a knife, and cut the old man's throat, and letting the old blood out, filled the dry veins with the juice. When Aeson had absorbed it, part through his mouth, and part through the wound, the white of his hair and beard quickly vanished, and a dark color took its place. At a stroke his leanness went, and his pallor and dullness of mind. The deep hollows were filled with rounded flesh, and his limbs expanded." So wrote Ovid in the Metamorphoses about Medea performing a crude transfusion to rejuvenate the father of her beloved Jason. The "barbarian witch" infused a concoction of "dark juices…. the wings and flesh of a vile screech-owl and the slavering foam of a sacrificed werewolf…. the scaly skin of a water-snake, the liver of a long-lived stag…."

This story from antiquity traces human fascination with blood—at first with the loss of blood and its connection with weakness and death, later with blood transference from strong persons or animals to the infirm as therapy, usually by mouth. Interest and reports of attempts at some form of transfusion continued throughout the Middle Ages and up until the 1600s and William Harvey's description of the human circulatory system, which ushered in a new era of attempts at transfusion. In addition to blood, experimentation involved beer, opium, and milk, infused directly into the veins and arteries of animals, which invariably died.

"What if we transfused the blood of an Archbishop into a Quaker," joked Samuel Pepys in a 1666 diary entry making light of a hot topic of his day: taking blood from a beast and putting it into another beast or into the veins and arteries of a person. But on a more serious note, he wrote, the practice might "if it takes, be of mighty use to man's health, for the amending of bad blood by borrowing from a better body." A fine record of the anxieties of its age, Pepys' diary documented unfolding historical events, becoming a valued primary source. The first person in England to receive a transfusion from a sheep was "indigent and 'looked upon as a very freakish and extravagant man'…. About 32 years of age…. He spoke Latin well … but his brain was a little too warm…. They purpose to let in about 12 ounces; which, they compute, is what will be let in in a minute's time by a watch."

The sensational nature of early transfusions made them a frequent topic of write-ups, now a substantial historical record. But the subject's appeal spilled into other areas, art among them. Jules Adler's *Transfusion of a Goat's Blood*, on this month's cover, is the pictorial representation of a transfusion that took place in 1890 and the circumstances surrounding it. Adler's undertaking showed that transfusion was perceived akin to other major historical themes of painting and part of the history of medicine.

The painting was commissioned by respected Paris physician Samuel Bernheim (1855–1915), a tuberculosis specialist who established a charity to send patients and their children to the seaside and other open areas as part of the sanatorium movement. Bernheim was depicted as the central figure standing above the patient. The procedure described involved transfusing some 200 g of blood from the goat to a female patient likely in an effort to strengthen immunity. The same year, Bernheim published the article "Transfusion of Goat's Blood and Lung Tuberculosis."

Adler was born in the commune Luxeuil-les-Bains in eastern France. Not much is known about his life, except that his talent was recognized early and his parents moved to Paris where he attended the École Nationale Supérieure

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des Arts Décoratifs and the École des Beaux-Arts. He also studied at the Académie Julian under William Bouguereau and Tony Robert-Fleury. Even though he is often categorized as an academic painter, Adler is better known as "the painter of the humble" for his affinity to the common people, whose plight he epitomized in his best works. While he moved within Paris Salon circles and espoused academic conventions, he took off on his own to create a naturalist style akin to social realism and attain broad audience appeal. He painted laborers (*The Strike at Creusot*) and the poor (*The Weary*), championed social causes, and addressed in his works anti-Semitism, injustice, and the alienation of modern life.

Transfusion of a Goat's Blood was shown in the Salon and won an award, but this success did not affect the popularity of the painting or the artist. The Paris École de Médicine, perhaps not wanting to draw attention to a discredited procedure, relegated the painting to a stairway. By this time, there had been plenty of evidence that human and animal blood were not compatible. But some physicians were using animal serum to treat diphtheria in children, and others wondered if animal fluids might cure various human diseases. In 1901, Karl Landsteiner would identify blood types and their role in safe human-to-human blood transfusions.

Adler's painting, like other illustrations of medical history, recorded procedures and related conventions through the artist's lens, which demystified some and chose to romanticize others. On the one hand, instead of a heroic physician, the painting showed a team, suggesting that the faces might change but not the procedure, which was guided by protocol. On the other hand, the depiction of blood was discrete, visible only against the extreme paleness of the patient and against the predominance of white in the room. Long dark hair frames the dying maiden's face against the white pillow and sheets, her vulnerable situation a stark contrast against that of the medicine men in suits, who seemed to have everything under control. Behind Bernheim, a goat was stretched out on an ordinary bench. The connection between the animal and the human patient was a piece of simple rubber tubing with a cannula at each end.

