

# EMERGING INFECTIOUS DISEASES<sup>®</sup>



Antimicrobial Resistance

September 2016

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September 2016



## On the Cover

Giovanni Paolo Panini (1691–1765), *Alexander the Great Cutting the Gordian Knot* (ca. 1718–1719).

Oil on canvas, 28 7/8 in × 23 1/2 in/ 73.3 cm × 59.7 cm. Public domain digital image courtesy of The Walters Art Museum, 600 N Charles St, Baltimore, Maryland, USA.

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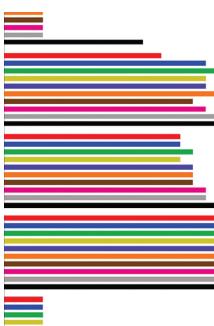
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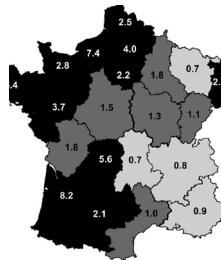
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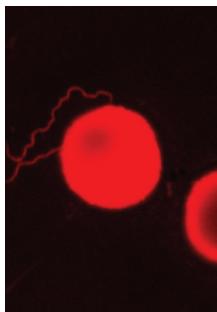
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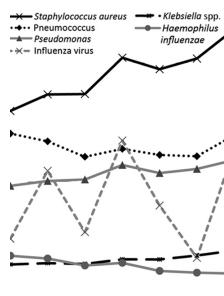
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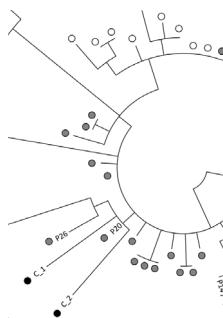
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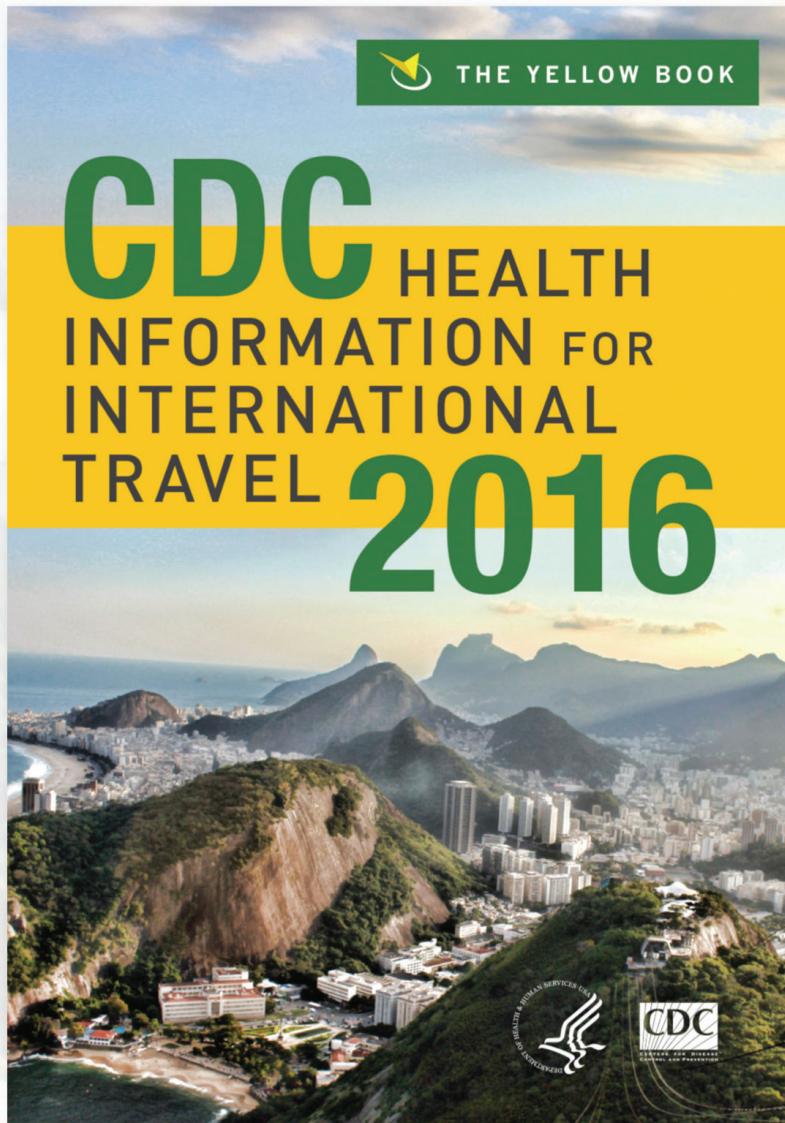
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# Treatment Outcomes for Patients with Extensively Drug-Resistant Tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa

Charlotte L. Kvasnovsky, J. Peter Cegielski, Martie L. van der Walt

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### Learning Objectives

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

- Compare extensively drug-resistant tuberculosis (XDR TB) and its treatment outcomes overall and for subgroups characterized by HIV status and treatment
- Determine predictors of favorable XDR TB treatment outcomes
- Identify predictors of unfavorable XDR TB treatment outcomes

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We analyzed data for a retrospective cohort of patients treated for extensively drug-resistant tuberculosis in 2 provinces in South Africa and compared predictors of treatment outcome in HIV-positive patients who received or had not received antiretroviral drugs with those for HIV-negative patients. Overall, 220 (62.0%) of 355 patients were HIV positive. After 2 years, 34 (10.3%) of 330 patients with a known

HIV status and known outcome had a favorable outcome. Multivariate analysis showed that predictors of favorable outcome were negative results for acid-fast bacilli by sputum microscopy at start of treatment and weight >50 kg. HIV-positive patients were more likely to have an unfavorable outcome. The strongest predictor of unfavorable outcome was weight  $\leq$ 50 kg. Overall outcomes were poor. HIV status was not a predictor of favorable outcome, but HIV-positive patients were more likely to have an unfavorable outcome. These results underscore the need for timely and adequate treatment for tuberculosis and HIV infection.

**T**uberculosis (TB) remains a major cause of illness and death in the 21st century. There were an estimated 9.6 million incident cases worldwide in 2014 (1). In addition, an estimated 3.3% of new cases and 20% of retreatment cases are multidrug-resistant TB (MDR TB), which is defined as TB resistant to at least rifampin and isoniazid, the 2 most powerful first-line drugs. This resistance threatens global TB control efforts. MDR TB patients need access to treatment, require longer treatment with toxic medications, and have a lower probability of cure.

Globally, MDR TB has a treatment success rate of 50% (1). Extensively drug-resistant TB (XDR TB) is MDR TB with additional resistance to at least a fluoroquinolone and any 1 of 3 injectable second-line drugs (amikacin, capreomycin, or kanamycin). By 2014, XDR TB had been reported in 105 countries, including 10 countries in sub-Saharan Africa (1).

Shortly after XDR TB was first described in 2005, patients in 1 hospital in KwaZulu-Natal Province were shown to have extremely rapid and high mortality rates in a setting with high HIV prevalence (2). Since that time, published case series have shown higher cure rates (48%–60%), and the World Health Organization (WHO) reported a treatment success rate of 22% for reported XDR TB cases worldwide (3,4). However, these studies included few HIV-positive patients.

We previously reported poor outcomes for patients with XDR TB after 1 year of treatment (5). Thus, we sought to collect the largest possible dataset for patients with XDR TB in an area of high HIV prevalence to assess predictors of favorable and unfavorable treatment outcomes among patients.

## Patients and Methods

We conducted a retrospective cohort study of patients initiating treatment for XDR TB in 2 contiguous provinces in South Africa. In Eastern Cape Province, all patients given a diagnosis of XDR TB by the provincial public laboratory were reported to the XDR TB treatment facility as described (6). Therefore, our sample in this province consisted of continuous patients who were given a diagnosis

of XDR TB during October 1, 2006–January 31, 2008. We also included continuous patients initiating treatment for XDR TB in this province during April 1–July 1, 2008, who were eligible for treatment with moxifloxacin after this drug became available in this province. In KwaZulu-Natal Province, new XDR TB case-patients were reported to individual clinics, which then contacted the sole XDR TB treatment facility to place their patients on a list to initiate treatment. Our sample in KwaZulu-Natal Province included all patients who initiated XDR TB treatment during October 1, 2006–January 31, 2008. Ethical approval to conduct this research was obtained from the Medical Research Council of South Africa, Eastern Cape Department of Health, and KwaZulu-Natal Department of Health.

In accordance with national and provincial policy during the study, patients with XDR TB initiated treatment only as inpatients at specialized referral hospitals. Inpatient XDR TB treatment was offered free of charge; however, this treatment was dependent on bed availability and often resulted in delays before treatment initiation (7). At provider discretion, generally upon completion of the intensive phase of treatment and after  $\geq$ 2 consecutive negative sputum samples, patients were discharged from the hospital. After discharge, ambulatory patients were followed up at local clinics in Eastern Cape Province and at a hospital-based clinic in KwaZulu-Natal Province.

## Case Definition

Patients were given a diagnosis of XDR TB if any sputum sample showed resistance to rifampin, isoniazid, a fluoroquinolone, and any injectable second-line drug. Drug-susceptibility testing was performed by local National Health Laboratory Services facilities for 1 or several samples. All patients had  $\geq$ 1 subsequent positive sputum culture. In Eastern Cape Province, samples were tested for resistance to rifampin, isoniazid, ethambutol, ethionamide, streptomycin, amikacin, ofloxacin, and, starting in early 2008, capreomycin. In KwaZulu-Natal Province, samples were tested for resistance to rifampin, isoniazid, ethambutol, streptomycin, kanamycin, and ciprofloxacin.

Voluntary counseling and testing for HIV was offered to all patients. In accordance with national policy during the study, HIV-positive, drug-resistant TB patients who were not receiving antiretroviral drugs (ARVs) were given these drugs either at completion of the 4-month intensive phase of drug-resistant TB treatment if they had a CD4 lymphocyte count <200 cells/mm<sup>3</sup> or as soon as their CD4 lymphocyte count was <200 cells/mm<sup>3</sup>. ARV treatment included 3 drugs in accordance with South African National Guidelines. Patients who initiated ARV therapy >30 days after XDR TB treatment initiation were analyzed

as patients not receiving ARVs at the time of starting XDR TB treatment.

### Outcome Definitions

We applied standard MDR TB case definitions for XDR TB: favorable treatment outcomes were cure and treatment completion, and unfavorable treatment outcomes were death, loss to follow-up, and treatment failure (4,8). We applied these outcomes on the basis of the status of the patient 2 years (730 days) after treatment initiation. Patients who had a poor prognosis and were subsequently lost to follow-up were included among poor outcomes.

Patients who had 2 consecutive negative sputum cultures obtained  $\geq 30$  days apart after treatment initiation were considered to have achieved culture conversion. Initial time to sputum culture conversion was calculated as the interval in days between the date of treatment initiation for XDR TB and the collection date for the first of 2 consecutive negative sputum cultures.

### Treatment of XDR TB

Patients were given individualized regimens composed of first-line and second-line TB drugs available within their province: ethambutol, pyrazinamide, high-dose isoniazid, capreomycin, para-amino salicylic acid, moxifloxacin, cycloserine, and terizidone. Cycloserine and terizidone were considered interchangeable. These drugs were supplemented by WHO Group 5 drugs (amoxicillin/clavulanic acid and clarithromycin) on an individual basis.

Patients were considered to be receiving effective treatment if they received  $>4$  drugs to which their TB could be considered susceptible per WHO guidelines (9). A drug was considered effective if 1) it was recognized as an agent for treatment of TB; 2) the patient had either never received it or received it for  $<3$  months before XDR TB treatment; and 3) patient isolates were not found to be resistant to the drug by drug-susceptibility testing.

While hospitalized, patients received directly observed therapy (DOT), although the quality of hospital-delivered DOT was unknown. Once discharged, many patients continued to receive DOT through various delivery models, and others were seen monthly for medication refill and to give sputum samples for smear and culture testing.

### Data Collection and Analysis

Clinical and treatment data were abstracted from patient medical records at XDR TB treatment hospitals from multiple-site visits; all data were censored on March 10, 2010. Hospital-based HIV treatment registers were also reviewed for any additional follow-up information.

Double-data entry was performed on a database created for this study in Epi Info version 3.3.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Data cleaning

was performed by using EpiInfo and Stata 10 (StataCorp LP, College Station, TX, USA). Analysis was performed by using SAS 9.1 and SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

Univariate and bivariate analyses were performed to identify differences between HIV-negative patients, HIV-positive patients receiving ARVs, HIV-positive patients not receiving ARVs at initiation of treatment, and HIV-positive patients with unknown status for ARVs. Categorical data were compared by using  $\chi^2$  or Fisher exact tests as appropriate. Continuous variables were compared across these 3 categories by using PROC GLM (SAS Institute, Inc.) and the F statistic.

For comparison of unadjusted deaths, we used a Kaplan–Meier plot and log-rank test that were stratified by HIV infection and ARV treatment status and excluded patients with unknown status for ARVs. We used death as the event variable and censored all other outcomes at 730 days.

We sought to further assess risk factors for favorable and unfavorable treatment outcomes. At treatment day 90, survival curves for HIV-positive patients receiving ARVs and those not receiving ARVs crossed, which visually violated the proportionality assumption necessary for Cox proportional hazards. We confirmed this finding by plotting Schoenfeld residuals over time, for which there was a relationship, and by creating a time-varying covariate among only HIV-positive patients, for which the interaction term was a significant predictor in the model (10,11).

However, when we compared only HIV-positive patients with and without ARV treatment, we found no difference in outcome, and models comparing these 2 groups did not predict treatment outcome. Thus, we combined HIV-positive patients with and without ARV treatment in the final model. In this instance, the proportionality assumption was met.

Because of covariance between effective treatment and previous MDR TB treatment  $>6$  months, we included only previous MDR TB treatment in the multivariate model. All tests were 2-sided, and  $p < 0.05$  was considered statistically significant.

## Results

### Baseline Characteristics

A total of 355 patients were included in the analysis: 229 (64.5%) from Eastern Cape Province and 126 (35.5%) from KwaZulu-Natal Province (Table 1). Most (194, 54.6%) patients were women, and median age was 35 years (interquartile range [IQR] 28–44 years) at start of treatment.

Eleven patients did not have a known HIV status, and 220 (62.0%) patients were HIV positive at start of treatment. Of these patients, 114 (51.8%) were receiving ARVs at start of TB treatment, and 79 (35.9%) were not. Of the 79 patients not receiving ARVs at start of TB treatment, 23

**Table 1.** Baseline characteristics of patients initiating treatment for extensively drug-resistant tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010\*

Characteristic	Total, n = 355	HIV negative, n = 124	HIV positive, receiving ARVs at start of treatment, n = 114	HIV positive, not receiving ARVs at start of treatment, n = 79	HIV positive, ARV status unknown, n = 27	p value†
Treated in KZN	124 (36.1)	28 (22.6)	57 (46.0)	34 (27.4)	5 (4.0)	<b>&lt;0.0001</b>
Treated in EC	220 (64.0)	96 (43.6)	57 (25.9)	45 (20.5)	22 (10.0)	
Male sex‡	155 (45.1)	74 (47.7)	41 (26.5)	29 (18.7)	11 (7.1)	<b>0.0007</b>
Age, y, at start of treatment	35 (28–44)	37 (24–48)	35 (30–41)	34 (30–42)	34 (28–42)	0.44
Weight, kg, at start of treatment	49 (43–55)	49 (43–55)	49 (44–57)	49 (43–54)	46 (42–53)	0.52
Weight >50 kg at start of treatment	176 (51.2)	65 (36.9)	59 (33.5)	37 (21.0)	15 (8.5)	0.83
Diabetes	14 (4.1)	10 (71.4)	1 (7.1)	3 (21.4)	0	<b>0.03</b>
Initial CD4 count, cells/mm <sup>3</sup>	220 (64.0)	0	114 (51.8)	79 (35.9)	27 (12.3)	<b>&lt;0.0001</b>
AIDS at start of treatment‡	193 (110–313)	NA	181 (97–238)	217 (126–370)	329 (162–413)	<b>0.002</b>
Months previous MDR TB treatment	4 (0–8)	6 (0–11)	2 (0–7)	1 (0–7)	5.5 (4–16)	0.62
Months previous TB treatment	13 (8–19)	13 (8–21)	12 (8–17)	12 (7–18)	16 (11–24)	0.44
Previous TB episodes	2 (2–3)	3 (2–3)	2 (2–3)	2 (2–3)	3 (2–4)	0.34
Previous episode of MDR TB	214 (63.3)	84 (39.3)	64 (29.9)	43 (20.1)	23 (10.8)	<b>0.004</b>
Cavitary disease at start of treatment	187 (57.5)	81 (43.3)	55 (29.4)	37 (19.8)	14 (7.5)	<b>0.05</b>
Smear positive at start of treatment	173 (53.1)	48 (27.8)	67 (38.7)	41 (23.7)	17 (9.8)	<b>0.03</b>
No. TB-resistant drugs	5 (4–6)	4 (4–5)	5 (4–6)	5 (4–6)	5 (5–6)	<b>0.005</b>

\*Values are no. (%) or median (interquartile range). p values in bold are statistically significant. ARVs, antiretroviral drugs; EC, Eastern Cape Province; KZN, KwaZulu Natal Province; MDR TB, multidrug-resistant tuberculosis; NA, not applicable; TB, tuberculosis.

†Calculation excluded 11 patients with unknown HIV status.

‡AIDS defined by CD4 count <200 cells/mm<sup>3</sup> at start of treatment or AIDS-defining illness other than TB.

(10.5%) initiated ARVs during XDR TB treatment, after a median of 224 days (IQR 89–507 days). An additional 27 (12.3%) patients had an unknown ARV status.

Most (97.7%) patients had ≥1 month of TB treatment before initiating XDR TB treatment. HIV-negative patients were similar to HIV-positive patients in this respect; they had ≈1 year of previous TB treatment before initiating XDR TB treatment. Patients had a median of 13 previous months (IQR 8–19 months) of TB treatment, including a median of 8 months (IQR 5–12 months) of category I TB treatment (2 months of rifampicin, isoniazid, ethambutol, and pyrazinamide [intensive phase], followed by 4 months of rifampicin and isoniazid alone [continuous phase], with extension per treating clinician) (12) for presumed drug-susceptible TB and 4 months (IQR 0–8 months) of MDR TB treatment. We found no difference in duration of category I treatment between groups (p = 0.86), but HIV-negative patients were more likely than HIV-positive patients to have had a previous episode of MDR TB (p = 0.004).

HIV-negative patients were least likely to have smear positive disease (42.1%) when compared with HIV-positive patients receiving ARVs (60.9%), HIV-positive patients

not receiving ARVs at start of treatment (54.7%), and HIV-positive patients with an unknown ARV status (63.0%; p = 0.02). Cavitary disease was more frequent in HIV-negative patients (67.5%) than in HIV-positive patients receiving ARVs (50.9%), HIV-positive patients not receiving ARVs at start of treatment (51.4%), and HIV-positive patients with unknown ARV status (56.0%; p = 0.05).

### Treatment Outcomes

After 2 years of treatment, 330 (95.9%) patients with a known HIV status had a known treatment outcome. Of these patients, 21 (6.4%) met the definition for cure and 13 (3.9%) met the definition for treatment completion; therefore, a total of 34 (10.3%) patients had a favorable treatment outcome (Table 2). An additional 61 (18.5%) patients were alive but showed treatment failure after 2 years. A total of 211 (63.9%) patients died, and 24 (7.3%) patients interrupted their treatment prematurely and were lost to follow-up.

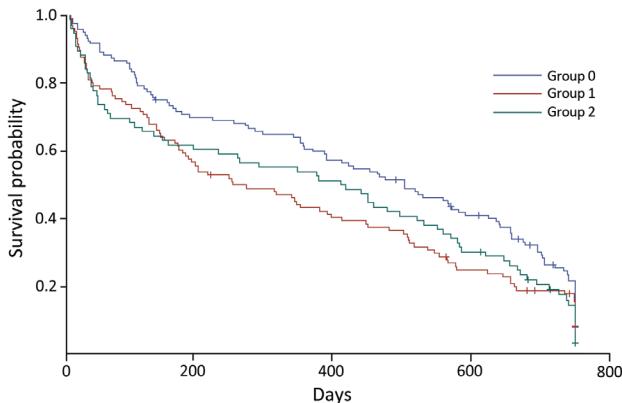
We found no significant difference in 2-year survival by Kaplan-Meier survival curves between the 3 groups (p = 0.07, by log-rank test) (Figure 1). There was also no

**Table 2.** Treatment and treatment outcomes for patients with extensively drug-resistant tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010\*

Characteristic	Total, n = 355	HIV negative, n = 124	HIV positive, receiving ARVs, n = 114	HIV positive, not receiving ARVs, n = 79	HIV positive, ARV status unknown, n = 27	p value†
No. drugs in treatment regimen	5 (5–6)	5 (5)	5 (5–6)	5 (5)	5 (5–6)	0.56
Effective drug treatment	109 (31.7)	29 (26.6)	46 (42.2)	32 (29.4)	2 (1.8)	<b>0.0004</b>
Any culture conversion	77 (22.4)	28 (22.6)	33 (29.0)	12 (15.2)	4 (14.8)	0.11
Alive after 2 y treatment	96 (27.9)	46 (37.1)	30 (26.3)	18 (22.8)	2 (7.4)	<b>0.007</b>
Favorable treatment outcome	34 (10.3)	15 (12.3)	13 (12.2)	5 (6.6)	1 (4.0)	0.37

\*Values are median (interquartile range) or no. (%). p values in bold are statistically significant. ARVs, antiretroviral drugs.

†Calculation excluded 11 patients with unknown HIV status.



**Figure 1.** Kaplan–Meier survival curves of 2-year survival probability (product limit survival estimates) for patients with extensively drug-resistant tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010. Group 0, HIV-negative patients; group 1, HIV-positive patients receiving antiretroviral drugs at start of treatment; group 2: HIV-positive patients not receiving antiretroviral drugs at start of treatment. +, censored value.

significant difference in 2-year survival between HIV-positive patients with or without ARV treatment ( $p = 0.89$ ). In pairwise comparisons, we found that the only difference between the 3 groups was that HIV-positive patients receiving ARVs at start of treatment had better survival rates than HIV-positive patients not receiving ARVs ( $p = 0.04$ ).

We compared patients who initiated treatment early in the cohort, immediately after XDR TB was first reported and explicitly treated (October 2006–February 2007), with patients treated in the last 6 months of the cohort. We found no improvement in sputum culture conversion ( $p = 0.46$ ) or favorable outcome ( $p = 0.70$ ).

HIV-positive patients who initiated ARV treatment during XDR TB treatment had a median baseline CD4 count of 192 cells/mm<sup>3</sup> (IQR 118–236 cells/mm<sup>3</sup>). We found no difference in favorable treatment outcomes among HIV-positive patients who initiated ARV treatment before or after start of XDR TB treatment ( $p = 0.59$ ).

### Culture Conversion

Of 78 patients who achieved culture conversion during the study, 71 had a known treatment outcome. Of these, 38 (53.5%) had culture conversion within 4 months of start of treatment, and an additional 20 (81.7%) patients had culture conversion within 8 months of start of treatment.

However, even patients who achieved sputum culture conversion had poor treatment outcomes overall: 39 (55.7%) patients had unfavorable treatment outcomes (11 patients died, 10 were lost to follow-up, and 18 were alive but showed treatment failure with persistently positive sputum cultures). We found no difference in favorable treatment outcome between patients with early culture

conversion (within either 4 months or 8 months of start of treatment) and patients with culture conversion after 8 months ( $p = 0.16$  and  $p = 0.35$ , respectively) (Figure 2).

### Multivariate Predictors of Favorable Treatment Outcome

Multivariate analysis showed that HIV status was not predictive of a favorable outcome (Table 3). Smear-negative patients were nearly 3-fold more likely to have a favorable treatment outcome (hazard ratio [HR] 2.69, 95% CI 1.29–5.63,  $p = 0.009$ ). Patients who weighed >50 kg at start of treatment were nearly 4-fold more likely to have a favorable outcome (HR 3.64, 95% CI 1.68–7.92,  $p = 0.001$ ).

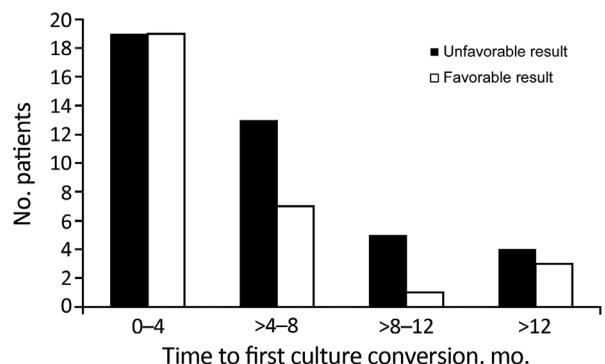
### Multivariate Predictors of Unfavorable Treatment Outcome

HIV-positive patients were more likely to have an unfavorable treatment outcome (adjusted HR 1.35, 95% CI 1.06–1.72,  $p = 0.02$ ) (Table 4). Weight <50 kg at start of treatment was the strongest predictor of an unfavorable treatment outcome for all patients (adjusted HR 1.56, 95% CI 1.23–1.98,  $p = 0.0003$ ).

### Discussion

For this large cohort of patients with XDR TB, we confirm previously reported poor outcomes in patients with or without HIV co-infection (1,3,13). Overall, 28.8% of patients were alive after 2 years of treatment, and 10.3% patients had a favorable outcome of cure or completion. This cohort survived a long duration of inadequate TB treatment before XDR TB treatment initiation, which is likely reflected in these results. Few patients with >6 months of previous MDR TB treatment were able to achieve a cure.

Patients in this study were in the first cohort of patients treated explicitly for XDR TB in South Africa. These patients were the first group to have access to additional second-line drugs, such as capreomycin and para-amino



**Figure 2.** Favorable and unfavorable treatment outcomes for patients with extensively drug-resistant tuberculosis, according to time-to-first sputum culture conversion, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010.

**Table 3.** Predictors of favorable outcome among patients initiating treatment for extensively drug-resistant tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010\*

Predictor	Unadjusted analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
HIV positive	1.08 (0.59–1.98)	0.81	1.29 (0.60–2.75)	0.52
Previous MDR TB treatment	0.59 (0.33–1.06)	0.08	0.77 (0.35–1.66)	0.50
Smear negative	2.97 (1.41–6.26)	<b>0.004</b>	2.69 (1.29–5.63)	<b>0.009</b>
Age	0.99 (0.97–1.02)	0.55	0.99 (0.96–1.02)	0.58
Weight >50 kg	1.93 (0.93–4.02)	0.08	3.64 (1.68–7.92)	<b>0.001</b>
KwaZulu Province	1.98 (1.12–3.52)	0.02	1.69 (0.81–3.53)	0.16
Male sex	0.57 (0.30–1.09)	0.09	0.77 (0.36–1.67)	0.51
Cavitary disease by chest radiograph	0.61 (0.33–1.10)	0.10	0.50 (0.24–1.03)	0.06

\*p values in bold are statistically significant. HR, hazard ratio; MDR TB, multidrug-resistant tuberculosis.

salicylic acid, as well as moxifloxacin in later instances. However, they were also survivors of previous failed or failing regimens for MDR TB treatment: patients were initiating XDR TB treatment a median of 4 months (IQR 0–8 months) after MDR TB treatment and an additional 8 months (IQR 5–12 months) after treatment for drug-susceptible TB (Table 1). As awareness has increased, patients have initiated appropriate TB treatment more rapidly. Furthermore, additional treatment options, such as linezolid, clofazimine, and bedaquiline and delamanid, have become available (14–16).

We found that patients with early culture conversion, within either 4 months or 8 months of treatment initiation, were not more likely to have a favorable outcome than patients with culture conversion after 8 months. Although overall treatment outcomes were poor, patients with culture conversion well into treatment (after 8–12 months) could still achieve cure. Conversely, patients without sputum culture conversion or with culture reversion could still survive 2 years of treatment and be counted as treatment successes. Although sputum culture conversion can be an appropriate surrogate endpoint for patients with drug-sensitive TB (17) and HIV-negative patients with MDR TB (18), it was not predictive in this study population.

When we assessed risk factors for favorable and unfavorable treatment outcomes, we found that HIV-positive patients receiving ARVs at start of treatment had similar treatment outcomes as patients not receiving ARVs. We take this finding as evidence that in this cohort of HIV-positive patients, ARVs were given too late in the disease

course of a patient and that these patients received an XDR TB treatment regimen that was insufficient, such that poor outcomes were seen regardless. Multivariate analysis showed that HIV-positive patients were more likely to have an unfavorable outcome, but that HIV status was not a predictor of a favorable treatment outcome. We speculate that this discrepancy might be caused by difficulty in achieving a favorable outcome for XDR TB in any patient, especially the malnourished population of patients with chronic TB included in this cohort.

It likely that given the chronicity of disease and paucity of TB treatment options available in South Africa for the study cohort at the time of the study, patients were often unable to achieve cure by the time they were able to initiate appropriate treatment. This situation was true for patients who initiated treatment in October 2006, when XDR TB was first treated, as it was for patients who initiated treatment more than a year later, in January 2008.

Anecdotally, medication adherence during this study was suboptimal. Hospitalized patients were not always observed while taking medications; medications were often dispensed in cups for patients to take with meals. The consistency of DOT in long-term hospitalized patients has not been reported, but hospitalization should not be equated with DOT. Although policy prescribes DOT for all discharged patients, this policy is not always practical and feasible. Treatment incentive and enablers might improve treatment adherence (19) but were not available. Drugs for treatment of drug-resistant infections can cause severe side effects. In the absence of treatment support, patients

**Table 4.** Predictors of unfavorable outcome among patients initiating treatment for extensively drug-resistant tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa, 2006–2010\*

Predictor	Unadjusted analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
HIV positive	1.37 (1.09–1.72)	<b>0.005</b>	1.35 (1.06–1.72)	<b>0.02</b>
Previous MDR TB treatment	0.96 (0.76–1.23)	0.76	1.04 (0.77–1.42)	0.78
Smear positive	1.34 (1.07–1.68)	<b>0.01</b>	1.25 (0.97–1.62)	0.09
Age	0.99 (0.98–1.00)	0.25	0.100 (0.100–1.01)	0.69
Weight <50 kg	1.66 (1.33–2.08)	<b>&lt;0.0001</b>	1.56 (1.23–1.98)	<b>0.0003</b>
KwaZulu Province	1.00 (0.78–1.28)	0.99	0.92 (0.67–1.26)	0.60
Male sex	0.92 (0.74–1.14)	0.43	0.99 *0.77–1.27)	0.94
Cavitary disease by chest radiograph	0.99 (0.78–1.26)	0.94	0.99 (0.76–1.30)	0.96

\*p values in bold are statistically significant. HR, hazard ratio; MDR TB, multidrug-resistant tuberculosis.

might be more noncompliant than treatment records show. Imperfect DOT and limited support offered to patients might result in treatment interruptions, which could help further explain poor treatment outcomes.

Although access to HIV care improved during this time (20), only 1 new, possibly effective, drug, moxifloxacin, was made available during the study. Our finding of similarly poor results in HIV-negative and HIV-positive patients with or without HIV treatment underscores the WHO recommendation that  $\geq 4$  effective drugs be used in TB treatment. Furthermore, continued integration of vertical and horizontal health systems incorporating care for multiple disease processes is essential (21).

As in our study, previous research in resource-limited settings showed higher mortality rates in HIV-positive patients with smear-negative disease (22,23). A US study of HIV-positive patients with TB reported lower mortality rates in smear-negative case-patients, which the authors attributed to early-stage disease, given normal chest radiographic results for these patients (24). In that cohort, patients had a long history of previous treatment for drug-sensitive TB and MDR TB, and no difference was found among smear-positive and smear-negative patients.

The strength of our study was the large number of patients treated for XDR TB, many of whom were also HIV positive. Furthermore, repeated follow-up site visits while patients continued to receive treatment enabled us to reduce the problem of missing data, which can adversely affect retrospective and prospective series in resource-limited settings. Our cohort also provides context to initial reports in 2006 of XDR TB as a near universally fatal disease in Tugela Ferry, KwaZulu-Natal Province, where 98% of patients died in a median of 16 days (2).

Our study included only patients who survived to initiate treatment at the provincial hospital but showed that cure is still possible. In KwaZulu-Natal Province, an estimated 50%–70% of patients with MDR TB did not initiate treatment (13,25), but our previous study in Eastern Cape Province showed that 23.7% of patients did not survive to start treatment (5). By studying consecutive patients initiating treatment in 2 provinces, we could demonstrate some favorable outcomes. Our finding of less previous TB treatment in HIV-positive patients not receiving ARVs probably reflects this finding because many patients probably died before they were able to initiate appropriate treatment for either disease.

A further limitation of our study was that we were only able to assess HIV status and use of ARVs at the time of XDR TB treatment initiation and as dichotomous variables. These limitations represent a range of immunologic compromise and risk for death during treatment for XDR TB. More recent guidelines from 2013 state that all HIV-positive patients with any TB diagnosis should be given ARVs, which might have improved survival in our cohort

(26). We found that HIV-positive patients receiving ARVs had better survival than patients not receiving ARVs, but HIV-positive patients were not more likely to achieve cure. The 2 HIV-positive patients not receiving ARVs who had a favorable treatment outcome initiated treatment when they had CD4+ counts  $>400$  cells/mm<sup>3</sup>. Gandhi et al. reported a step-wise increase in mortality rates for HIV-positive patients with at least MDR TB and CD4 counts  $<50$  cells/mm<sup>3</sup>, 50–200 cells/mm<sup>3</sup>, and  $>200$  cells/mm<sup>3</sup> (13).

Since 2008, when the last patients included in this research initiated treatment, many lessons have been learned in the management of XDR TB, resulting in programmatic and treatment changes. Since 2011, treatment for XDR TB has been decentralized in South Africa, and as of June 2015, a total of 400 sites were treating patients who had drug-resistant TB (27). Treatment regimens have been fortified by the addition of bedaquiline, delamanid, linezolid, clofazamine, and levofloxacin (28). Therefore, patients with XDR TB might be able to receive 3–5 effective drugs. Patients co-infected with drug-resistant TB and HIV are immediately given treatment for TB and HIV infection (29).

Our results demonstrate the need for rigorous follow-up of patients receiving treatment for TB. Just as MDR TB is caused by systemic failures in treatment for drug-susceptible TB, XDR TB develops when MDR TB is inadequately treated. Without  $\geq 4$  effective drugs in a TB treatment regimen and consistent adherence to medications, treatment cure is rare. These results, as well as those from Tugela Ferry in 2006 (2), illustrate worst-case scenarios in health systems. When TB or HIV infection are inadequately treated, diseases might spread rapidly and have lethal results.

C.L.K. collected data; C.L.K. and J.P.C. analyzed data; and the 3 co-authors designed the study, wrote the article, and approved the final version.

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# Clinical Features of and Risk Factors for Fatal Ebola Virus Disease, Moyamba District, Sierra Leone, December 2014–February 2015

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The 2013–2016 outbreak of Ebola virus disease (EVD) in West Africa infected >28,000 people, including >11,000 who died, and disrupted social life in the region. We retrospectively studied clinical signs and symptoms and risk factors for fatal outcome among 31 Ebola virus–positive patients admitted to the Ebola Treatment Center in Moyamba District, Sierra Leone. We found a higher rate of bleeding manifestations than reported elsewhere during the outbreak. Significant predictors for death were shorter time from symptom onset to admission, male sex, high viral load on initial laboratory testing, severe pain, diarrhea, bloody feces, and development of other bleeding manifestations during hospitalization. These risk factors for death could be used to identify patients in need of more intensive medical support. The lack of fever in as many as one third of EVD cases may have implications for temperature-screening practices and case definitions.

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Since its discovery in 1976 (1), Ebola virus has caused small sporadic outbreaks with high case-fatality rates (CFRs), mostly in rural areas in central and eastern Africa (2). The outbreak of Ebola virus disease (EVD) in West Africa that began in Guinea in December 2013 (3,4) and subsequently spread to Sierra Leone and Liberia (5) is the largest known outbreak to date: >28,000 cases and >11,000 deaths were reported (6). Weak health systems, deep-rooted traditional burial customs, high population mobility, and early spread of disease in urban areas contributed to the unprecedented extent of the epidemic (5,7,8). The high death toll among healthcare workers further undermined weak existing health systems (9). Apart from Médecins Sans Frontières and a few other organizations, the international community responded slowly (8,10). However, a turning point came with a United Nations resolution on August 8, 2014, that led to creation of UNMEER (UN Mission for Ebola Emergency Response) on September 19. Leading public health agencies predicted that the epidemic could spiral out of control and estimated that, in a worst-case scenario, 1.4 million persons could become infected (11). In response, an increasing number of international stakeholders became involved, and several countries made direct monetary contributions. The epidemic peaked in September–October 2014 in Liberia and in December 2014 in Sierra Leone and Guinea; a gradual decline in the number of new cases followed (6,8).

The sporadic nature of previous Ebola outbreaks in remote areas of Africa has hampered the collection of clinical and laboratory data. In the 13 previously described outbreaks of Zaire EVD, the average CFR was 81% (1,123 deaths/1,390 cases) (12). The natural history of the 2013–2016 EVD outbreak in West Africa may vary from that in previous outbreaks. The World Health Organization (WHO)

reported an overall CFR of 40% (11,314 deaths/28,634 cases) for the outbreak, which is probably an underestimate, and a CFR of 58% (513 deaths/881 cases) among healthcare workers (6). However, a large WHO-led study across the region found an average CFR of 71% (13).

Specific treatment for EVD is not available, but aggressive supportive treatment has resulted in increased survival (14–17). Identification of patient groups with a higher risk for death could help target comprehensive supportive therapy to those most in need and could ultimately improve outcomes. Considering the evidence of Ebola virus persistence and delayed sexual transmission (18), late relapse of EVD in survivors (19), reemergence of EVD in Liberia 2 months after the country was declared Ebola-free (20), and the likely natural reservoir of Ebola virus in bats (4), it is imperative that health systems draw on lessons learned during the West Africa outbreak to prepare for future EVD outbreaks (21,22). We studied the clinical features of and risk factors for death among patients admitted to the Ebola treatment center (ETC) in Moyamba District, Sierra Leone, during mid-December 2014–March 2015.

## Methods

### Study Design

We performed a retrospective, descriptive study of clinical data from all patients admitted to the ETC in Moyamba District, Sierra Leone, one of the countries hardest hit by the West Africa Ebola epidemic. Moyamba District, located on the Atlantic Coast southeast of Freetown, the capital of Sierra Leone, has a rural population dispersed across 14 chiefdoms. The Moyamba ETC, one of 23 ETCs in the country, was established by the UK Department for International Development, administrated by the nongovernmental organization Médicos del Mundo, and manned by healthcare workers from Sierra Leone, Spain, France, United Kingdom, and Norway (23). We collected available data from all patients admitted to the ETC from its opening on December 19, 2014, until its closure on March 31, 2015; we obtained data on demographics, potential exposure situations, symptoms, findings, PCR test results, treatment, and outcome. The last patient with confirmed EVD was discharged on February 17, 2015. We retrospectively defined severe pain as pain clinically assessed to be severe enough as to lead the clinician to prescribe opiates. Laboratory resources (e.g., the ability to measure electrolytes) were not available at the ETC. Data were systematically compiled from multiple sources, including triage forms, patient records, and laboratory registries, and were plotted anonymously in EpiData 2.0 (EpiData Association, Odense, Denmark). The study was approved by the Sierra Leone Ethics and Scientific Review Committee (expedited review approved April 28, 2015) and the Western

Norwegian Regional Committee for Medical and Health Research Ethics (reference no. 2015/538).

### Diagnostic Methods

Through January 11, 2015, diagnostic services (Ebola Zaire virus nucleoprotein real-time reverse transcription PCR [rRT-PCR]) were provided in Bo, Sierra Leone, by a US Centers for Disease Control and Prevention laboratory. On-site diagnostics were provided, beginning January 12, 2015, by the US Department of Defense (via the Defense Threat Reduction Agency's Cooperative Biological Engagement Program) MEDaC (Moyamba Ebola Diagnostic Center) laboratory, which used 2 Ebola Zaire rRT-PCR assays provided by the Department of Defense Critical Reagent Program: a glycoprotein gene assay and a nucleoprotein gene assay (TaqMan minor groove protein binder). In addition, the laboratory ran a third PCR, the human RNase P assay, on every sample as a control for nucleic acid extraction and amplification. Genetic material was extracted robotically from whole blood by using the EZ1 Advanced XL instrument (QIAGEN, Hilden, Germany); rRT-PCR was subsequently performed using the AB 7500 Fast Dx Real-Time PCR Instrument (Applied Biosystems, Carlsbad, CA, USA). Positive template and negative extraction controls were included in every RT-PCR run; no-template controls for master mix and sample addition were also included. Negative controls during nucleic acid extraction and PCR set-up were used to screen for false positive stray template or potential contamination. This analysis provides semiquantitative results expressed as cycle thresholds ( $C_t$ s). As requested from Ministry of Health and Sanitation, a malaria rapid test (SD. BIOLINE malaria Ag P.f [HRP-II]; Alere Standard Diagnostics, Yongin, South Korea) was added to the diagnostic workup for each patient beginning in February 2015.

### Statistical Analysis

We expressed the magnitude and statistical significance of risk factors as odds ratios with 95% CIs and p values; 2-tailed  $p < 0.05$  was used as the cutoff for statistical significance. Statistical analyses were performed in Stata 14 (StataCorp, College Station, TX, USA). To assess differences between proportions, we used the Fisher 2-tailed exact mid-p test (syntax file fishermidp.ado provided by M.W. Fagerland, Oslo Centre for Biostatistics and Epidemiology, Research Support Services, Oslo University Hospital, Oslo, Norway); to calculate odds ratios and 95% CIs, we used the Baptista–Pike or Cornfield mid-p interval with the mercii command. For comparison of continuous variables, including time variables, we used the 2-sample Fligner–Policello robust rank order test with the fprank command in Stata. For comparison of ordered groups (e.g., age groups), we used the nonparametric test for trend

across ordered groups with the `np trend` command in Stata. Kaplan–Meier survival plots were developed using the `sts` graph function in Stata.

## Results

### Patients

We performed PCR on samples for 82 (93%) of the 88 patients admitted to the Moyamba ETC; 31 (38%) were positive for Ebola virus. Of these 31 patients, 28 (90%) reported contact with confirmed or suspected EVD case-patients, most of whom were household members (64%, 18/28); 11 (35%) had participated in burials for suspected EVD patients. No healthcare workers were among the 31 persons with confirmed EVD. Most of the Ebola virus–positive patients (87%, 27/31), including all who died, came from Ribbi chiefdom; 14 (45%) were male and 17 (55%) female (Table 1). Eighteen (58%) patients were 21–45 years of age (median 30 years, range 3 months–85 years). Overall CFR was 58% (18/31 died) (Figure 1, panel A), but the CFR was significantly higher among male than female patients (86% [12/14] vs. 35% [6/17];  $p = 0.007$ ) (Figure 1, panel B). No significant correlation was found between age and fatal outcome.

### Incubation Period

Because of the retrospective study design and because patients in critical condition at admission could not give detailed histories, the time of exposure could be established for only 10 of the 18 patients who died and 10 of the 13 patients who survived. For those 20 patients, the median incubation period was 8 (range 1–17) days; no significant difference was found between fatal and nonfatal cases (Table 1). However, the median time from onset of symptoms to admission was significantly shorter for patients who died than for those who survived ( $p = 0.006$ ). For the 18 patients who died, the median time from symptom onset to death was 6 (range 2–18) days; most (16/18 [89%]) died 4–11 days after symptom onset. EVD survivors were discharged a median of 19.5 (range 12–45) days after symptom onset, when they were asymptomatic and had negative Ebola PCR results ( $C_t > 36$ ).