Attempts at sharing blood and other tissues across species have not abated, though without Medea's flamboyant concoctions and recipient fear of incurring animal traits or the donor's religious beliefs. Challenges persist because of transfusion and organ and cell transplant–associated diseases. Demand is high, but the supply of allografts is limited. Immunologic rejection remains a problem. And not the least are infectious disease threats: allograft- and xenograft-derived zoonotic and nonzoonotic infections and infections introduced during tissue processing, the transplant procedure, or post-operative hospitalization.

Threat identification and response related to donor and recipient are improving the process. But problems remain with the specificity and sensitivity of tests, unknown pathogens, and faulty histories. Many zoonotic infections, from hepatitis E and human granulocytic anaplasmosis to solid organ transplant-associated lymphocytic choriomeningitis, can cause severe complications in recipients who, unlike Medea's charge, are not so fortunate.

A long history of close cohabitation speaks for a far closer connection between animals and humans than shown by the simple rubber tubing in Adler's painting. This history is celebrated in poetry too, which examines, among other subjects, the interface of their health and common fate—the never-ending calamity of death. W.B. Yeats, pondering his own declining health and weak aging body, was able to see beyond the literal cannula of transfusion. In a precocious "one health" stance, in the poem "Sailing to Byzantium" (1928), he conjured the immense damage to the body from illness and physiologic decline. The spirit, imagination and intellect, which he posed as the only way to remain vital in the face of this decline, also fuel continued medical efforts to improve health and prolong life—literally through transfusion and solid tissue transplants and metaphorically when the perennially young human heart finds itself "fastened to a dying animal," the body.

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Article Title

Vaccination of Health Care Workers to Protect Patients at Increased Risk for Acute Respiratory Disease

CME Questions

1. Several employees in your health care system are complaining about a policy of universal influenza vaccination announced several years ago. They feel that mandatory vaccinations (with substantial precautions for workers who opt out of vaccinations) are unfair and may not be effective.

You need to craft an answer to their complaints. What should you consider regarding influenza infection among health care workers?

A. More than half of health care workers have clinical infection with influenza during a given season
B. Nearly all young adults infected with influenza will have clinical symptoms of infection
C. Influenza vaccination has not been effective in protecting against influenza B strains
D. Universal influenza vaccination of health care workers is likely to be cost-effective

2. Regarding the current systematic review by Dolan and colleagues, what should you consider regarding the methods of research into vaccination of health care workers and patient protection from illness?

A. Vaccination rates among staff in the intervention groups were nearly 100% across all studies
B. Most research was conducted in long-term care facilities
C. Vaccine coverage among patients was limited between 5% and 20%
D. All studies demonstrated good blinding of participants and study personnel

3. Based on the current systematic review, what can you tell your colleagues regarding the effects of influenza vaccination for health care workers on patient cases and consultations for ILI (ILI)?

A. There is no effect on health care worker vaccination on patients' risk of ILI or consultation for ILI
B. Vaccination of health care workers reduces patients' risk of ILI but not rates of consultation for ILI
C. Vaccination of health care workers reduces patients' rates of consultation for ILI but not ILI itself
D. Vaccination of health care workers reduces patients' risk of ILI as well as rates of consultation for ILI

4. Which of the following outcomes among patients appears to be most improved with influenza vaccination of health care workers?

A. All-cause mortality
B. Death due to respiratory causes
C. Death due to pneumonia
D. Hospitalization for respiratory causes

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Paragonimus kellicotti Flukes in Missouri, USA

CME Questions

1. You are seeing a 24-year-old man with a 4-week history of cough, fever, and malaise. He was seen in an urgent care center 10 days ago and prescribed a course of macrolide antibiotics, which did not improve his symptoms. On questioning, the symptoms began after a camping trip in which the patient and his friends experimented with eating raw meat and seafood.

You are concerned regarding the possibility of paragonimiasis in this patient. What should you consider regarding the epidemiology and microbiology of paragonimiasis?

A. Most cases are reported in North America
B. *P. kellicotti* requires snail and crustacean intermediate hosts
C. Humans usually are infected after eating escargot
D. *P. kellicotti* infects only humans

2. What should you consider regarding the clinical presentation of paragonimiasis in the current case series as you evaluate this patient?

A. All patients were female
B. Cough and fever were the most common clinical symptoms
C. Vomiting and malaise were the most common clinical symptoms
D. The onset of symptoms occurred within 48 hours of ingesting food contaminated with *P. kellicotti*

3. What should you consider regarding the management of paragonimiasis in the current case series as you evaluate this patient?