### Clinical Features

The most frequent symptoms among patients at admission were weakness (97%), diarrhea (68%), fever (62%), loss of appetite (62%), vomiting (58%), pain in muscles (62%) and joints (55%), headache (55%), abdominal pain (45%), and red eyes (42%) (Table 1). At admission, diarrhea was significantly more common among patients who died than those who survived (83% vs. 46%;  $p = 0.036$ ).

Bleeding was present in 35% (11/31) of patients at admission and occurred in 55% (17/31) at any time during

their hospital stay; bleeding occurred significantly more frequently among patients who died than those who survived (78% vs. 23%;  $p = 0.002$ ) (Figure 1, panel C). Bloody feces was the most frequent hemorrhagic manifestation and a predictor of fatal outcome: 28% (5/18) of patients who died and none of those who survived had bloody feces at admission ( $p = 0.033$ ), and 72% (13/18) of patients who died and none of those who survived had bloody feces at any time during their hospital stay ( $p < 0.001$ ). Bleeding from the mouth ( $p = 0.031$ ) or puncture sites ( $p = 0.019$ ) during hospitalization was also associated with death.

Pain was a prominent clinical feature and was often severe. All 18 patients who died reported pain, compared with 85% (11/13) of patients who survived ( $p = 0.084$ ). The ETC was well stocked with analgesic medication, such as paracetamol and morphine, and did not experience shortages. Pain requiring opiate analgesia was significantly more frequent among patients who died than those who survived (89% [16/18] vs. 23% [3/13];  $p < 0.001$ ). The mean daily morphine doses given to patients who eventually died (5.9 mg [range 2.3–15.0 mg]) and those who survived (4.4 mg [range 2.5–7.5 mg]) did not differ significantly ( $p = 0.175$ ).

Major neurologic symptoms were infrequent. Among 4 patients with possible neurologic signs at or early after admission, 2 with hiccups died and 1 of 2 patients with seizures died.

### Laboratory Findings

The median time from symptom onset to first Ebola PCR test did not differ significantly for patients who died (3.0 days) and those who survived (3.5 days) ( $p = 0.202$ ). However, on the first PCR after admission, viremia was significantly higher in patients who died (median  $C_t$  20.5 [range 15–23]) than those who survived (median  $C_t$  26.5 [range 22–36.5]) ( $p < 0.001$ ) (Table 1; Figure 1, panel D; Figure 2). All patients with an initial  $C_t < 22$  (high viremia) died, but all patients with an initial  $C_t > 23$  (low viremia) survived.

### Treatment

Oral rehydration solution was administered liberally, and patients were encouraged to drink abundantly. Intravenous fluids were given to 26 (84%) of the 31 patients with confirmed EVD. In total, 29 (94%) patients received anti-malarial treatment. Antimicrobial drugs were used empirically and administered to 26 (84%) patients with confirmed EVD. Among the patients who died, 78% (14/18) and 44% (8/18) received intravenous ceftriaxone and metronidazole, respectively. The difference in medications given to patients with fatal and nonfatal disease was not statistically significant (Table 2).

**Table 1.** Characteristics of patients with confirmed Ebola virus disease admitted to the treatment center in Moyamba District, Sierra Leone, December 19, 2014–February 17, 2015\*

Characteristic	Patients†			OR (95% CI)	p value‡
	Total, N = 31	Died, n = 18	Survived, n = 13		
<b>Demographic characteristics</b>					
Sex					
M	14 (45)	12 (67)	2 (15)	11.0 (2.1–57.0)	0.007
F	17 (55)	6 (35)	11 (65)	0.009 (0.02–0.5)	
Age group, y					
<15	6 (19)	4 (22)	2 (15)	2.0 (0.25–16.3)	0.565§
15–44	19 (61)	11 (61)	8 (62)	1.4 (0.26–7.1)	NA
≥45	6 (19)	3 (17)	3 (23)	1	NA
Healthcare workers	0 (0)	0	0	NA	NA
Length of incubation, median d (range)¶	8 (1–17)	7 (1–10)	8.5 (5–17)	NA	0.059#
Time from symptom onset to admission, median d (range)**	3 (0–23)	2 (0–17)	4.5 (1–23)	NA	0.006#
Time from symptom onset to death/discharge, median d (range)**	10 (2–45)	6 (2–18)	19.5 (12–45)	NA	<0.001#
<b>Signs and symptoms</b>					
Weakness	30 (97)	18 (100)	12 (92)	NA	0.210
Diarrhea	21 (68)	15 (83)	6 (46)	5.8 (1.2–25.0)	0.036
Fever	19 (61)	12 (67)	7 (54)	1.7 (0.42–7.4)	0.597
Loss of appetite	19 (61)	12 (67)	7 (54)	1.7 (0.42–7.4)	0.597
Vomiting	18 (58)	12 (67)	6 (46)	2.3 (0.60–11.0)	0.216
Red eyes	13 (42)	9 (50)	4 (31)	2.3 (0.55–8.3)	0.378
Nausea	8 (26)	4 (22)	4 (31)	0.64 (0.16–2.7)	0.551
Dysphagia	8 (26)	5 (28)	3 (23)	1.3 (0.22–5.7)	0.845
Hiccups	2 (6)	2 (11)	0	NA	0.332
Pain					
Overall	29 (94)	18 (100)	11 (85)	NA	0.084
Muscle pain	19 (61)	10 (56)	9 (69)	0.56 (0.15–2.2)	0.373
Joint pain	17 (55)	10 (56)	7 (54)	1.1 (0.27–4.1)	0.858
Headache	17 (55)	11 (61)	6 (46)	1.8 (0.48–7.7)	0.378
Abdominal pain	14 (45)	9 (50)	5 (38)	1.6 (0.42–7.3)	0.599
Chest pain	7 (23)	3 (17)	4 (31)	0.45 (0.10–2.1)	0.302
Pain requiring opiates	19 (61)	16 (89)	3 (23)	27 (3.9–144.0)	<0.001
Bleeding manifestations					
At admission	11 (35)	8 (44)	3 (23)	2.7 (0.61–11.0)	0.202
In feces	5 (16)	5 (28)	0	NA	0.033
From mouth	2 (6)	1 (6)	1 (8)	0.71 (0.04–14.0)	0.748
From eyes	1 (3)	0	1 (8)	0.0 (0–6.5)	0.210
From genitals	3 (10)	1 (6)	2 (15)	0.32 (0.02–3.2)	0.401
From puncture sites	1 (3)	1 (6)	0	NA	0.710
Any time during hospitalization	17 (55)	14 (78)	3 (23)	12 (2.3–50.0)	0.002
In feces	13 (42)	13 (72)	0	NA	<0.001
From mouth	9 (29)	8 (44)	1 (8)	9.6 (1.2–114.0)	0.031
From eyes	4 (13)	3 (17)	1 (8)	2.4 (0.31–33.0)	0.452
From genitals	6 (19)	4 (22)	2 (15)	1.6 (0.29–9.3)	0.838
From puncture sites	7 (23)	7 (39)	0	NA	0.019
Cycle threshold, median (range)††	22 (15.0–36.5)	20.5 (15–23)	26.5 (22.0–36.5)	NA	<0.001#

\*NA, not applicable; OR, odds ratio.

†Data are no. (%) except as indicated.

‡By Fisher 2-tailed exact mid-p test, except for ordered groups and continuous variables.

§By nonparametric test for trend across ordered groups (i.e., age groups).

¶Incubation time was unknown for 8 patients with fatal and 3 with nonfatal disease.

#By 2-sample Fligner-Policello robust rank order test for continuous variables (i.e., time variables, PCR values).

\*\*Time was unknown for 1 patient with nonfatal disease.

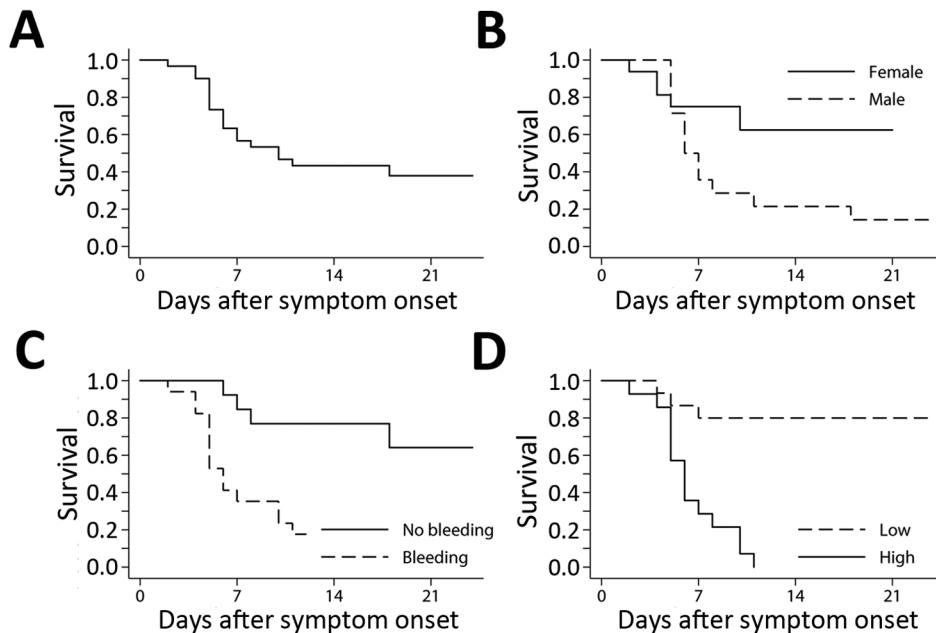
††Determined by real-time reverse transcription PCR; value was unknown for 1 patient with fatal disease.

## Discussion

Our principal findings from this study were that EVD patients in Moyamba District had a higher rate of bleeding manifestations than reported elsewhere; a third of patients did not have fever at admission; and predictors for fatal outcome were shorter time from onset to admission, male sex, high viral load on initial laboratory test, severe pain,

diarrhea or bloody feces at admission, and development of bleeding manifestations during hospital stay.

The main limitations of the study were the retrospective study design and the relatively small number of patients. Some data were incomplete because patients were in a critical condition when admitted and the hands-on time with patients was short because of infection control



**Figure 1.** Survival analysis for patients with confirmed Ebola virus disease admitted to the treatment center in Moyamba District, Sierra Leone, December 19, 2014–February 17, 2015. Survival among A) all patients; B) male and female patients; C) patients with and without bleeding manifestations at admission; and D) patients with initial PCR results showing high- and low-level viremia, as defined by cycle thresholds <22 and ≥22, respectively.

considerations. Cultural and linguistic differences among staff and patients may have represented a challenge for data collection and patient management. Laboratory testing was limited to PCR confirmation of EVD. In addition, the catchment area for the Moyamba ETC included some poorly accessible areas with strong traditional medicine influence (24), so ETC admissions could have been biased toward persons with severe disease and patients who sought care late in the disease course.

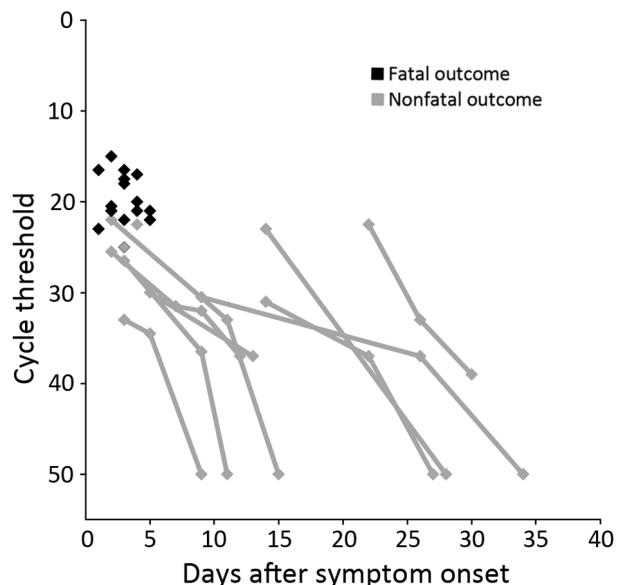
Strengths of the study were the availability of highly dedicated local staff; skilled international healthcare workers, including the almost continuous presence of medical doctors; availability of intravenous fluids and other supportive treatment; and consistent access to semiquantitative PCRs for Ebola virus testing. Due to the limited number of patients, clinicians could establish trusted associations with patients and meticulously register clinical features and responses to supportive treatment. The continuous presence of clinical staff enabled consistent data registration over time, decreasing the risk of registration bias.

Most patients were infected through contact with household members or burials for EVD patients. In contrast with findings from other treatment facilities during this and past outbreaks (24–26), none of the confirmed cases in Moyamba were among healthcare workers. The Moyamba ETC opened late in the epidemic; thus, lessons learned from other ETCs may have helped healthcare workers avoid virus transmission.

At the Moyamba ETC, the overall CFR was 58% for confirmed EVD patients; this rate is lower than that reported across Sierra Leone, Liberia, and Guinea (71% CFR) (13) but similar to that reported for healthcare workers in

the region (6). The Moyamba ETC started operations in December 2014; at that late stage in the epidemic, most communities were better informed about EVD, and ill persons knew where to report for suspected EVD. Thus, unlike patients with mild symptoms earlier in the epidemic, patients with mild symptoms at this later stage may have sought healthcare. The Moyamba ETC was well equipped and staffed with competent local and international staff who could provide good care for the relatively few hospitalized patients. Of note, other ETCs, especially the one in Hastings, Sierra Leone, have documented CFRs as low as 23% in patients given comprehensive supportive treatment (14). However, the higher CFR in Moyamba could partly be explained by a bias favoring selection of severe cases from some of the less accessible areas that rely extensively on traditional medicine; in such areas, milder cases would have been treated locally rather than in Moyamba. Factors such as education level, cultural and socioeconomic factors, and health-seeking behavior may also have contributed to the differences in CFRs.

No significant difference was seen in incubation time between fatal and nonfatal groups, but at admission, patients who eventually died had a clinical picture of rapidly progressing illness. The median time from symptom onset to admission was shorter in patients who died than in survivors. Most patients with fatal disease died 4–11 days after symptom onset. This time coincides with that observed in other West Africa centers during the outbreak (17,26,27). Young and old age have been associated with increased CFRs in other studies (17,26), but we found no such association. However, the mortality rate was higher among male (86%) than female (35%) patients ( $p = 0.007$ ),



**Figure 2.** Ebola viral load for patients with confirmed Ebola virus disease admitted to the treatment center in Moyamba District, Sierra Leone, December 19, 2014–February 17, 2015. Viral loads were determined by semiquantitative PCR and are expressed as cycle thresholds for patients with fatal (n = 18) and nonfatal (n = 13) disease.

consistent with the finding among male (40%) and female (29%) patients in Kerry Town, Sierra Leone (p = 0.181) (27). The reasons for this difference are unknown but might be due to an immunosuppressive effect of androgens and immunostimulatory effect of estrogen (28,29).

Fever is a major symptom in the WHO Ebola case definition, and thermometers have been used widely by healthcare workers and government officials to screen for EVD cases among persons in hospitals, shops, and airports and at road check points. However, as many as a third of the patients at the Moyamba ETC had no fever at admission. Several other reports have also noted the lack of fever among EVD patients (30,31), and there are indications that fever has been present in fewer EVD patients during the 2013–2016 West Africa outbreak compared with previous outbreaks (1,24). Most patients (61%) referred to the Moyamba ETC had negative Ebola PCR results, indicating

that the WHO case definition is not sufficiently specific in identifying EVD cases and, consequently, Ebola-negative patients might be at risk for nosocomial Ebola virus transmission in ETC settings (31,32). A newly developed Ebola prediction score has shown promising results in risk-stratifying suspected EVD patients, but further validation is needed before this method can be put to use (33). The prediction score is based on 6 variables: contact with a sick person; presence of diarrhea, anorexia, or muscle pain; difficulty swallowing; and absence of abdominal pain. Clinical features alone are not sufficiently sensitive or specific to detect EVD cases, emphasizing the urgent need for an effective point-of-care test (31,33). A rapid diagnostic antigen test, which was recently tested in Sierra Leone, may become an efficient tool for excluding EVD among suspected cases in the future (34).

In general, bleeding manifestations were seen more frequently in the Moyamba patients than in those from other treatment facilities in Sierra Leone (17,25), but bleeding was less frequent in Moyamba patients than in patients during the 1976 outbreak (1). At Kenema Government Hospital in Kanema, Eastern Province, Sierra Leone, evidence of bleeding was found in only 1 of 106 EVD patients during the 2013–2016 outbreak (17). At the ETC in Moyamba, some of the patients with bleeding manifestations were from the same household and admitted during the same period. In the ETC, patients were not given any drugs that could have increased the risk of bleeding (e.g., nonsteroidal antiinflammatory drugs). However, we do not know if, before ETC admission, the patients took such medicines or any traditional medicine that might cause bleeding. Because of limitations on laboratory services, we had no information on whether patients might have concurrent diseases (e.g., malaria, Lassa fever, dengue fever) that might increase the tendency to bleed. In addition, many patients came from the same family, so we cannot exclude some form of genetic predisposition for bleeding manifestations. Furthermore, we lack knowledge about the different Ebola virus strains and routes of transmission in Moyamba District and cannot exclude the possibility that properties of the virus may have evolved during the epidemic.

Diarrhea and bloody feces on admission were significant risk factors for fatal outcome. Most patients who died

**Table 2.** Treatment given to patients with confirmed Ebola virus disease admitted to the treatment center in Moyamba District, Sierra Leone, December 19, 2014–February 17, 2015\*

Treatment	No. (%) patients			OR (95% CI)	p value†
	Total, N = 31	Died, N = 18	Survived, N = 13		
Intravenous fluids	26 (84)	17 (94)	9 (69)	7.6 (0.90–97.0)	0.096
Antimalarial drugs	29 (94)	17 (94)	12 (92)	1.4 (0.07–28.0)	0.748
Ceftriaxone	23 (74)	14 (78)	9 (69)	1.6 (0.37–6.4)	0.551
Metronidazol	13 (42)	8 (44)	5 (38)	1.3 (0.33–5.8)	0.864
Albendazol	5 (16)	1 (6)	4 (31)	0.1 (0.01–1.1)	0.096
Zinc	29 (94)	17 (94)	12 (92)	1.4 (0.07–28.0)	0.748

\*OR, odds ratio.

†p values calculated by the Fisher 2-tailed exact mid-p test.

(72%), but no survivors, experienced bloody feces during their hospital stay. The occurrence of bleeding manifestations overall as well as bleeding from the mouth or puncture sites during hospitalization also predicted fatal outcome. Bloody diarrhea may be attributable to severe enterocolitis caused by Ebola virus; disseminated intravascular coagulation; or concurrent bacterial, viral, or malarial infection (24,35). Chest pain was frequent and may suggest upper gastrointestinal tract involvement, particularly in combination with dysphagia. However, previous studies have suggested that pericarditis and myocarditis may cause chest pain in EVD (24). Although rhabdomyolysis has been postulated as a contributing factor to progressive renal failure and death in EVD (17,27), muscle pain was common in our study but was not a risk factor for death. Abdominal pain in EVD is probably of multifactorial etiology, and underlying pancreatitis has been proposed as a cause (1,24,35). Although a frequent finding, abdominal pain did not predict death in this study.

Almost all EVD patients reported pain, but severe pain requiring opiate treatment was significantly more frequent among patients with fatal disease. Attention should be given to palliation of severe pain in EVD. However, in this study, the short hands-on time with patients because of personal protection procedures limited the possibility of adequately treating pain with parenteral opiates. Transdermal administration of opiates, such as fentanyl-containing patches, was not available in the Moyamba ETC but would be a safer alternative for providing pain relief to EVD patients.

High viremia on admission was a strong predictor for death (100% fatality among patients with  $C_t$ s <22), and low viremia was a good prognostic sign (100% survival among patients with  $C_t$ s >23). This finding supports those from other studies (17,24,26,27,36,37) and should be kept in mind when interpreting trials of experimental treatments for EVD (38).

Systematic, comprehensive supportive therapy, including antimalarial, antimicrobial, and antihelminthic treatment, has been suggested to improve the prognosis for EVD patients (14,27). Although all patients admitted to the Moyamba ETC were treated according to the protocols developed by WHO and health authorities in Sierra Leone, the retrospective study design and the small sample size may have impeded the assessment of associations between treatment and outcome (26).

In summary, our findings are in agreement with those from other studies, but bleeding manifestations appeared to be more common in Moyamba than elsewhere and associated with fatal outcome. Awareness of risk factors for death, including short time from symptom onset to admission, male sex, diarrhea, bloody feces and other bleeding manifestations, severe pain, and high viral load, could be

used to group patients at greatest risk into dedicated wards with more intensive medical support. Selective use of intravenous fluid therapy could be a rational approach when resource constraints and infection control considerations prevent delivery of fluid therapy to all patients. Severe pain was common, particularly among moribund patients, calling attention to the need for adequate and safe pain relief (e.g., with transdermal administration of opiates) for EVD patients. The lack of fever in as much as one third of EVD patients and the finding that 61% of admitted patients tested negative for EVD may have implications for screening practices, case definitions, and isolation strategies. The sharing of clinical experiences regarding EVD, a hitherto rare disease, may help prepare for more effective patient care in future outbreaks.

### Acknowledgments

We thank all doctors, nurses, hygienists, logisticians, and other persons from Sierra Leone, Norway, Spain, France, the United Kingdom, and elsewhere who cared for patients in the Moyamba ETC. We also express our deep-felt sympathy and condolences to the families and societies affected by EVD in Moyamba District and elsewhere.

Dr. Haaskjold worked with the coordinating group for the Norwegian National Ebola Response hosted at Haukeland University Hospital and currently serves as resident doctor in the Department of Medicine at the same hospital. His areas of interest include nephrology, infectious diseases, and global health.

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# Travel- and Community-Based Transmission of Multidrug-Resistant *Shigella sonnei* Lineage among International Orthodox Jewish Communities

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Shigellae are sensitive indicator species for studying trends in the international transmission of antimicrobial-resistant *Enterobacteriaceae*. Orthodox Jewish communities (OJCs) are a known risk group for shigellosis; *Shigella sonnei* is cyclically epidemic in OJCs in Israel, and sporadic outbreaks occur in OJCs elsewhere. We generated whole-genome sequences for 437 isolates of *S. sonnei* from OJCs and non-OJCs collected over 22 years in Europe (the United Kingdom, France, and Belgium), the United States, Canada, and Israel and analyzed these within a known global genomic context. Through phylogenetic and genomic analysis, we showed that strains from outbreaks in OJCs outside of Israel are distinct from strains in the general population and relate to a single multidrug-resistant sublineage of *S. sonnei* that prevails in Israel. Further Bayesian phylogenetic analysis showed that this strain emerged approximately 30 years ago, demonstrating the speed at which antimicrobial drug-resistant pathogens can spread widely through geographically dispersed, but internationally connected, communities.

Antimicrobial-resistant (AMR) *Enterobacteriaceae* are recognized as a global public health threat (1,2). Understanding the emergence of these pathogens and

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tracking transmission across international borders is vital for informing public health surveillance, intervention, and management (3). *Shigella* spp. are *Enterobacteriaceae* that cause severe, acute diarrhea resulting in mortality rates second only to rotaviruses as known agents of diarrheal disease (4). Shigellae cause disease in both low- and high-income nations (5), and  $\geq 10$  organisms can initiate disease (6). Shigellae are also increasingly resistant to antimicrobial drugs (7–10). Because of the large global burden of shigellosis, the low infective dose, highly visible disease syndrome, and ability to acquire AMR, shigellae are a relevant and sensitive indicator species for studying trends in the global transmission and emergence of AMR enteric bacteria.

Of the recognized *Shigella* spp., the distribution of *S. sonnei* makes it particularly relevant for studying international transmission because it is the most commonly isolated species in middle- to high-income nations (5) and causes a substantial disease incidence in low-income nations; for example, 23.7% of all documented shigellosis cases causing moderate to severe diarrhea in children <5 years of age in Africa and Asia (11). Moreover, *S. sonnei* prevalence increases as nations develop economically (12–15). To examine the underlying processes of such broad epidemiologic phenomena over medium- to long-term scales in bacterial populations, robust, high-resolution molecular subtyping is used. Subtyping of *S. sonnei* by using whole-genome sequencing has defined a global population structure that is divided into 4 lineages; the third lineage, global III, disseminated globally after acquiring multidrug resistance (MDR) (16). This advanced subtyping and established global context was used to show that the rise of *S. sonnei* in Vietnam was attributable to the point introduction and subsequent expansion of a single sublineage in the 1980s (17), demonstrating the effectiveness of this approach for characterizing epidemiologic phenomena.

Similarly, assessing the global burden of a widespread pathogen such as *S. sonnei* calls for use of a patient group in which the effects of illness are international. One such risk group for *S. sonnei* is Orthodox Jewish communities (OJCs) (5). These communities are highly susceptible to shigellosis because of densely populated living conditions, high numbers of young children per family, and intracommunity transfer facilitated by large holiday gatherings (18–20). *S. sonnei* shigellosis is highly endemic to Israel; its incidence there since the early 1990s has primarily been driven by biennial epidemics within OJCs in Israel (primarily in the 0–4-year age group, in whom the incidence is  $\approx 7$  cases/1,000 population/y [19]). In addition to incidence in OJCs in Israel, outbreaks ranging in size from 27 culture-confirmed cases to >13,000 cases of *S. sonnei* shigellosis have been reported in OJCs in Europe and North America (18,20–24). These outbreaks are attributable to highly clonal organisms, determined by using pulsed-field gel electrophoresis (20,22,24) and, in the case of an outbreak in Belgium, linked to prevailing strains from Israel (22). Thus, characterizing the international connectivity of OJC-associated *S. sonnei* represents an opportunity to assess the effects of travel- and community-based associations on the transmission of AMR *Enterobacteriaceae*.

We generated whole-genome sequences from >400 clinical isolates of *S. sonnei* collected over 22 years from OJCs within Israel, OJCs outside of Israel, and non-OJCs in the United Kingdom. We then combined these data with the established genomic global context for *S. sonnei*. By analyzing phylogenetic relationships, we investigated the distinction of strains from OJC outbreaks from other locally circulating strains (i.e., among non-OJCs) and explored the possible epidemiologic relationship of outbreaks in OJCs outside of Israel and endemic shigellosis in Israel. We also sought to determine the relationship of these epidemiologic processes with AMR determinants in *S. sonnei*.

## Materials

We performed whole-genome sequencing on 437 *S. sonnei* isolates as part of this study. These isolates were from patients associated with OJCs outside of Israel ( $n = 171$ ), from 221 patients in Israel (200 OJC, 21 of unknown ethnicity), or from patients in the United Kingdom not associated with OJCs ( $n = 45$ ) (online Technical Appendix 1, <http://wwwnc.cdc.gov/EID/article/22/9/15-1953-Techapp1.xlsx>). The isolates were collected from 6 countries (Israel, the United Kingdom, France, Belgium, the United States, and Canada) during 1992–2014 (Figure 1). The collection included isolates from most previously reported OJC-associated outbreaks of *S. sonnei* shigellosis; we defined cases as being OJC-associated separately for each public health agency (Table 1).

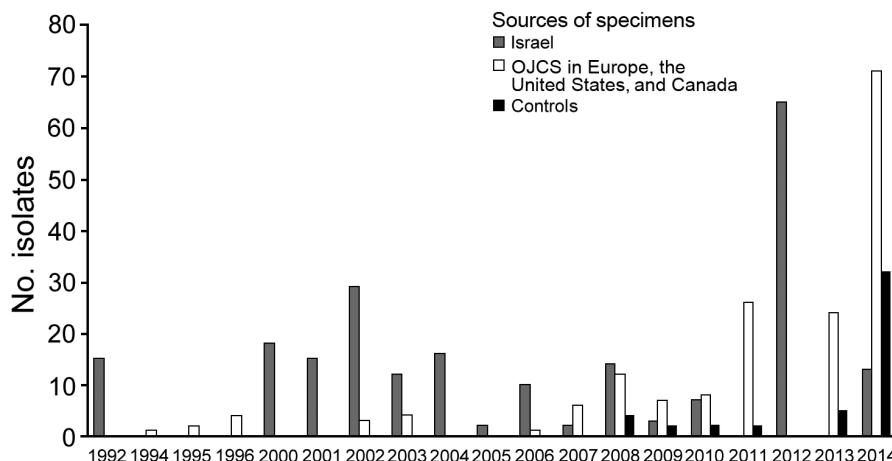
### Samples from Israel

The 221 samples of *S. sonnei* in Israel were collected during 1992–2014 in local hospital and health maintenance organization laboratories, including the national sentinel laboratory-based surveillance program (19). As described by Cohen et al. (19), 90% of the *S. sonnei* shigellosis isolates collected in Israel are from Jewish patients, and the isolates from Israel sequenced in this study were primarily ( $n = 200$ , 90%) derived from OJCs (online Technical Appendix 2 Table 1, <http://wwwnc.cdc.gov/EID/article/22/9/15-1953-Techapp2.pdf>).

### Samples from Outside of Israel

#### United Kingdom

A total of 146 *S. sonnei* samples were used from the Gastrointestinal Bacteria Reference Unit at Public Health England (London, United Kingdom). These samples included 22 from a small OJC-associated outbreak (23) and an additional 79 from outbreaks during 2006–2014 that were epidemiologically confirmed to be associated with OJCs by interviews and questionnaires as part of public



**Figure 1.** Origin and year of collection for 437 clinical isolates collected and sequenced from different countries and patient communities as part of study of travel- and community-based transmission of multidrug-resistant *Shigella sonnei* among international OJCs. Non-OJC samples were isolated from samples in the United Kingdom that were phage-type and temporally matched to isolates from OJCs in the United Kingdom (online Technical Appendix 2 Figure 1, <http://wwwnc.cdc.gov/EID/article/22/9/15-1953-Techapp1.pdf>). OJCs, Orthodox Jewish communities.

**Table 1.** Origins of *Shigella sonnei* isolates used to track travel- and community-based transmission of multidrug-resistant *Shigella sonnei* among international Orthodox Jewish communities\*

Region/community	Country	Year(s)	Details	References	No. isolates
Europe OJCs	Belgium	2008	Outbreak	(22)	3
	France	1996–2014	Multiple outbreaks	This study, (21)	64
	United Kingdom	2006–2014	Multiple outbreaks	This study, (23)	101
Europe non-OJCs (controls)	United Kingdom	2008–2014	Matched (time and phage-type) non-OJC cases	This study	45
United States and Canada OJCs	United States	1994–1995	Outbreak	(24)	3
Israel†	Israel	2000–2014	Sentinel laboratory surveillance	This study, (19)	221
Global context	Multiple	1943–2008	Used for background	(16)	118
<b>Total</b>					<b>555</b>

\*OJC, Orthodox Jewish communities.

†90% known OJC ethnicity.

health investigations. Also included were a set of 45 isolates from patients with no known OJC association (non-OJC). These background isolates were contemporaneously collected and selected on the basis of phage typing; that is, including diverse phage types, but focused on representing phage types associated with OJC outbreaks (online Technical Appendix 2 Figure 1).

#### France

A total of 64 isolates from OJC-associated outbreaks in France (21) were submitted to the French National Reference Center for *E. coli*, *Shigella*, and *Salmonella* at the Pasteur Institute collected during 1996–2014. These isolates included those from a small OJC-associated outbreak in 2007 (21).

#### Belgium

Three *S. sonnei* isolates were provided from Belgium. These isolates were collected during a small OJC-associated outbreak in Antwerp in 2008 (22).

#### United States and Canada

Three representative isolates were collected during a large, homogenous, OJC-associated outbreak of *S. sonnei*. This outbreak occurred across the United States and Canada during 1994–1996 (24).

#### Comparison Dataset

We analyzed the clinical isolates of *S. sonnei* alongside an existing global dataset compiled by KE Holt et al. (n = 118) (online Technical Appendix 1) (16). In brief, this global context dataset comprises temporally (collected during 1943–2008, 70% collected after 1992) and geographically diverse (from 4 continents) *S. sonnei* isolates previously used to define the population structure of this pathogen. The dataset includes 1 sample collected in Israel in 2003.

#### Methods

DNA was extracted at multiple sites by using the Wizard genomic DNA extraction kit (ProMega, Madison, WI,

USA) according to manufacturer's instructions. DNA was sequenced by using the MiSeq and HiSeq 2000 platforms (Illumina, San Diego, CA, USA) at multiple institutes according to in-house protocols (online Technical Appendix 1) (25–27). Sequencing data for all isolates described passed internal quality control and were assembled into <687 contiguous sequences with a total length of <5.0 MB. Sequence data are available in the European Nucleotide Archive (<http://www.ebi.ac.uk/ena>; accession numbers in online Technical Appendix 2).

Analysis of sequencing data was similar to that previously described (28). Multiple sequence alignment for phylogenetic analysis was generated by mapping to reference isolate *S. sonnei* Ss046 (GenBank accession no. CP000038), then masking mobile and repetitive elements (16) and stripping sites of recombination (29). Analysis of remaining variable sites was performed by using maximum-likelihood analysis in RAXML version 7.8.6 to create phylogenetic trees (30).

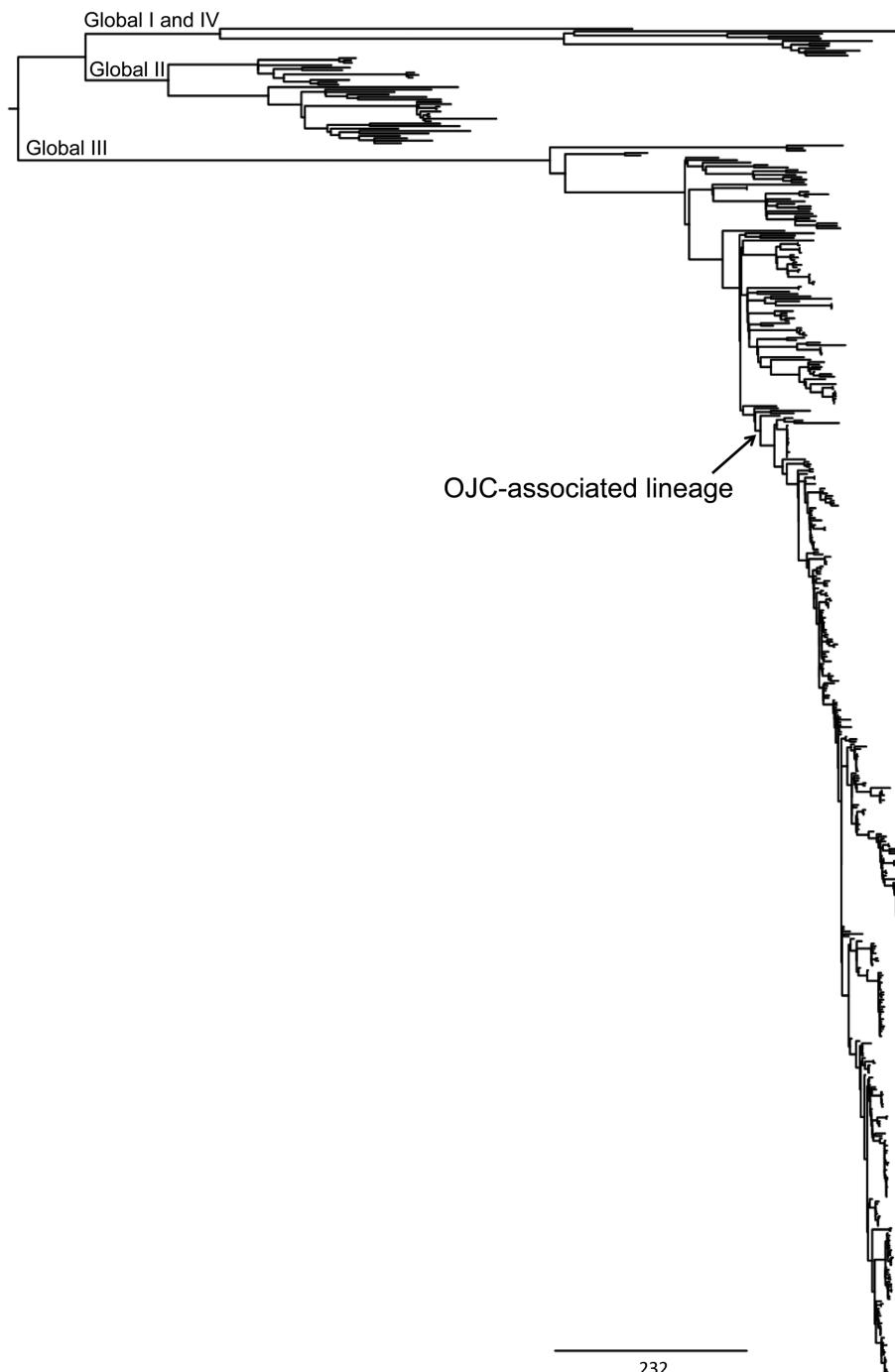
For isolates in the OJC-associated lineage for which the sampling date was known (n = 333; online Technical Appendix 1), BEAST version 1.8 software (<http://beast.bio.ed.ac.uk/>) was used to estimate the emergence date of the lineage (31). Root-to-tip distances were generated by using Path-O-Gen version 1.4 (32). BEAST results shown are from 4 chains of 100 million Markov chain Monte Carlo generations run according to a general time reversible plus gamma substitution model, with a relaxed normal clock and Bayesian Skyline Population model, previously used for this pathogen (16,17). Chains were sampled every 1,000 generations with a 10% initial burn-in for root-height (time to most recent common ancestor) analysis. The maximum clade credibility tree was generated with a 10% burn-in and sampling every 100,000 generations. These results were consistent with those generated similarly by using a constant population growth model (online Technical Appendix 2 Table).

De novo assembly, annotation, and antimicrobial resistance gene detection in the isolates was done as previously described (28) (accession numbers for annotated

draft genome assemblies in online Technical Appendix 1). Contiguous sequences containing antimicrobial resistance genes were extracted from assemblies and the presence of plasmid incompatibility groups on these contiguous sequences was determined by using Plasmid-Finder (33). The presence of the Tn7/Int2 cassette was confirmed by mapping, and synteny detected by using ACT (34).

## Results

To determine the relationships among *S. sonnei* from OJCs inside and outside of Israel, we constructed a phylogenetic tree including whole-genome sequence data from 437 isolates of *S. sonnei* alongside the 118 isolates from the global context dataset (Table 1; Figure 2). This analysis showed the existence of a large, unique monophyletic sublineage (n = 396 isolates) of the global III lineage that was almost



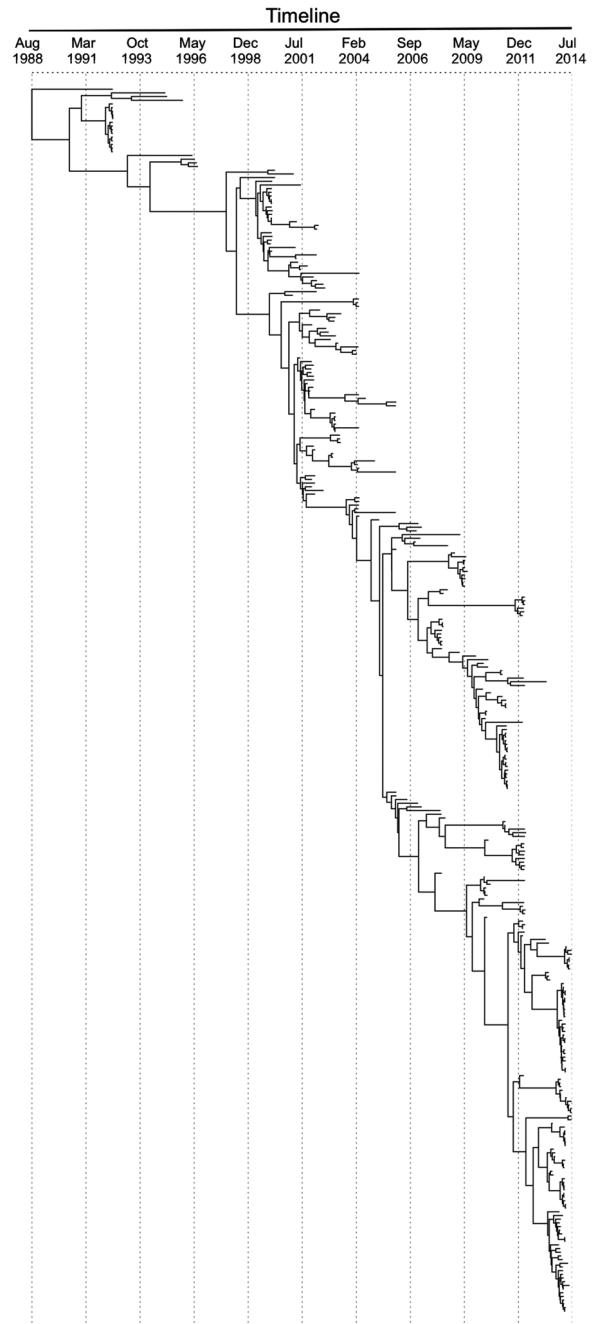
**Figure 2.** The OJC-associated lineage of multidrug-resistant *Shigella sonnei* in context with other global lineages. These background (non-OJC) isolates were contemporaneously collected and selected on the basis of phage typing; that is, including diverse phage types, but focused on representing phage types associated with OJC outbreaks. The midpoint rooted maximum-likelihood phylogenetic tree shows the relationships of 437 sequences from study of travel- and community-based transmission of multidrug-resistant *S. sonnei* among international OJCs compared with 118 isolates from a global context database of previously defined lineages of *S. sonnei*. Lineages are labeled along branches. OJCs, Orthodox Jewish communities. Scale bar indicates single-nucleotide polymorphisms. An expanded version of this figure with additional details is available online (<http://wwwnc.cdc.gov/EID/article/22/9/15-1953-F2.htm>).

exclusively (388/396, 98%) composed of isolates from OJC-associated outbreaks and samples from persons in Israel (OJC-associated lineage; Figure 2; online Technical Appendix 2 Figure 2). This lineage contained nearly all isolates (217/221, 98%) sequenced from Israel and collected during 1992–2014 (Figure 2); 170/171 (99%) of those identified from samples collected during the same time frame from OJCs in the United States, Canada, France, Belgium, and the United Kingdom; and the 1 isolate from the global context dataset that originated in Israel (Figure 2; online Technical Appendix 1). The clustering of most (387/392, 99%) of the strains from Israel and the other OJC-associated strains in the OJC-associated lineage is remarkable considering that the lineage represented approximately 10% of the diversity of the *S. sonnei*: the largest intra-lineage pairwise distance was 8.8-fold less in the OJC-associated lineage relative to the remainder of the tree (Figure 2).

The inclusion of isolates from patients in the United Kingdom that were not associated with OJCs provided further illumination of the association of this lineage with OJCs. These non-OJC samples were almost entirely (37/45, 82%) located outside the OJC-associated lineage, distributed elsewhere in the global III and II lineages (Figure 2). Ultimately, this resulted in a statistically significant association of lineage with sample designation (i.e., OJC or non-OJC) among UK isolates ( $p < 0.0001$  by Fisher exact test). This association correlated better with phylogenetic position than phage type, which was a comparatively poor indicator of genome level phylogeny (online Technical Appendix 2 Figure 3). The relative phylogenetic positions of non-OJC and OJC isolates from the United Kingdom when viewed in an international context, i.e., including the other strains (Figure 2) showed that strains from UK OJCs were more likely to be related to strains from Israel than to strains circulating in non-OJCs. For example, strains from OJCs sampled in the United Kingdom in 2014 were phylogenetically adjacent to strains from Israel sampled in 2014, rather than to non-OJC strains sampled in the United Kingdom in 2014 (Figure 2.)