A. The median time from symptom onset to the correct diagnosis was 12 weeks
B. Only half of patients had received antibiotic therapy
C. No patients had received corticosteroids
D. Most patients failed to respond even to appropriate antiparasitic therapy

4. Which of the following tests is most likely to be abnormal if this patient has paragonimiasis?

A. Serum neutrophil counts
B. Serum eosinophil counts
C. Serum sodium levels
D. Electrocardiogram

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.				
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Article Title

Increasing Resistance to Ciprofloxacin and Other Antimicrobial Drugs in *Neisseria gonorrhoeae*, United States

CME Questions

1. You are a consultant to a US public health department regarding development of antibiotic resistance in Neisseria gonorrhoeae. Based on the analysis of data from the Gonococcal Isolate Surveillance Project (GISP) by Dr. Goldstein and colleagues, which of the following statements about overall patterns of drug resistance stratified by sexual orientation is most likely correct?

A. Between 1998 and 2007, ciprofloxacin resistance grew faster for heterosexual men than in men who have sex with men (MSM)
B. The fastest growing class of ciprofloxacin-resistant types in MSM was the type resistant to ciprofloxacin, tetracycline, and penicillin (triply resistant)
C. The mono-resistant type (resistant to ciprofloxacin; sensitive to tetracycline/penicillin) declined overall during the study period
D. In heterosexuals, the mono-resistant type was the most common form of ciprofloxacin resistance since mid 2001

2. Based on the analysis of data from GISP by Dr. Goldstein and colleagues, which of the following statements about recent travel and resistance in MSM and heterosexuals is most likely correct?

A. In MSM, triple resistance prevalence was positively associated with recent travel
B. In heterosexuals, triple resistance prevalence was negatively associated with recent travel
C. The positive association between mono-resistance and recent travel was borderline statistically significant for heterosexuals
D. In MSM, there was a strong, statistically significant association between mono-resistance and recent travel

3. Based on the analysis of data from GISP by Dr. Goldstein and colleagues, which of the following statements about the first appearance of resistance in heterosexuals and MSM would most likely be correct?

A. The timing of first appearance of ciprofloxacin resistance in heterosexuals and in MSM in different GISP sites was not correlated
B. Median delay in timing of first appearance between MSMs and heterosexuals was 5.5 months in either direction, despite a 5-year range of times of first appearance across different sites
C. Correlations were limited to the same geographic regions at the same times
D. The data suggest that prevention efforts should target only MSM

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.				
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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

Upcoming Issue

Hepatitis E, a Vaccine-Preventable Cause of Maternal Deaths

Evaluation of Immigrant Tuberculosis Screening in Industrialized Countries

Trends in Meningococcal Disease in the United States Military

Prevention and Control of Fish-borne Zoonotic Trematodes in Fish Nurseries, Vietnam

Diagnostic and Therapeutic Approach for Patients with Suspected Influenza A(H1N1)pdm09

Effectiveness and Timing of Vaccination during School Measles Outbreak

Surveillance for Influenza Viruses in Poultry and Swine, West Africa

Controlled Fluoroquinolone Resistance through Successful Regulation, Australia

Multiple Synchronous Outbreaks of Puumala Virus, Germany, 2010

MRSA Harboring *mecA* Variant Gene *mecC*, France

Influenza Virus Infection in Nonhuman Primates

Oral Human Papillomavirus Infection among Young Adults, Sweden

Demographic Shift of Influenza A(H1N1)pdm09 During and After Pandemic, Rural India

Hospitalizations Associated with Disseminated Coccidioidomycosis, Arizona and California

Reemergence of Sudan Ebola Virus in Uganda, 2011

Francisella tularensis Subspecies *holarctica* in Tasmania, Australia

Lack of Evidence for Chloroquine-Resistant *Plasmodium falciparum* Malaria, Leogane, Haiti

Infectious Diseases in Children and Body Mass Index in Young Adults

Inadequate Antibody Response to Rabies Vaccine in Immunocompromised Patient

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 25–29, 2012
2012 Infectious Disease Board Review Course
Ritz-Carlton, Tysons Corner
McLean, VA, USA
http://www.IDBoardReview.com

September 5–8, 2012
Incidence, Severity, and Impact Conference
Munich, Germany
http://www.isirv.org

September 9–14, 2012
XVIIIth International Pathogenic Neisseria Conference (IPNC) 2012
Maritim Hotel, Würzburg, Germany
http://www.ipnc2012.de

October 17–21, 2012
IDWeek Annual Meeting
San Diego, CA, USA
http://www.IDWeek.org

October 23–24, 2012
"Emerging Viruses: Disease Models and Strategies for Vaccine Development"
A symposium in honor of CJ Peters, MD
Galveston, TX, USA
http://www.utmb.edu/wrce

October 24-26, 2012
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Edinburgh, Scotland, UK
www.escaide.eu

October 27–31, 2012
APHA 140th Annual Meeting & Expo
San Francisco, CA, USA
http://www.apha.org/meetings/AnnualMeeting

Announcements

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Announcements may be posted on the journal Web page only, depending on the event date.

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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.

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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 figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif 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.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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.

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.

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.