Consistent with this finding, phylogenetic relationships within the OJC-associated lineage were defined more by time than geography (Figure 2; online Technical Appendix 2 Figures 2, 4) Bayesian phylogenetic analysis showed that the OJC-associated lineage emerged in 1988 (95% highest posterior distribution 1985–1990) (Figure 3). Since that time, contemporaneously collected isolates were phylogenetically proximate with subsequent evolution, resulting in strain replacement rather than coexistence over time (Figure 3; online Technical Appendix 2 Figure 2). Contrasting with the clear time signature in the lineage, geographic admixing of isolates occurred within the lineage (Figure 3). For example, >5 and 7 monophyletic clusters of isolates from OJC-associated outbreaks in France and the

United Kingdom, respectively, were encompassed within the diversity of strains characterized in Israel (Figure 3). Samples from a single epidemiologic OJC-associated



**Figure 3.** The OJC-associated lineage of multidrug-resistant *Shigella sonnei* across time. The Bayesian-inferred phylogenetic tree shows the evolutionary relationships of 333 isolates (those for which a fixed date was available) in the OJC-associated lineage since its emergence in the late 1980s. Tree tips overlay the collection date of the isolates. OJCs, Orthodox Jewish communities. An expanded version of this figure showing associated antimicrobial drug resistance is available online (<http://wwwnc.cdc.gov/EID/article/22/9/15-1953-F3.htm>).

outbreak in Belgium were separated from each other by 44 single-nucleotide polymorphisms, making them phylogenetically distinct (Figure 2; online Technical Appendix 2 Figure 2). This finding is consistent with contemporaneous OJC-associated outbreaks in different geographic areas representing real-time transmission events.

Because of the potential consequences of this intercontinental transmission to the transfer of AMR to *S. sonnei*, we determined the AMR characteristics of the OJC-associated lineage. We found that the lineage had a unique AMR profile relative to isolates outside of the OJC-associated lineage (Table 2). The OJC-associated lineage belonged to the global III lineage of *S. sonnei*, and every isolate in the OJC-associated lineage contained a Tn7/Int2 cassette

that encoded the MDR thought to have facilitated the global dispersal of global III (16). This cassette contains the *aadA1*, *sat2*, and *dfrA1* genes that confer resistance to aminoglycosides, streptothricin, and trimethoprim and was chromosomally integrated adjacent to the *glmS* gene in these isolates (indicating a single acquisition event). This region is identical to a Tn7-like island, also adjacent to the *glmS* gene, in the newly emerging Xv serotype of *S. flexneri* (reference strain 2002017 [35]; online Technical Appendix 2 Figure 5). No mutations known to confer quinolone resistance were found in *gyrA* or *parC* sequences of isolates in the OJC-associated lineage, and no plasmid-encoded quinolone resistance genes were detected (online Technical Appendix 1).

To consider AMR mechanisms that had potential to mobilize among bacteria, we further examined antimicrobial resistance genes that were inconsistently present (in 5%–95% of isolates) (Figure 3; online Technical Appendix 1; online Technical Appendix 2 Figure 2). The 7 genes found to be inconsistently present across the lineage were second copies of *aadA1* present in some isolates; the aminoglycoside resistance–conferring *strA* and *strB* genes; the sulphonamide resistance genes *sul1* and *sul2*; the tetracycline resistance gene *tetA*; and the ampicillin resistance gene *bla<sub>TEM</sub>* (Figure 3; online Technical Appendix 2 Figure 2). Attempts were made to determine the coinheritance and genetic carriage elements of these genes (within the limitations of genome assembly). In isolates that had additional copies of *aadA1*, the gene was typically co-inherited with the *sul1* gene (Figure 3; online Technical Appendix 1; online Technical Appendix 2 Figure 2); this combination was found on plasmids of 2 different incompatibility groups, I1 and P, as well as on contiguous sequences where no plasmid incompatibility groups were identified, shown as unknown (Figure 3; online Technical Appendix 2 Figure 2). Similarly, *strA*, *strB*, and *sul2* were frequently co-inherited and found on plasmids of 4 different incompatibility groups. Isolates collected earlier in the lineage's evolution tended to carry *strA/strB/sul2* on B/O/K/Z, Q1, and P incompatibility group plasmids, whereas later isolates carried the genes on I1 plasmids (Figure 3; online Technical Appendix 2 Figure 2). Similarly, the *tetA* gene appeared to have had 2 major introductions into the lineage, earlier on a P group plasmid and later on an I1 plasmid (Figure 3; online Technical Appendix 2 Figure 2). Last, *bla<sub>TEM</sub>* genes were found in 86% of isolates in the lineage, compared with 14% outside of the lineage (Table 2; online Technical Appendix 1); these genes were carried on plasmids of 5 different incompatibility groups, with sporadic coinheritance patterns with other resistance genes (Figure 3; online Technical Appendix 2 Figure 2).

**Table 2.** Antimicrobial resistance determinants among isolates sequenced in study of travel- and community-based transmission of multidrug-resistant *Shigella sonnei* among international OJCs\*

Antimicrobial resistance determinant	Within OJC-associated lineage, n = 395	Outside OJC-associated lineage, n = 42
<i>bl2d_oxa1</i>	0.00	0.02
<i>catA1</i>	0.00	0.02
<i>tetB</i>	0.00	0.05
<i>dfrA5</i>	0.00	0.07
<i>bl2b_tem</i>	0.00	0.00
<i>dfrA16</i>	0.00	0.00
<i>aac3iia</i>	0.01	0.00
<i>dfrA17</i>	0.01	0.00
<i>bl2b_tem1</i>	0.01	0.00
<i>mphA</i>	0.02	0.00
<i>dfrA14</i>	0.03	0.02
<i>sul1</i>	0.12	0.07
<i>tetA</i>	0.18	0.79
<i>strB</i>	0.53	0.88
<i>sul2</i>	0.53	0.90
<i>strA</i>	0.53	0.88
<i>arnA</i>	0.95	0.95
<i>mdtP</i>	0.97	1.00
<i>mdtO</i>	0.97	0.98
<i>mdtN</i>	0.97	1.00
<i>bacA</i>	0.99	0.98
<i>emrE</i>	0.99	1.00
<i>mdfA</i>	0.99	1.00
<i>mdtK</i>	0.99	1.00
<i>aadA1</i>	0.99	0.81
<i>macB</i>	0.99	0.98
<i>mdtL</i>	0.99	0.98
<i>mdtE</i>	0.99	1.00
<i>mdtF</i>	0.99	1.00
<i>mdtG</i>	1.00	1.00
<i>mdtH</i>	1.00	1.00
<i>dfrA1</i>	1.00	0.90
<i>acrA</i>	1.00	1.00
<i>acrB</i>	1.00	1.00
<i>bcr</i>	1.00	1.00
<i>bl1_ec</i>	1.00	1.00
<i>ksgA</i>	1.00	1.00
<i>tolC</i>	1.00	1.00
<i>bla<sub>TEM</sub>†</i>	0.86	0.14

\*Excludes global context isolates. OJC-associated lineage defined in Technical Appendix Figure 1 (<http://wwwnc.cdc.gov/EID/22/9/15-1953-TeChapp1.pdf>). OJC, Orthodox Jewish communities.

†Detected separately.

## Discussion

We used whole-genome sequencing to develop a high-resolution picture of the international transmission of *S. sonnei* and its AMR determinants among OJCs over several decades. These analyses offer insight for the epidemiology of shigellosis inside and outside of Israel as well as for the broader transmission of AMR enteric pathogens. We showed that, in countries outside of Israel, outbreak strains in OJCs were distinct from strains circulating in the general population and that OJC-associated strains were more closely affiliated with outbreaks associated with OJCs in other countries (irrespective of geographic distance) and strains circulating in Israel. Strains from Israel and strains from nearly all previously reported OJC outbreaks elsewhere formed a distinct OJC-associated sublineage that emerged  $\approx 30$  years ago. Unlike other described emergent *Shigella* sublineages (17,28), the OJC-associated lineage lacked a defining association with AMR.

Isolates collected during outbreaks of *S. sonnei* in OJCs outside Israel were phylogenetically linked to contemporaneous isolates from Israel. This finding was suspected from previous studies that used pulsed-field gel electrophoresis, the results of which supported that samples from outbreaks among OJCs in the United States and Belgium were distinct from samples of *S. sonnei* circulating locally in non-OJCs (22,24) and were related to strains from Israel (22). We confirmed this link in an analysis of specimens collected in the United Kingdom that showed that strains from OJC-associated outbreaks were distinct from other circulating strains in that country but related to contemporaneous strains from Israel (Figure 2). The broader analysis, expanded in time and geography, showed that local epidemics in OJCs in France, Belgium, and North America (21,22,24) were also linked with contemporaneous isolates from Israel (Figure 3; online Technical Appendix 2 Figure 2). This pattern of phylogenetic clustering by community affiliation was also recently demonstrated for *S. flexneri* 3a strain transmission among a global epidemiologic community of men who have sex with men, through which a unique MDR sublineage spread during  $\approx 20$  years (28). These studies demonstrate the speed with which AMR *Enterobacteriaceae* can be transmitted among persons in an internationally linked community rather than by contiguous geographic spread.

These findings also have implications for the epidemiology of shigellosis within Israel. The isolates from Israel in this study derive primarily from OJCs, which drive cyclic *S. sonnei* epidemics in Israel (19); here, they were shown to belong to a single, low-diversity sublineage. The lineage was monophyletic and had a strong time signature, consistent with a point introduction and subsequent epidemic emergence. This pattern was similar to that observed in Vietnam after the introduction of another global III *S.*

*sonnei* sublineage (15,17). The date of the emergence of the OJC-associated lineage (1988 [95% highest posterior distribution 1985–1990]) is consistent with that estimated in a previous study where a 2-isolate lineage emerged in the Middle East during 1983 (16). Considering the timing and context of the emergence, it is possible that the OJC-associated lineage emerged from the large waterborne epidemic that occurred in Israel in the mid-1980s (19) or was potentially introduced from the first reported OJC-associated outbreak of shigellosis outside of Israel, which was an outbreak of  $>13,000$  cases across the United States that also occurred in the 1980s (18). Samples from this period were not available to explore these origins of these outbreaks, but it is clear that the OJC-associated lineage is now endemic to OJCs in Israel and is causative of OJC-associated outbreaks elsewhere.

The AMR profile of the OJC-associated lineage is consistent with the phenotypic information and is likely influenced by antimicrobial resistance selection pressures in the 0–4-year age group, which is primarily affected by shigellosis in OJCs. Sulfonamide and tetracycline resistance determinants were in flux across the lineage (Figure 3), and these antimicrobial classes have been reported as being phenotypically dynamic over time among *S. sonnei* isolated in Israel (19). Furthermore, trimethoprim resistance was chromosomally encoded in all isolates, and plasmid-mediated ampicillin resistance was a common finding (86% of isolates) (Figure 3). These antimicrobial classes are key for the treatment of children with shigellosis. Similarly, resistance to tetracyclines, which can be used in children  $>7$  years of age (8), and macrolides (*mphA* gene [21]) (Table 2) were also found. Despite being reported in other global III *S. sonnei* strains (16,17), resistance to quinolones was not found in the OJC-associated lineage, possibly because the use of quinolones is contraindicated in children.

The acquisition of the Tn7/Int2-encoded MDR in this lineage may have facilitated its epidemic emergence, as has been hypothesized for the broader global III lineage (16) and as is possible for *S. flexneri* Xv (35), although these possibilities cannot be explored fully by using these data. The presence of this gene cassette throughout the lineage and other phylogenetic clusters of antimicrobial resistance genes demonstrates that AMR can spread through this geographically dispersed, but closely associated, community. However, in contrast to the emergence of *S. flexneri* 3a among men who have sex with men or of *S. sonnei* in Vietnam, further acquisition of AMR does not appear to have shaped the subsequent evolution of the lineage. The presence of additional resistance genes (Figure 3) was not correlated with later time points, and the same genes were carried on distinct mobile genetic elements, consistent with sporadic reintroduction, rather than maintenance of the additional resistance genes in the population. This absence of

a defining association with AMR suggests that it is probably primarily the epidemiologic suitability of OJCs to the transmission of *S. sonnei* that supports its maintenance in these communities. This likelihood is consistent with previous studies of OJC-associated shigellosis, which suggest that transmission is largely driven by communal childcare arrangements in a host population that is young and densely structured (19,20,22).

This study documents the speed with which MDR enteric bacteria can transmit intercontinentally through travel within a geographically dispersed, but closely linked, community. Awareness of this mode of sustained, geographically noncontiguous transmission must inform public health practice, including targeted control and reduction of consequences of the pathogen through developing effective relationships with affected communities; identifying specific risk factors; and designing, piloting, and eventually implementing specific culturally appropriate interventions with the participation and support of the community while avoiding stigmatization. Effective ongoing surveillance is also vital, and we demonstrated the resolution required for that purpose and the need for effective data sharing to track these otherwise silent transmission phenomena. Furthermore, the repeated application of high-resolution tools supports identification of parallels and contrasts with previous genomic epidemiology studies, supporting construction of a complete picture of the global transmission of AMR enteric pathogens, advancing our position for tackling this global public health issue.

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## EID Podcast: Nipah Virus Transmission from Bats to Humans Associated with Drinking Traditional Liquor Made from Date Palm Sap, Bangladesh, 2011–2014



Nipah virus (NiV) is a paramyxovirus, and *Pteropus* spp. bats are the natural reservoir. From December 2010 through March 2014, hospital-based encephalitis surveillance in Bangladesh identified 18 clusters of NiV infection. A team of epidemiologists and anthropologists investigated and found that among the 14 case-patients, 8 drank fermented date palm sap (*tari*) regularly before their illness, and 6 provided care to a person infected with NiV. The process of preparing date palm trees for *tari* production was similar to the process of collecting date palm sap for fresh consumption. Bat excreta was reportedly found inside pots used to make *tari*. These findings suggest that drinking *tari* is a potential pathway of NiV transmission.

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# Feasibility of Using Convalescent Plasma Immunotherapy for MERS-CoV Infection, Saudi Arabia

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We explored the feasibility of collecting convalescent plasma for passive immunotherapy of Middle East respiratory syndrome coronavirus (MERS-CoV) infection by using ELISA to screen serum samples from 443 potential plasma donors: 196 patients with suspected or laboratory-confirmed MERS-CoV infection, 230 healthcare workers, and 17 household contacts exposed to MERS-CoV. ELISA-reactive samples were further tested by indirect fluorescent antibody and microneutralization assays. Of the 443 tested samples, 12 (2.7%) had a reactive ELISA result, and 9 of the 12 had reactive indirect fluorescent antibody and microneutralization assay titers. Undertaking clinical trials of convalescent plasma for passive immunotherapy of MERS-CoV infection may be feasible, but such trials would be challenging because of the small pool of potential donors with sufficiently high antibody titers. Alternative strategies to identify convalescent plasma donors with adequate antibody titers should be explored, including the sampling of serum from patients with more severe disease and sampling at earlier points during illness.

Middle East respiratory syndrome coronavirus (MERS-CoV) was initially identified in September 2012 when a patient in Saudi Arabia with a severe, acute

respiratory infection and acute renal failure died (1). As of June 19, 2016, more than 1,733 MERS-CoV cases and at least 628 associated deaths had been identified; >80% of the cases occurred in Saudi Arabia (2). More than 20 countries outside of the Arabian Peninsula have reported MERS-CoV cases, and the 2015 outbreak in South Korea with attendant mortality has reinforced concerns about international outbreaks (3). No specific treatment has been proven effective for MERS-CoV infection.

Convalescent plasma containing MERS-CoV-specific antibodies from recovered patients has been suggested as a potential therapy for infected persons (4). Convalescent plasma has been used to treat several other viral infections, including those caused by the severe acute respiratory syndrome coronavirus (SARS-CoV), avian influenza A(H5N1) virus, and influenza A(H1N1)pdm09 virus (5–10). A recent metaanalysis of studies using passive immunotherapy for treatment of severe acute respiratory infections of viral etiology suggests that the timely use of convalescent blood products, particularly those with neutralizing antibodies, results in a reduced death rate (11). Public Health England and ISARIC (the International Severe Acute Respiratory and Emerging Infection Consortium) published a decision-making support tool on potential therapies for MERS-CoV that highlights convalescent plasma and other neutralizing antibody-containing immunotherapeutics (e.g., hyperimmune immunoglobulins and monoclonal antibodies) as the most promising potential treatments for serious MERS-CoV illness and deserving of evaluation in human clinical trial(s) (4).

However, no data support the feasibility of obtaining convalescent plasma from patients who have been exposed to MERS-CoV or recovered from infection with the virus. Camels are the likely source for most animal-to-human transmission and appear to have long-lasting antibody responses; in preclinical models, such antibodies appear effective in reducing the severity of pathologic

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<sup>1</sup>This author is a member of ISARIC (the International Severe Acute Respiratory and Emerging Infection Consortium).

changes in infected lungs (12). However, the antibody response to MERS-CoV infection in humans is poorly defined. Thus, we planned a 2-phase study to 1) determine the feasibility of collecting high-titer convalescent plasma from MERS-CoV patients and contacts and, if successful, to 2) conduct a pilot therapeutic study using convalescent plasma in symptomatic MERS-CoV patients with moderate to severe illness. Herein, we report on the feasibility study.

## Methods

In collaboration with the King Abdullah International Medical Research Center, the Gulf Cooperation Council Infection Control Center, and the World Health Organization (WHO)–International Severe Acute Respiratory and Emerging Infection Consortium MERS-CoV Working Group, we developed a study protocol to screen potential donors, collect high-titer convalescent plasma, and administer the plasma in a clinical trial (13). The study was approved by the Ministry of the National Guard Health Affairs Institutional Review Board (approval no. IRBC/233/14, June 9, 2014) and registered in ClinicalTrials.gov (NCT02190799). We conducted the study at King Abdulaziz Medical City, a 1,100-bed tertiary care center in Riyadh, Saudi Arabia. The hospital is accredited by the Joint Commission International, and the hospital's Department of Pathology and Laboratory Medicine is accredited by the College of American Pathologists and the American Association of Blood Banks.

## Study Population

We screened potential convalescent plasma donors from 3 cohorts: 1) patients with acute respiratory illness who were suspected of having MERS-CoV or who were confirmed MERS-CoV–positive by real-time reverse transcription PCR (rRT-PCR) of upper or lower respiratory secretions; 2) healthcare workers exposed to a laboratory-confirmed MERS-CoV patient, as identified by ongoing active surveillance of the hospital Infection Prevention and Control Department; and 3) household contacts of patients with laboratory-confirmed MERS-CoV infection. We obtained written informed consent for MERS-CoV serologic testing from all healthcare workers and household contacts. Medical teams ordered serologic testing as part of the clinical care for patients with suspected or confirmed MERS-CoV infection; no additional informed consent was required. Healthcare workers completed a self-administered survey that asked questions about the nature, duration, and degree of exposure to patients with laboratory-confirmed MERS-CoV infection. For all study participants, we documented the time that had elapsed from symptom onset or exposure to the collection of samples for testing.

## Study Procedures

During July–October 2015, we screened serum samples from study participants by using a spike protein subunit 1 (S1)–based ELISA. To confirm results of ELISA-reactive samples, we used indirect immunofluorescent antibody (IFA) and microneutralization (MN) assays (14–16). For MERS-CoV patients with a nonreactive ELISA result, we collected a follow-up sample 14–21 days later for repeat ELISA.

Study participants were considered candidates for plasma donation if they 1) had a reactive ELISA result; 2) had a MN assay titer of  $\geq 100$ ; 3) had no clinical or laboratory evidence of ongoing MERS-CoV infection; and 4) met the eligibility for plasma donation according to the institutional criteria, which were in accordance with WHO guidelines (17). Persons who met all criteria were eligible for plasma donation according to the position paper of the WHO Blood Regulators Network (18).

## Outcome Measures

The primary outcome of this first phase of the study was the feasibility of conducting the second phase. Feasibility was measured by our ability to screen and identify a sufficient number of potential plasma donors to provide enough high-titer, fresh-frozen plasma to enroll and provide transfusions to 20 patients over 12 months. Each phase 2–enrolled patient would require 2 fresh-frozen plasma units (250–350 mL/unit).

## Laboratory Procedures

We first conducted testing for MERS-CoV by rRT-PCR. We extracted RNA from respiratory specimens (nasopharyngeal swab, tracheal aspirate, bronchoalveolar lavage) using the MagNA Pure 96 Viral NA Kit (Roche Applied Science, Indianapolis, IN, USA). We tested the extracted nucleic acids by rRT-PCR targeting the upstream envelope protein gene (upE) and open-reading frame 1a (ORF1a) regions of the MERS-CoV genome on a LightCycler 480 System (Roche Diagnostics, Mannheim, Germany) real-time PCR (19). A positive control for ORF1a and upE rRT-PCR was performed according to the manufacturer's instructions. To be consistent with the cutoff used by the Saudi Arabia Ministry of Health reference laboratory, we considered a cycle threshold ( $C_t$ ) of  $<35$  the cutoff for upE and ORF1a. For  $C_t \geq 35$ , we repeated the testing using different samples, preferably from the lower respiratory tract, to avoid false-positive results.

We detected MERS-CoV antibodies by ELISA (Euroimmun AG, Lubeck, Germany), using wells coated with MERS-CoV S1 antigen (20,21). Serum samples were diluted (1:100) and incubated with antigens according to the ELISA manufacturer's instructions. Positive and negative control serum and calibration samples were included.

Antibodies were detected by adding peroxidase-labeled rabbit anti-human IgG (Euroimmun AG, Lubeck). Results were reported as the optical density (OD) ratio, which was calculated as the OD value of the patient's sample divided by the calibrator OD value. We used cutoff values recommended by the ELISA kit manufacturer: a ratio of  $<0.8$  was considered negative,  $\geq 0.8$  to  $<1.1$  was considered borderline, and  $\geq 1.1$  was considered positive.

We used an IFA (Euroimmun AG) according to the manufacturer's instructions to detect MERS-CoV antibodies. Serum samples were diluted in doubling dilutions, starting with 1:10 and ending with 1:1,280, in sample buffer and then incubated with Vero B4 cells infected with HCoV-EMC (Euroimmun AG). MERS-CoV IgG was detected by adding FITC-labeled goat anti-human IgG (Euroimmun AG); positive and negative controls were included. Samples with an IFA titer of  $\geq 1:10$  were considered reactive according to the IFA manufacturer's instructions. Our original protocol used an IFA cutoff of  $\geq 160$  to define suitable donors for plasma (13). In the course of the study, MN became available, and we revised the criteria for plasma donation to be based on MN assay results.

The presence of neutralizing MERS-CoV antibodies was also assessed using a MN assay (16). In brief,  $2 \times 10^4$  Vero cells/well were plated onto a 96-well microtiter plate. After 24 h, 2-fold serial dilutions of serum samples (heat-inactivated at 56°C for 30 min) were incubated with an equal volume of the MERS-CoV strain Jordan-N3/2012 (200 TCID<sub>50</sub> [50% tissue culture infectious doses]) for 1 h at 37°C (16). Medium was aspirated from the microtiter plate, and 200  $\mu$ L of the serum-virus mixture was added to the wells in triplicate. The plate was incubated for 48 h at 37°C in a humidified chamber with 5% carbon dioxide, after which the serum-virus mixture was aspirated and the cells were fixed by adding 100  $\mu$ L of a 1:1 mixture of cold ethanol and methanol. The plate was then incubated at -80°C for 30 min, washed 5 times with PBS, and processed as described above for ELISA, using rabbit anti-coronavirus spike protein antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Plates were developed using ABTS substrate (KPL Inc., Gaithersburg, MD, USA); OD was measured at 405 nm. Controls consisted of uninfected cells and cells infected with 200 TCID<sub>50</sub> of MERS-CoV. The highest dilution of serum sample that resulted in a  $\geq 50\%$  reduction in OD, compared with the control containing no antibody, was reported as the 50% virus neutralization titer.

rRT-PCR, ELISA, and IFA testing for MERS-CoV were performed at the King Abdulaziz Medical City laboratory. MN was performed at the Naval Medical Research Center (Silver Spring, MD, USA).

### Statistical Analysis

We used descriptive statistics (i.e., numbers and proportions, means  $\pm$  SD, and medians with quartile 1 [Q1] and Q3 values) for measurements for eligible donors and participants with seroreactive test results. We used the Pearson correlation to test for correlations between ELISA OD and IFA and MN titers.

### Ethical Considerations

The identity of study participants with MERS-CoV was known only to investigators listed on the approved King Abdullah International Medical Research Center protocol. All samples were delinked from any identifiable personal information when provided to nonlisted investigators.

## Results

### Serologic Findings for Healthcare Workers

We contacted 692 healthcare workers who had a history of exposure to or a diagnosis of MERS-CoV infection. Of those 692 healthcare workers, 230 (33%) consented to serum sampling and were tested (Table 1); 11 had a history of laboratory-confirmed MERS-CoV infection, and 219 had a history of exposure but were MERS-CoV rRT-PCR negative during their asymptomatic or potential immediate postincubation period. Only 4 (36.7%) of 11 healthcare workers who had a history of laboratory-confirmed MERS-CoV infection had ELISA-reactive serum samples after a median of 381 days (Q1 246 days, Q3 485 days) after infection (Figure 1). The confirmatory IFA was reactive for all 4 of those healthcare workers, and MN was reactive for 3 (Table 2). However, only 1 healthcare worker (participant no. 9) had a high MN titer (800) (Table 2), but she was not considered a candidate for plasma donation because of a previous pregnancy. Exposed healthcare workers who had negative MERS-CoV rRT-PCR results also had nonreactive ELISA results.

### Serologic Findings for Patients with Suspected or Laboratory-Confirmed MERS-CoV Infection

A total of 196 patients with suspected or laboratory-confirmed MERS-CoV infection were tested; 183 (88.8%) were hospitalized in the emergency department, 11 (5.8%) in the intensive care unit, and 2 (0.97%) in the medical wards (Table 1). Two (40%) of 5 patients with laboratory-confirmed MERS-CoV and 6 (3%) of 191 who were MERS-CoV rRT-PCR negative had ELISA-reactive serum samples.

IFA and MN assay results were positive for 6 (75%) of 8 patients who had ELISA-reactive serum samples; the 2 patients who had nonreactive IFA results also had nonreactive MN results. One of the 6 patients (no. 7) had high IFA (1:1,280) and MN (400) titers (Table 2). This patient, a 69-year-old man, was admitted to the intensive care unit

**Table 1.** Characteristics of participants in a study for the feasibility of collecting convalescent plasma from persons who had been infected with or exposed to MERS-CoV, Saudi Arabia, July–October 2015\*

Characteristic	Value
Healthcare workers exposed to laboratory-confirmed MERS-CoV patients, N = 230	
Median age, y (Q1, Q3)	35 (29, 42)
Sex	
M	34 (14.8)
F	196 (85.2)
Work-associated exposure	
Intubation	52 (22.6)
Bronchoscopy	22 (9.6)
Tracheal suctioning or inhalation therapy	72 (31.3)
Patient care	117 (50.9)
Reported total duration of exposure†	
≤24 h	66/199 (33.2)
>24 h	133/199 (66.8)
Reported exposure intensity‡	
Mild	108/200 (54.0)
Moderate	60/200 (30.0)
Severe	31/200 (15.5)
Laboratory-confirmed MERS-CoV infection	11 (4.8)
ELISA-reactive serum sample	4 (1.7)
Median time from exposure to testing positive, d (Q1, Q3)	381 (246, 485)
Patients with suspected or laboratory-confirmed MERS-CoV infection, N = 196	
Median age, y (Q1, Q3)	65 (49, 76)
Sex	
M	97 (49.5)
F	99 (50.5)
Hospitalization admission area	
Intensive care unit	11 (5.8)
Emergency room	183 (88.8)
Ward	2 (0.97)
Laboratory-confirmed MERS-CoV infection	5 (2.6)
ELISA-reactive serum sample	8 (4.1)
Median time to testing positive, d (Q1, Q3)	7 (4, 12)
Household contacts of confirmed MERS-CoV patients, N = 17	
Median age (range), y	37 (26, 46)
Sex	
M	6 (35.3)
F	11 (64.7)
Laboratory-confirmed MERS-CoV infection	0
ELISA-reactive serum sample	0
Median time to antibody testing, d (Q1, Q3)	34 (34, 34)

\*Unless otherwise specified, data are no. (%). Q1 and Q3, quartiles 1 and 3, respectively; MERS-CoV, Middle East respiratory syndrome coronavirus.

†Data from a self-administered survey question answered by 199 healthcare workers.

‡Data from a self-administered survey question answered by 200 healthcare workers.

with MERS-CoV infection resulting in acute respiratory distress syndrome, acute kidney injury, and shock. He required mechanical ventilation, renal replacement therapy, and vasopressors (Figure 2). The high titer occurred while he was in intensive care, 32 days after symptom onset. His serologic titers by ELISA, IFA, and MN declined progressively as he recovered clinically; ELISA and IFA were nonreactive by 8 months after hospital admission (Figure 2). Of the 6 patients, 5 (nos. 1–5) had MN titers  $\geq 100$  (Table 2), but these patients did not meet clinical criteria for plasma donation because of age, concurrent conditions, or previous pregnancy.

Of note, 3 patients with laboratory-confirmed MERS-CoV infection had a nonreactive ELISA; these 3 samples were collected 3, 6, and 36 days after symptom onset. Two of the patients died before the test was repeated. For the

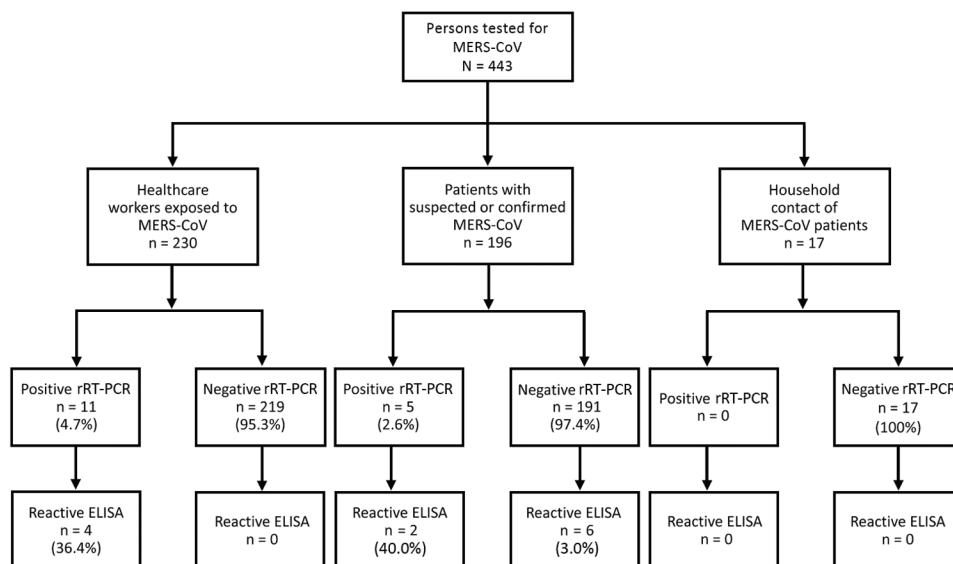
third patient, repeat ELISAs at 2 and 4 weeks after the first nonreactive ELISA were negative.

#### Serologic Findings for Household Contacts

A median of 34 days (Q1 34 days, Q3 34 days) after 2 patients received a laboratory diagnosis of MERS-CoV infection, we tested 3 household contacts for 1 of the patients and 14 for the other (Table 1). Serum samples for all 17 contacts were nonreactive by ELISA (Figure 1; Table 2).

#### Correlation of ELISA, IFA and MN Titers

ELISA and MN results were highly correlated (Pearson correlation coefficient 0.70,  $p = 0.001$ ) (Figure 3). However, ELISA and IFA results showed only a modest correlation (Pearson correlation coefficient 0.55,  $p = 0.015$ ), and IFA and MN results were not statistically



**Figure 1.** Antibody test results for 443 persons in a study determining the feasibility of using convalescent plasma immunotherapy for Middle East respiratory coronavirus (MERS-CoV) infection, Saudi Arabia. rRT-PCR, real-time reverse transcription PCR.

significantly correlated (Pearson correlation coefficient 0.38,  $p = 0.12$ ).

**Discussion**

Our results indicate that it would be possible to obtain quantities of convalescent plasma large enough to use in therapeutic studies or in a large number of MERS-CoV patients; however, large-scale screening would be required because of the limited availability of eligible potential donors with sufficient levels of antibody. Our findings suggest that recently recovered MERS patients may be suitable potential donors, provided they meet other plasma donation criteria. Of note, none of the seropositive persons in our study met our clinical and laboratory criteria for plasma donation.

Our findings show that serum antibody to MERS-CoV was infrequently reactive by ELISA; however, reactivity may have been affected by the timing of sample collection or severity of the illness. Most of the small subset of participants with ELISA-reactive serum samples had MERS-CoV antibodies as assessed by IFA and MN. ELISA results and MN titers were highly correlated; IFA and MN were not. One healthcare worker had high MN titers, but she did not meet the clinical criteria for plasma donation. Another critically ill patient had high antibody titers by the 3 assays, but antibody titers declined quickly as the patient recovered clinically, and he was not eligible to donate plasma.

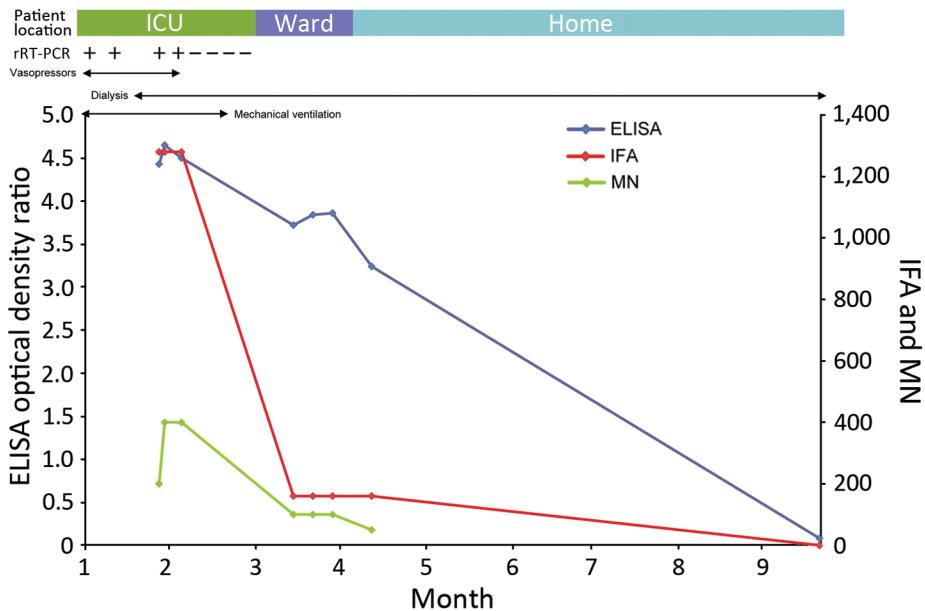
In accordance with WHO and US Centers for Diseases Control and Prevention guidelines, we used ELISA

**Table 2.** Characteristics and findings for participants with MERS-CoV antibodies detected by ELISA in a study determining the feasibility of using convalescent plasma immunotherapy for MERS-CoV infection, Saudi Arabia\*

Participant no.	Age, y/sex	Symptom at first medical visit	Admitted to ICU	MERS-CoV rRT-PCR	Days from symptom onset or exposure to serum sampling	OD ratio	IFA	MN titer
<b>Patient</b>								
1	70/M	ARI	No	-	35	2.00	1:10	100
2	61/F	ARI	No	+	10	1.12	Nonreactive	200
3	40/F	ARI	No	-	4	3.66	1:20	100
4	63/M	ARI	No	-	27	3.95	1:80	200
5	76/M	ARI	No	+	13	2.59	1:20	200
6	73/M	ARI	No	-	4	1.62	Nonreactive	Nonreactive
7	69/M	ARI	Yes	+	87	4.70	1:1,280	400†
8	71/M	ARI	No	-	9	1.86	Nonreactive	Nonreactive
<b>Healthcare worker</b>								
9	46/F	ARI	Yes	+	24	5.51	1:40	800
10	27/M	None	Yes	+	273	2.33	1:20	50
11	31/M	ARI	No	+	365	1.46	1:10	Nonreactive
12	33/F	None	No	+	365	2.34	1:10	50

\*ARI, acute respiratory infection; ICU, intensive care unit; IFA, indirect immunofluorescent antibody; OD ratio, optical density value of patient sample/optical density value of calibrator; MERS-CoV, Middle East respiratory syndrome coronavirus; MN, microneutralization assay; rRT-PCR, real-time reverse transcription PCR; -, negative; +, positive.

†Serial tests were performed for the patient (Figure 2). Values shown are the highest values for the patient.

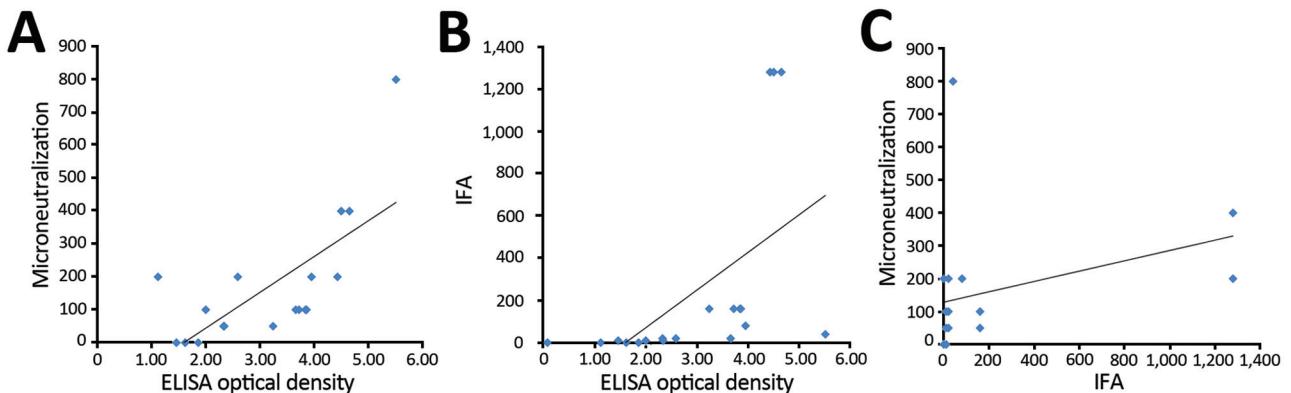


**Figure 2.** Clinical and laboratory timeline for a Middle East respiratory coronavirus–infected patient with high ELISA, indirect immunofluorescent antibody (IFA), and microneutralization (MN) titers. The highest titers were measured while the patient had active infection and was critically ill. The ELISA optical density ratio and IFA and MN titers declined as the patient recovered. ICU, intensive care unit; rRT-PCR, real-time reverse transcription PCR; ward, hospital ward; –, negative; +, positive.

to screen for MERS-CoV IgG and IFA and MN assays to confirm positive results (14,15). The ELISA is based on the virus S1 protein as antigen, and the IFA is based on detection of virus-specific antibodies, using cell cultures infected with the virus. The MERS-CoV spike protein is a glycoprotein that forms the spikes of the virus, and the N terminal component (S1) is believed responsible for the first step of virus entry into the host cell (22). Our original protocol used IFA as a confirmatory test; however, we switched to MN when that assay became available. Our findings showed a high correlation between ELISA and MN results but not between IFA and MN results, which may indicate that MN is a better confirmatory test. However, for 1 patient in our study, the 3 tests showed similar results (Figure 2), and other studies have shown good

correlation between the tests (20), which may indicate that the lack of correlation shown between IFA and MN in our study was associated with sample size.

Our findings suggest that the low prevalence of seroreactivity for MERS-CoV, even among persons with confirmed or suspected infection, may be a reflection of a short-lasting antibody response. It is possible that some of the study participants were seronegative at the time of testing because the window for positive serologic results had passed. A short-lasting immune response may also partly explain why negligible or low levels of MERS-CoV seroreactivity have been detected in persons at risk for the disease (i.e., camel and abattoir workers) in Saudi Arabia and elsewhere (23–26). A seroprevalence study conducted during December 2012–December 2013 showed MERS-CoV



**Figure 3.** Correlation between ELISA optical density and antibody assay results in a study determining the feasibility of using convalescent plasma immunotherapy for Middle East respiratory coronavirus infection, Saudi Arabia. A) Correlation between ELISA and microneutralization assay results (Pearson correlation coefficient 0.70,  $p = 0.001$ ). B) Correlation between ELISA and indirect immunofluorescent antibody (IFA) assay results (Pearson correlation coefficient 0.55,  $p = 0.015$ ). C) Correlation between IFA and microneutralization assay results. (Pearson correlation coefficient 0.38,  $p = 0.12$ ).

antibodies in only 0.15% of the general population ( $n = 10,009$ ) in all 13 provinces in Saudi Arabia (21). Seroprevalence was also low among camel shepherds (2.3%,  $n = 87$ ) and slaughterhouse workers (3.6%,  $n = 140$ ), albeit higher than in the general population (21). The clinical relevance of antibody titers in protecting against subsequent MERS-CoV infection is uncertain.

Similar findings have been described with other coronaviruses. Cao et al. (27) studied specific and neutralizing antibody titers in 56 patients who recovered from SARS-CoV infection. Their findings showed that SARS-CoV IgG and neutralizing antibodies peaked at 4 months and then began diminishing, reaching undetectable levels in 25.6% (IgG) and 16.1% (neutralizing antibodies) of patients at 36 months. Xie et al. (28) showed that SARS-CoV IgG decreased over 1 year in recovering SARS patients. In an experiment of intranasal inoculation of CoV 229E in human volunteers, Callow et al. (29) studied the time course of specific antibody response and found that those antibodies peaked 1 week after the inoculation and then began declining. Furthermore, it appears that the antibody immune response to MERS-CoV in humans differs from that in camels. Alagaili et al. (30) showed that 74% of 150 camels from different parts of Saudi Arabia have antibodies to MERS-CoV by ELISA, and the prevalence of antibodies is higher in older camels (95%).

Two patients in the 2015 MERS-CoV outbreak in South Korea were reported to have received convalescent plasma collected from recovered patients (31). It is unclear whether the plasma was tested for the presence of MERS-CoV antibodies. Our study demonstrates that such testing should be mandatory for donated convalescent plasma because of the low prevalence of MERS-CoV antibodies, even in patients with past laboratory-confirmed MERS-CoV infection. Without such testing, the presence of antibodies to MERS-CoV cannot be confirmed, and the convalescent plasma may not be associated with a protective effect. Our study also highlights the need for prospective serology studies to better understand the humoral response to MERS-CoV infection.

The strengths of our study are that we screened a large number of persons, including patients with laboratory-confirmed MERS-CoV infection, and used screening and confirmatory antibody assays. A study limitation was the small number of household contacts who were screened, although, based on our findings and those of others (20), a small proportion of household contacts are likely to show seroreactivity. Only one third of invited healthcare workers participated in the study. We used an S1-based ELISA for screening, and our study did not address the magnitude and duration of other antibody isotypes in the immune response. The interval between illness and recovery was prolonged in most of the exposed healthcare workers (median

interval  $>1$  year). It is possible that earlier sampling would have resulted in the detection of more reactivity and higher antibody titers. Our study, which was designed to screen for antibodies in convalescent plasma, was not designed to characterize the immune response to MERS-CoV infection or to identify clinical correlates of the presence or absence of MERS-CoV antibodies.

Further testing is needed to determine whether the antibodies in convalescent plasma are clinically effective against MERS-CoV infection. Our findings suggest the need to explore other passive immunotherapeutic approaches, such as monoclonal or polyclonal human antibodies from transchromosomal bovines (16,32) and, possibly, polyclonal antibodies from camels (12). Our findings also raise questions about whether naturally occurring infections and potential MERS-CoV vaccines will offer long-lasting immunity.

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# Prediction of Protection against Asian Enterovirus 71 Outbreak Strains by Cross-neutralizing Capacity of Serum from Dutch Donors, the Netherlands

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Outbreaks of human enterovirus 71 (EV-71) in Asia are related to high illness and death rates among children. To gain insight into the potential threat for the population of Europe, we determined the neutralizing activity in intravenous immunoglobulin (IVIg) batches and individual serum samples from donors in the Netherlands against EV-71 strains isolated in Europe and in Asia. All IVIg batches and 41%, 79%, and 65% of serum samples from children  $\leq 5$  years of age, women of childbearing age, and HIV-positive men, respectively, showed high neutralizing activity against a Dutch C1 strain, confirming widespread circulation of EV-71 in the Netherlands. Asian B3–4 and C4 strains were efficiently cross-neutralized, predicting possible protection against extensive circulation and associated outbreaks of those types in Europe. However, C2 and C5 strains that had few mutations in the capsid region consistently escaped neutralization, emphasizing the importance of monitoring antigenic diversity among circulating EV-71 strains.

Enterovirus 71 (EV-71) is a member of the genus *Enterovirus*, family *Picornaviridae*, and is a major causative agent of hand, foot and mouth disease in children  $\leq 5$  years of age. The virus may also invade the central nervous system and cause severe neurologic disease, including paralysis and brainstem encephalitis (1). On the basis of nucleotide sequence diversity in the viral protein (VP)1 capsid gene, 7 EV-71 genogroups (A–G) have been defined (2,3). Genogroup A contains a prototype strain that was isolated in the United States in 1969; additional strains of this genogroup were identified in China in May 2008 (4). Genogroup B and C viruses have been circulating more widely and contain 6 and 5 genotypes, respectively, defined as B0–B5 and C1–C5. Within genotype C4, 2 additional subgenotypes have been classified, C4a and C4b (2). The

genogroups D and G in India and genogroups E and F in Africa were identified more recently (2,3).

The incidence of EV-71 infection has greatly increased in the Asia–Pacific region since 1997. Multiple countries within this region have documented massive outbreaks of EV-71, reporting thousands of cases of severe illness and death among children (1). The increased incidence coincided with the identification of new genotypes (B3–5, C3–5) (5). Although strains of genotypes C4 and B5 have been isolated from patient samples in Europe, widespread circulation of new genotypes and associated massive outbreaks are restricted to the Asian Pacific region (5–10). Most EV-71 strains circulating in Europe belong to genotypes C1 and C2, and presence of herd immunity conferred by cross-protective antibodies induced by these types could explain the limited spread of new genotypes. However, this hypothesis has not been formally studied. Multiple studies have reported cross-neutralization, but antigenic diversity among different EV-71 genotypes has also been observed (11–17). These studies have mostly been conducted by using serum samples from Asian donors or animals immunized with Asian outbreak strains.

Intravenous administration of human immunoglobulin (IVIg) is currently the only option to treat persons with severe enterovirus infections. For that, the determination of the neutralizing capacity of IVIg batches against locally circulating strains is of clinical importance. Furthermore, IVIg used in the Netherlands contains plasma from  $>1,000$  healthy Dutch donors and so represents the immunologic profile of the general population against specific pathogens. To gain more insight into the potential threat of Asian EV-71 outbreak strains for the European population and the potential treatment efficacy of IVIg, we determined the cross-neutralizing capacity of IVIg batches composed of plasma from the general population of the Netherlands during 2005–2014 against EV-71 subtypes circulating in Europe or Asia and compared results to IVIg batches from

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Japanese and Vietnamese donors. Furthermore, we determined neutralizing antibody (nAb) titers against EV-71 in serum samples from Dutch donors representing groups that are vulnerable for EV-71 infection but that are not or might not be present in large numbers in the IVIg pools. These include children  $\leq 5$  years of age, the main target group for enterovirus infections, and women of childbearing age who had a high probability of exposure by contact with young children. We also included serum samples from HIV-infected men in the analyses to determine EV-71 seroprevalence in a background population with an estimated average exposure to enteroviruses and without increased risk for enterovirus infections. Analysis of the complete capsid encoding regions of EV-71 strains included in the serologic analyses provided possible explanations for observed differences in nAb titers.

## Materials and Methods

### Cells and Virus Strains

Serum neutralization assays were performed by using rhabdomyosarcoma (RD), human colorectal adenocarcinoma (HT-29) and African green monkey kidney (Vero) cells (American Type Culture Collection, <https://www.atcc.org/>). Cell lines were cultured at 37°C, 5% carbon dioxide (CO<sub>2</sub>) in Eagle's minimum essential medium (EMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. EV-71 strains C1 91–480 and C2 07–2485 (provided by the National Institute for Public Health and the Environment, Bilthoven, The Netherlands), were isolated from clinical specimens in 1991 and 2007, respectively, by the national enterovirus surveillance system in the Netherlands (10,18). Strains C2 2105–1721 and C2 2105–2503 were isolated as part of primary diagnostics at the Academic Medical Center (AMC) in Amsterdam in 2010. The B3 SK-EV006 and B4 C7-Osaka strains were isolated in Malaysia and Japan in 1997. Strain C4 75-Yamagata was isolated in Japan in 2003, and strain C5 209-VN in Vietnam in 2006.

### IVIg Batches and Serum Samples

IVIg batches composed of plasma from Dutch or Asian donors were tested for their cross-neutralizing capacity against EV-71 strains isolated in the Netherlands and Asia. Six batches from Dutch donors were included (Nanogam, Sanquin, the Netherlands): 1 each from 2005, 2009, and 2014 and 3 from 2010. Batches from Asian donors were 2 from Japan (Teijin Institute for Bio-Medical Research, Hino, Tokyo, Japan; year of manufacture unknown) and 1 from Vietnam from 2011 (Green Cross Corporation, Pymepharco, Vietnam). Along with the IVIg

batches, a previously described rabbit polyclonal serum against EV-71 C1 91–480 was included in the neutralization assays (16). Individual human serum samples used for this study were collected and stored at –20°C as part of primary virus diagnostics in the Laboratory of Clinical Virology at AMC during 2010–2014. We defined 2 groups vulnerable for EV-71 infection: children  $\leq 5$  years of age, and women of childbearing age who were admitted to the obstetrics ward and had a high probability of exposure to young children. HIV-positive men receiving treatment who regularly attended the outpatient clinic for HIV care were included to study seroprevalence in a background population. A total of 177 samples (on average 12 serum samples/year) were randomly selected from these groups; those from children  $\leq 5$  years of age showed a proportional distribution of ages for each year. None of the patients selected had been diagnosed with hand, foot and mouth disease or had positive results for routine EV diagnostic tests.

According to laws in the Netherlands, no ethical approval is required for anonymous use of biobanked specimens. The study was conducted according to the Dutch code of conduct for responsible use of human tissue for medical research 2011 (<http://www.federa.org/code-goed-gebruik-van-lichaamsmateriaal-2011>) and the AMC Research Code (<https://www.amc.nl/web/AMC-website/Research-Code/>).

### Serum Neutralization Assays

EV-71 nAb titers of IVIg batches and individual human serum samples were determined by using a serum neutralization assay. Human serum samples were heat inactivated at 56°C for 30 min. A 2-fold serial dilution of the serum samples was subsequently incubated with an equal volume of chloroform-treated 100 50% cell culture infectious doses of virus at 37°C in 5% CO<sub>2</sub> for 1 h. HT29, RD, or Vero cells in EMEM supplemented with 10% FBS were subsequently added and plates were incubated at 37°C in 5% CO<sub>2</sub> for 5 d. The neutralizing titer was calculated on the basis of the number of wells showing cytopathogenic effect by using the Spearman-Kärber method and reported as the reciprocal titers of serum dilutions that exhibited 50% neutralization. A neutralizing titer of  $\geq 1:16$  was used as a threshold for seropositivity, because this titer has been correlated with protection against EV-71–associated disease in phase III clinical trials with EV-71 vaccines (2).

### Plaque Assay

Preliminary data indicated that several EV-71 strains escaped neutralization by IVIg batches and individual human serum samples. To exclude presence of non-EV-71 virus strains in our virus stocks, 3,000 50% cell culture infectious doses of the EV-71 virus strains escaping neutralization

and a strain that was neutralized as a positive control was incubated with a 1:4 dilution of the rabbit polyclonal serum against EV-71 C1 91–480, a selection of the IVIg batches, or plain medium (control) at 37°C in 5% CO<sub>2</sub> for 1 h. In total, 200 mL of a 0.5 log<sub>10</sub> serial dilution of the virus and serum mixtures was transferred to a monolayer of Vero cells in a 6-well format and incubated at 37°C in 5% CO<sub>2</sub> for 1 h. Cells were subsequently covered with 0.9% agarose mixed with 2× concentrated MEM supplemented with 4% fetal calf serum (1:1 ratio). Plates were incubated at 37°C at 5% CO<sub>2</sub> for 48–72 h. Single plaques were harvested and amplified on Vero cells before RNA isolation by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Zwijndrecht, the Netherlands) according to the manufacturer's instructions. The viral RNA was subsequently subjected to an enterovirus genotyping PCR as described previously (19).

### Capsid Sequence Analysis

Viral RNA was extracted from 50 µL of EV-71 isolates cultured on RD, Vero, or HT-29 cell lines, by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions. Viral RNA was eluted in 50 µL elution buffer. The capsid-encoding regions were PCR amplified in 4 overlapping regions by using genogroup B- and C-specific primers and a PCR amplification protocol described previously (20). Sequencing of the PCR products was performed by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.2 (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (Applied Biosystems). Editing of the sequence data and generation of consensus sequences of the capsid region by assembling overlapping DNA sequences determined from both strands was performed by using the ClustalW method implemented in BioEdit version 7.2.5 (21). Nucleotide sequences of EV-71 strains used in the current study are accessible in the Genbank/EMBL/DDBJ nucleotide sequence databases under accession numbers AB552982.1 (C1 91–480), AB552987.1 (C2 2485), KU697333 (C2 1721), KU697334 (C2 2503), KU697335 (C4 75-Yamagata), KU697336 (C5 209-VN), AB469182.1 (B3 SK-EV006), and AB550336.1 (B4 C7-Osaka).

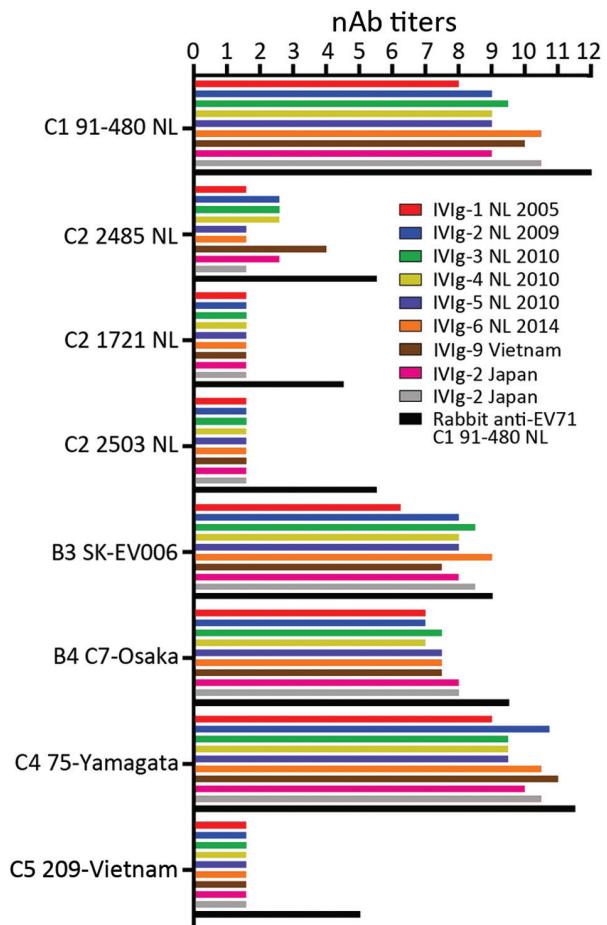
### Statistical Analysis

Differences in titers among the study groups were analyzed by using the Kruskal-Wallis One-Way ANOVA test in GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) at a significance level of  $p < 0.05$ . The 95% CIs of the proportions of seropositive persons were calculated according to the E.B. Wilson method, using the VassarStats Web site for Statistical Computation (<http://vassarstats.net/index.html>).

## Results

### EV-71 Cross-neutralizing Capacity of IVIg Batches

As human immunoglobulin batches represent the immunological profile of the general population, we studied the cross-neutralizing capacity of IVIg batches composed of plasma from Dutch, Japanese, and Vietnamese donors against EV-71 strains isolated in Europe and Asia (Figure 1). All batches had high nAb titers against the Dutch C1 strain (mean titer 1:776) and the Asian C4, B3, and B4 strains (mean titers 1:1176, 1:274, and 1:181, respectively). However, no neutralizing efficacy was observed against the Dutch C2 strains (isolated in 2007 and 2010) and the Asian C5 strain in any of the batches, including the Vietnamese IVIg batch. The hyperimmune rabbit polyclonal serum against EV-71 C1 91–480 did neutralize the strains, but with several-fold lower titers than observed for C1, C4, B3, and B4 strains (Figure 1). The results remained similar when virus strains were



**Figure 1.** EV-71 nAb titers in IVIg batches composed of plasma from Dutch (6 batches), Japanese (2 batches), and Vietnamese (1 batch) donors and in a polyclonal rabbit serum against EV-71 C1 91–480. nAb titers are presented as log<sub>2</sub> values. EV-71, enterovirus 71; IVIg, intravenous immunoglobulin; nAb, neutralizing antibody; NL, the Netherlands.

pretreated with chloroform to remove potential virus aggregates or when a different cell line (Vero) was used (data not shown). To exclude presence of non-EV-71 strains in our virus stocks that caused the cytopathogenic effect in the neutralization assays, we plaque-purified viruses incubated with or without the polyclonal serum from rabbits and a selection of the IVIg batches. Genetic characterization confirmed the presence of the EV-71 C2 and C5 genotypes in the virus stocks that escaped neutralization.

### EV-71 Neutralizing Antibody Titers in Serum from Dutch Donors

To determine the level of protection in groups at risk for EV-71 infection, nAb titers against the Dutch C1 strain were determined in serum samples from 61 children (median age 2 years, interquartile range [IQR] 1–4 years) and 56 women of childbearing age (median age 28.6 years, IQR 24.4–34.3 years), collected in the Amsterdam area during 2010–2014. Additionally, serum samples from 60 HIV-positive men (median age 38.2 years, IQR 30–51.7 years), collected during the same years in this region, were included to study seroprevalence in a background population. On average, 41% (25/61) of the children had nAb titers ( $\geq 1:16$ ) against EV-71 C1 (Table 1; Figure 2). Of the 61 children, 3 were  $<0.5$  years of age; 2 of those had nAb titers against EV-71, most likely reflecting presence of maternal antibodies. The remaining 58 children were  $\geq 0.5$ –5 years of age; the percentage of seropositive children was highest in the age group  $>2$ –5 years (60.7% vs. 20% in the age category 0.5–2 years). The percentages of seropositive children varied per year. In 2011, 4 (57%) of the 7 children 0.5–2 years of age who were tested were found to be seropositive, compared to none in 2010 and 2014. The nAb titers among children 0.5–2 years of age (median titer 1:6) were significantly lower than those for children  $>2$ –5 years of age (median titer 155;  $p < 0.05$ ) (Figure 3).

Among women of childbearing age, 44 (79%) of 56 were seropositive (median titer 1:64) (Figure 2). Titers

within this category significantly differed from those of children 0.5–2 years of age ( $p < 0.05$ ) but not from titers of children  $>2$ –5 years of age.

Of 60 HIV-positive men, 39 (65%) were seropositive for EV-71 (median titer 1:32). Although titers were lower within this group, they did not significantly differ from those observed among children  $>2$ –5 years of age or among women of childbearing age.

### Cross-neutralizing Capacity of Human Serum

A selection of individual serum samples with nAb titers against the EV-71 C1 strain were tested for cross-neutralizing activity against heterotypic EV-71 genotypes (Table 2). A total of 17 seropositive serum samples (from 7 children, 4 women, and 6 men) with nAb titers ranging from 1:91 to 1:2,896 (mean 1:997), were selected for inclusion on the basis of the volume available for testing multiple EV-71 strains and the titer being high enough to detect several folds lower heterologous nAb titers. Four seronegative serum samples with nAb titers of  $\leq 1:8$  from 2 children and 2 men were included to study whether serum samples with no nAbs against EV-71 C1 potentially contained nAbs against other EV-71 types. In line with nAb titers of the IVIg batches, all serum samples from the 17 EV-71 seropositive donors showed nAb titers against the C4 strain in the range of those observed for the C1 strain (maximum 2-fold differences, mean titer 1:1,024). High nAb titers, but several-fold lower than those for C4 strains, were observed for B3 and B4 strains (mean 1:239 and 1:158, respectively). However, the Dutch C2 1721 strain and Vietnamese C5 strain could not be neutralized by any of the serum samples. Serum samples of the 4 donors with nAb titers of  $\leq 1:8$  against the Dutch C1 strain did not neutralize any of the strains tested.

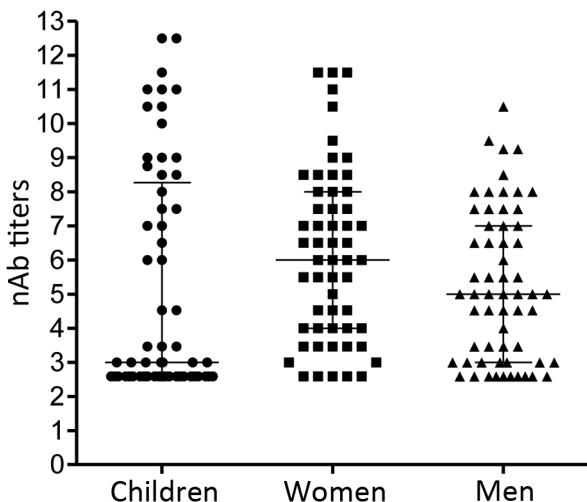
### Amino Acid Sequence Comparison

To find explanations for the escape of C2 and C5 strains from neutralization, the complete capsid encoding regions

**Table 1.** Percentages of children  $\leq 5$  y of age, women of childbearing age, and HIV-positive men with nAb titers against the Dutch enterovirus 71 strain C1 91–480, the Netherlands\*

Year	No. positive/no. tested (% , 95% CI)						
	Children, age, y			Total $\leq 5$	Adults, characteristics		M, HIV-positive
	$<0.5$	0.5–2	$>2$ –5		F, childbearing age		
2010	1/1 (100, 20.6–100)	0/7 (0, 0–35.4)	3/5 (60.0, 23.1–88.2)	4/13 (30.8, 12.7–57.6)	10/13 (76.9, 49.7–91.8)	9/14 (64.3, 38.8–83.7)	
2011	1/2 (50.0, 9.5–90.6)	4/7 (57.1, 25.0–84.2)	3/6 (50.0, 18.8–81.2)	8/15 (53.3, 30.1–75.2)	8/10 (80.0, 49.0–94.3)	5/10 (50.0, 23.7–76.3)	
2012	0/0	1/8 (12.5, 2.2–47.1)	5/6 (83.3, 43.7–97.0)	6/14 (42.9, 21.4–67.4)	9/12 (75.0, 46.8–91.1)	10/14 (71.4, 45.4–88.3)	
2013	0/0	1/3 (33.3, 6.2–79.2)	4/8 (50.0, 21.5–78.5)	5/11 (45.5, 21.3–71.2)	6/8 (75.0, 40.9–92.9)	10/14 (71.4, 45.4–88.3)	
2014	0/0	0/5 (0, 0.0–43.5)	2/3 (66.7, 20.8–93.9)	2/8 (25.0, 7.2–59.1)	11/13 (84.6, 57.8–95.7)	5/8 (62.5, 30.6–86.3)	
Total	2/3 (66.7, 20.8–93.9)	6/30 (20.0, 9.5–37.3)	17/28 (60.7, 42.4–76.4)	25/61 (41.0, 29.5–53.5)	44/56 (78.6, 66.2–87.3)	39/60 (65.0, 52.4–75.8)	

\*nAb, neutralizing antibody.



**Figure 2.** Enterovirus 71 nAb titers in serum collected from Dutch children  $\leq 5$  years of age, women of childbearing age, and HIV-positive men during 2010–2014. nAb titers are presented as  $\log_2$  values. Median titers (wide horizontal lines) with interquartile ranges (error bars) are indicated for each category. nAb, neutralizing antibody.

of strains included in this study were sequenced and used for amino acid sequence analysis (Figure 4, <http://wwwnc.cdc.gov/EID/article/22/9/15-1579-T4.htm>) (22–25). In total, 19 residues differed between the genogroup B and C strains, of which 3 were located in a linear epitope: residues 164, 240, and 241 of VP1 (Figure 4) (22–25). The C2 and C5 strains differed from strains that could be neutralized in residues 145 of VP1 (145E vs. 145G/Q) and 93 of VP3 (93S vs. 93N/D). The C2 strains had an additional mutation of residue 22 of VP1 (22R vs. 22Q) and the C5 strain of residue 262 of VP1 (262V vs. 262I).

## Discussion

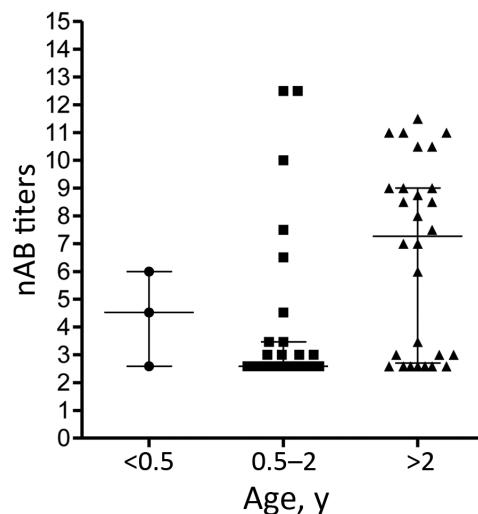
We determined the nAb titers against EV-71 in IVIg batches and individual serum samples from Dutch donors and tested their cross-neutralizing capacity against strains responsible for outbreaks in Asia. The high percentage of EV-71 seropositive serum samples suggests widespread circulation of EV-71 in the Netherlands, which is likely sustained by the presence of a relatively large cohort of susceptible infants (80% in the age category 0.5–2 years). In 2010, EV-71 caused an elevated number of enterovirus infections in the Netherlands, likely explaining the high number of seropositive children in 2011 (18) and in the age category  $>2$ –5 years in 2012 in this study.

The rates of seropositivity observed in this study are comparable to those observed among the German population (26,27). The prevalence, however, seems not be uniform across Europe, because seroprevalence levels among children in Finland ( $<11$  years of age) and in

European regions of Russia (3–5 years of age) were only 1.6% and 19%–27%, respectively (6,28). The seroprevalence levels observed in the Netherlands are in the range of those observed in Asia before outbreaks (29–33). Although this finding points toward the possibility of large outbreaks in Europe as well, widespread circulation of Asian EV-71 genotypes and associated outbreaks seem to be restricted to the Asian region.

In this study, we showed that IVIg batches and serum samples with nAb titers against the Dutch C1 strain efficiently neutralized Asian C4, B3, and B4 strains. This result implies that administration of IVIg from the Netherlands could benefit recovery of a patient infected by an Asian EV-71 strain and could explain why the multiple introductions of genotype C4 strains in Europe have not resulted in widespread circulation (6,9,10). In agreement with this finding, the transient occurrence of C4 and B5 infections in Europe coincided with low population sizes of C1 and C2 (34). However, a relatively large group of susceptible children is still at risk for severe EV-71 infections; therefore, monitoring introduction of new genotypes into Europe remains of importance.

A limitation of our study is that it is not possible to differentiate between neutralization and cross-neutralization. The high neutralizing activity against C4 strains could reflect actual exposure to C4 virus rather than cross-neutralization of antibodies induced by C1 or C2 strains. This possibility, however, is thought to be negligible, because the systematic, nationwide enterovirus surveillance system in the Netherlands has only reported circulation of C1 and C2 strains (10). Additionally, we showed neutralization of the



**Figure 3.** Enterovirus 71 nAb titers in serum collected from Dutch children ( $<0.5$  years, 0.5–2 years and  $>2$ –5 years of age) during 2010–2014. nAb titers are presented as  $\log_2$  values. Median titers (wide horizontal lines) with interquartile ranges (error bars) are indicated for each category. nAb, neutralizing antibody.

**Table 2.** Cross-neutralization of heterotypic Asian and Dutch enterovirus 71 strains by serum from Dutch donors, the Netherlands\*

Serum sample no.	Category	nAb titers against					
		C1 91-480 NL	C2 1721 NL	C4 75-Yamagata	C5 209-VN	B3 SK-EV006	B4 C7-Osaka
1	Children	1,448	NT	1,448	NT	362	64
2	Children	362	NT	362	NT	128	45
3	Children	2,048	NT	1,448	NT	724	362
4	Children	1,448	NT	1,024	NT	512	256
5	Children	2,896	NT	2,048	NT	ND	ND
6	Children	2,048	NT	5,793	NT	ND	ND
7	Children	181	NT	ND	ND	ND	ND
8	Women	2,896	NT	1,448	NT	181	256
9	Women	91	NT	91	NT	45	23
10	Women	724	NT	724	NT	128	362
11	Women	181	NT	256	NT	32	32
12	Men	724	NT	512	NT	181	64
13	Men	609	NT	362	NT	64	91
14	Men	609	NT	512	NT	ND	ND
15	Men	256	NT	181	NT	ND	ND
16	Men	256	NT	181	ND	ND	ND
17	Men	181	NT	128	NT	ND	ND
18	Men	8	NT	NT	NT	ND	ND
19	Men	8	NT	NT	NT	ND	ND
20	Children	8	NT	NT	ND	ND	ND
21	Children	8	NT	NT	NT	ND	ND

\*ND, not determined; NT, no titer.

C4 strain by a polyclonal rabbit serum against EV-71 C1, which confirms that nAbs elicited against C1 are cross-protective against C4. We did not have access to antigenically divergent genotype B5 strains, which is unfortunate, as B5 strains were isolated from clinical specimens in Denmark in 2007 and in France in 2013 (8).

Neither the C2 strains nor the C5 strain could be neutralized by IVIg batches or individual serum samples. Because C2 has been persistently circulating in Europe since 1997 (10), it is unlikely that there has not been exposure to this genotype. In fact, a serum sample from our biobank obtained from a patient proven to be infected with EV-71 C2 could not neutralize the Dutch C2 strains either (data not shown). Large variations in nAb titers against C2, even in serum from children proven to be infected with C2, were observed by other research groups as well (11,12,35,36). Variation in neutralizing activity could be explained by antigenic diversity among strains included in the analyses and strains to which patients or populations were exposed (23,37). The C2 and C5 strains escaping neutralization in the current study differed from the neutralizable strains in VP3 residue 93 and VP1 residues 22 and 145 (C2) or 145 and 262 (C5). VP1 residue 145 has previously been identified as a key antigenic determinant, of which substitution can significantly affect the neutralizing activity (23,38). Furthermore, VP1 22, 145, and 262 are among residues of which substitution through time has been suggested to be necessary for EV-71 persistence by generating antigenic novelty (5). Because the evolution of EV-71 genotypes is shaped by a continuous replacement of viral lineages over time, it will be of importance to further characterize the role of the identified mutations in determining antigenic diversity and to study whether the observed low nAbs titers against C2 and C5 reflect a real

low seroprevalence or are a test artifact due to inclusion of antigenically divergent strains.

Cross-neutralizing activity against C5 in serum of C4-infected humans has been reported, and from that perspective it is remarkable that the C5 strain escaped neutralization by the IVIg isolated in Vietnam that had high nAb titers against C4 (12,39,40). However, it is complicated to compare results from studies that used different virus strains and serum from persons with different exposure histories, considering the co-circulation of different genotypes and the continuous evolution of EV-71 types with potentially novel antigenicity (5).

In conclusion, we showed the presence of high cross-nAb titers against EV-71 in IVIg batches and serum samples from Dutch donors. This finding implies that administration of Dutch IVIg could support recovery of a patient infected with an Asian EV-71 strain and that herd immunity induced by locally circulating strains could be cross-protective against widespread circulation and associated outbreaks of Asian strains in Europe. The identification of viruses that escape neutralization, however, warrants further research on antigenic diversity among EV-71 strains and emphasizes the importance of monitoring both the genetic and antigenic diversity of circulating strains.

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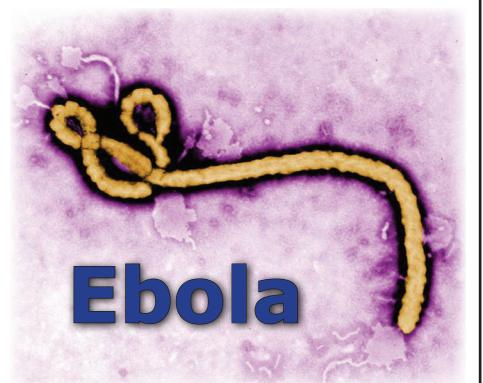
## SPOTLIGHT

Ebola, previously known as Ebola hemorrhagic fever, is a rare and deadly disease caused by infection with one of the Ebola virus strains. Ebola can cause disease in humans and nonhuman primates (monkeys, gorillas, and chimpanzees).

Ebola is caused by infection with a virus of the family *Filoviridae*, genus *Ebolavirus*. There are five identified Ebola virus species, four of which are known to cause disease in humans. Ebola viruses are found in several African countries, and was first discovered in 1976

near the Ebola River in what is now the Democratic Republic of the Congo. Before the current situation, outbreaks have appeared sporadically in Africa.

The natural reservoir host of Ebola virus remains unknown. However, on the basis of evidence and the nature of similar viruses, researchers believe that the virus is animal-borne and that bats are the most likely reservoir. Four of the five virus strains occur in an animal host native to Africa.



<http://wwwnc.cdc.gov/eid/page/ebola-spotlight>

# *Staphylococcus aureus* Regulatory RNAs as Potential Biomarkers for Bloodstream Infections

Valérie Bordeau, Anne Cady, Matthieu Revest, Octavie Rostan, Mohamed Sassi, Pierre Tattevin, Pierre-Yves Donnio, Brice Felden

*Staphylococcus aureus* is a commensal bacterium and pathogen. Identifying biomarkers for the transition from colonization to disease caused by this organism would be useful. Several *S. aureus* small RNAs (sRNAs) regulate virulence. We investigated presence and expression of 8 sRNAs in 83 *S. aureus* strains from 42 patients with sepsis or septic shock and 41 asymptomatic colonized carriers. Small pathogenicity island sRNAs sprB and sprC were clade specific. Six sRNAs had variable expression not correlated with clinical status. Expression of RNAIII was lower in strains from septic shock patients than in strains from colonized patients. When RNAIII was associated with expression of sprD, colonizing strains could be discriminated from strains in patients with bloodstream infections, including patients with sepsis and septic shock. Isolates associated with colonization might have sRNAs with target expression different from those of disease isolates. Monitoring expression of RNAIII and sprD could help determine severity of bloodstream infections.

*Staphylococcus aureus* causes many community-acquired, healthcare-related, and nosocomial infections in humans. This bacterium is a commensal organism (part of the normal microflora), but it can also infect the body at various sites. Diseases caused by *S. aureus* differ greatly, ranging from skin lesions to invasive infections. A total of 20%–30% of the healthy population is colonized with *S. aureus* in the nostrils (1), and a substantial percentage of *S. aureus* bacteremia originates from endogenous colonies in the nasal mucosa (2,3).

Clinical expression of sepsis covers a continuum of manifestations; the most serious form is septic shock. In this state, vascular offense and systemic inflammation lead to endangered cardiac function and decrease in blood

pressure that cause impaired oxygen delivery, organ failure, and death. Sepsis-related deaths and lack of mitigating clinical approaches attest to our limited understanding of the complex host–*S. aureus* interactions.

*S. aureus* has high rates of transmission and increased levels of antimicrobial drug resistance and produces many virulence factors (4). To coordinate expression of virulence genes during infection, *S. aureus* uses 2-component systems, transcription factors (5) and regulatory or small RNAs (sRNAs), which function as positive (6) or negative (7) virulence determinants. There are ≈160 sRNAs in the Staphylococcal Regulatory RNA database (8). Although their functions have not been extensively investigated, some sRNAs are known to regulate virulence factors. Quorum sensing is mediated by the accessory gene regulator *agr*, and RNAIII is the effector (9). Staphylococcal infection severity is based on host factors and bacterial pathogenesis (10). We investigated differences in sRNA gene content and expression levels in *S. aureus* strains isolated from patients with bloodstream infections and from asymptomatic carriers.

## Materials and Methods

### Isolates and Sample Collection

We obtained clinical isolates from a prospective study of all patients given a diagnosis of *S. aureus* bloodstream infections in 2006 at the Rennes University Hospital (Rennes, France), a tertiary referral hospital in western France. We selected patients with nonsevere sepsis or septic shock (11). Patients with severe sepsis (sepsis with organ dysfunction or tissue hypoperfusion improving after fluid therapy and not requiring vasopressors) were not included because their clinical status might too closely resemble nonsevere sepsis or shock. To prevent other confounding factors, immunodeficient patients were excluded: those infected with HIV; those with congenital immunodeficiency, malignant hemopathy, organ or stem cell transplants; those receiving systemic corticosteroid therapy for >3 weeks; and those undergoing another immunosuppressive treatment.

We extracted data from medical records. A nosocomial bloodstream infection was defined as either a bloodstream

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infection diagnosed in a patient hospitalized for >48 hours before symptom onset or a bloodstream infection in a patient receiving chronic hemodialysis or peritoneal dialysis. For each patient, we calculated at admission the Charlson Comorbidity Index and the Simplified Acute Physiology Score (12). We also collected 41 isolates from asymptomatic carriers: 23 from medical students in Rennes; 7 from healthcare workers sampled during their medical visit at a hospital in Lausanne, Switzerland; and 11 from the National Reference Laboratory for Staphylococci in Lyon, France. The study was approved by review board at Rennes University Hospital.

### Multilocus Sequence Typing and *S. aureus*

#### Protein A Typing

*S. aureus* protein A (*spa*) typing was performed by using primers *spa*-1113f (5'-TAAAGACGATCCTTCG-GTGAGC-3') and *spa*-1514r (5'CAGCAGTAGTGCC-GTTTGCTT-3'). Sequences were determined by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a 3730xl DNA analyzer (Applied Biosystems). The *spa* repeats and types were determined by using BioNumerics (Applied Maths, Sint-Matens-Latem, Belgium) and the Ridom *Spa* Server (<http://www.spaserver.ridom.de/>). The *spa* types with similar profiles were grouped within similar lineages. Multilocus sequence typing (MLST) was performed according to procedures of Enright et al. (13). The PCR products were sequenced by using a 3730xl DNA analyzer, and sequence types (STs) were determined by using BioNumerics and the MLST database (<http://www.mlst.net/>). *MecA1* and *MecA2* primers were used to amplify a 1,102-bp gene fragment, which was used to identify *mecA*. Isolates were screened for *tst* and *pvl* by using real-time PCR. PCR was used to detect small pathogenicity island (PI) RNAs (*spr*); *sprA1/2*, *sprB*, *sprC*, *sprD*, *sprX*, *ssrA*, 6S RNA, and *rsaE*. Primers used are listed in online Technical Appendix Table 1 (<http://wwwnc.cdc.gov/EID/article/22/9/15-1801-Techapp1.pdf>). All PCR products were analyzed by using 2% agarose gel electrophoresis.

#### Bacterial Cultures, RNA Isolation, and Expression Analysis

*S. aureus* strains were grown in Luria-Bertani medium and then harvested. Cells were isolated by centrifugation and dissolved in a solution of 33 mmol/L sodium acetate, 17 mmol/L sodium dodecyl sulfate, and 1 mmol/L EDTA (pH 5.5). The cells were then mixed with glass beads and lysed by using a Fast Prep Apparatus (MP Biochemicals, LLC, Santa Ana, CA, USA).

RNAs were isolated by using water-saturated phenol (pH 5.0). RNAs were precipitated and washed with ethanol. Northern blotting of RNA markers was conducted by

loading 10 µg of total RNA onto 8 mol/L urea, 8% polyacrylamide gels. Gels were subjected to electrophoresis and blotted onto nylon membranes at 30 V for 1.5 h in 0.5× Tris-HCl, borate, EDTA buffer.

Prehybridization and hybridization were performed by using ExpressHyb solution (Clontech, Mountain View, CA, USA) and <sup>32</sup>P-labeled DNAs (online Technical Appendix Table 2). Signals were detected by using phosphorimaging (BioCompare, South San Francisco, CA, USA) and quantified. Expression levels of sRNAs in strains were monitored by using quantitative PCR and specific primers (online Technical Appendix Table 3). cDNAs were produced by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Using the comparative cycle threshold method (Applied Biosystems), we normalized sRNA counts against transfer-messenger RNA (tmRNA) and *S. aureus* reference strain L102.

#### Bacterial Protein Extracts and Western Blotting

For preparation of protein extracts, bacteria were grown until the desired optical density (OD) at 600 nm was reached. Cells were centrifuged at 8,000 × *g* for 10 min at 4°C and suspended in lysis buffer (10 mmol/L Tris-HCl [pH 7.5], 20 mmol/L NaCl, 1 mmol/L EDTA, and 5 mmol/L MgCl<sub>2</sub>) in the presence of a protease inhibitor cocktail tablet containing 0.1 mg/mL lysostaphin. The cells were then dissolved in 1× Laemmli buffer containing 10% β-mercaptoethanol and heated at 90°C for 5 min. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% polyacrylamide gels and transferred to polyvinylidene fluoride membranes at 100 V for 1 h. Membranes were blocked in Tris-buffered saline containing 5% milk.

Second immunoglobulin-binding protein (Sbi), which is an immune evasion protein, was detected by using specific antibodies as described (14), and SaeR protein was detected by using specific antibodies. Incubation with primary antibodies against Sbi (diluted 1:10,000) or antibodies against SaeR protein (diluted 1:5,000) was performed at room temperature for 2 h. Blots were incubated with antirabbit IgG peroxidase-conjugated secondary antibodies for 1 h; washed in Tris-buffered saline, 0.05% Tween; developed in ECL Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA); and exposed on an ImageQuant LAS4000 Imaging System (GE Healthcare Bio-Sciences). Quantifications were performed by using the ImageQuant System. Levels of Sbi or SaeR protein were normalized against levels of total proteins.

All statistical tests and graphic representations were performed by using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Quantitative values were compared by using the Mann-Whitney U test. A *p* value <0.05 was considered significant.

## Results

### Characteristics of Patients with *S. aureus*

#### Bloodstream Infections

A total of 42 patients (17 with septic shock and 25 with nonsevere sepsis) were included in this study (Table 1). Patients with nonsevere sepsis were more likely than those with septic shock to have nosocomial bloodstream infections ( $p = 0.02$ ) and a lower Simplified Acute Physiology Score ( $p = 0.01$ ). The mortality rate was also significantly higher for patients with septic shock than for patients with nonsevere sepsis (41.2% vs. 8.0%;  $p = 0.01$ ). The clonal distribution of isolates (Table 2, <http://wwwnc.cdc.gov/EID/article/22/9/15-1801-T2.htm>) was similar to that reported for France in the European Antimicrobial Resistance Surveillance System (15).

#### Genotyping of Strains from Patients with Invasive Diseases and Asymptomatic Carriers

We used MLST and *spa* typing to analyze 83 *S. aureus* isolates from 42 blood cultures for patients with bloodstream infections or 41 nasal samples from asymptomatic carriers. Isolates clustered into 17 STs (Figure 1, panel A, <http://wwwnc.cdc.gov/EID/article/22/9/15-1801-F1.htm>). Of 83 strains analyzed, none had genes encoding the Pantone-Valentine leukocidin, which is associated with increased virulence of certain strains. Toxic shock syndrome toxin genes were detected in infectious and methicillin-susceptible *S. aureus* colonizing strains that belonged to ST5 and ST30.

Among 37 methicillin-susceptible *S. aureus* colonizing strains, ST398 was the most common ( $n = 6$ ) (online Technical Appendix Table 4). Only 4 strains isolated from nasal samples were methicillin-resistant *S. aureus* (MRSA), and these belonged to ST8 and ST22 (Table 2). Most ( $n = 10$ ) MRSA isolates were ST8 (Table 2). MRSA prevalence was  $\approx 10\%$  in healthy colonized healthcare workers and students and  $\approx 2\%$  in the general population (17). The prevalence of MRSA in infectious samples ( $\approx 21\%$ ), all positive for *mecA*,

was consistent with the overall prevalence of staphylococcal infections in France (18). We detected a predominance of the ST8 MRSA clone, which is the predominant pandemic MRSA clone in France and has *sea* and *lukED* genes. Isolates from patients with bloodstream infections and carriers were evenly distributed among the STs (Table 2). As reported by Feil et al. (19), genetic distances between the 8 group 1 STs (mean  $\pm$  SD 25.  $\pm$  10.6) were longer than those between the 9 group 2 STs (20.0  $\pm$  7.4), which is consistent with earlier emergence of group 1 isolates.

#### Specificities of sRNAs for Bacterial Clades

We used PCR to identify a subset of sRNAs that were specific for conserved sequences. Eight sRNAs were selected from core and accessory genomes for determining their distribution among strains. We chose sRNAs according to their presence in the accessory genome because this presence implies variability in their presence/absence among strains and their putative roles in virulence. We included the few sRNAs ubiquitously detected in bacteria. House-keeping tmRNA and 6S RNA genes, which were detected in many bacterial species, were found in all strains. All strains also contained RNIII, which is the quorum-sensing effector (9).

In addition, we detected 5 sRNAs expressed on PIs: *sprA* (*srn\_3580*), *sprB* (*srn\_3600*), *sprC* (*srn\_3610*), *sprD* (*srn\_3800*), and *sprX* (*srn\_3820*) (8). Because of absence of PIs *phiSa3* and *vsaf* in group 1, all 5 sRNAs on PIs were detected only in group 2 STs (Table 2). We found that *sprA* was rarely detected in ST398 strains, *sprB* was not detected in all group 1 strains and STs, *sprC* was not detected in group 1 STs except ST398 strains, *sprD* was detected in all but 5 strains from both groups, and *sprX* was detected in all strains except ST398. Detection of *sprD* and *sprX* in most STs from both groups reflects evolution of *S. aureus*, which has been punctuated by successive acquisitions and losses of genetic elements. The presence of *sprB* and *sprC* among *S. aureus* infectious isolates indicates *S. aureus* phylogeny

**Table 1.** Clinical characteristics of 42 patients with *Staphylococcus aureus* bloodstream infections admitted to Rennes University Hospital, Rennes, France\*

Characteristic	Sepsis, n = 25	Septic shock, n = 17	p value
Male sex, %	84	84	1.00
Age, y	62.7 (15–97)	68.1 (33–84)	0.30
Nosocomial bacteremia	80	41.1	0.02
MRSA	32	5.8	0.06
Diabetes mellitus	16	29.4	0.45
Alcohol abuse	12	35.3	0.12
Charlson Comorbidity Index	1.4 (0–5)	2.1 (0–5)	0.06
Endovascular device	52	29.4	0.21
SAPS II	43.2 (14–61)	60.9 (38–126)	0.01
Delayed antibiotherapy	4.6 (0–42)	3.1 (0–10)	0.42
Infective endocarditis	12	17.6	0.70
C-reactive protein	186.4 (32–427)	250.5 (67–445)	0.17
Polynuclear neutrophils	14,588 (4,700–33,000)	14,977 (4,230–26,000)	0.84
Mortality rate	8	41.2	0.01

\*Values are % or no. (range). MRSA, methicillin-resistant *S. aureus*; SAPS II, Simplified Acute Physiology Score.

and strain clonality (Figure 1, panel B). Strain genotyping showed that the sample analyzed reflected diversity of staphylococcal infections at the national level in France.

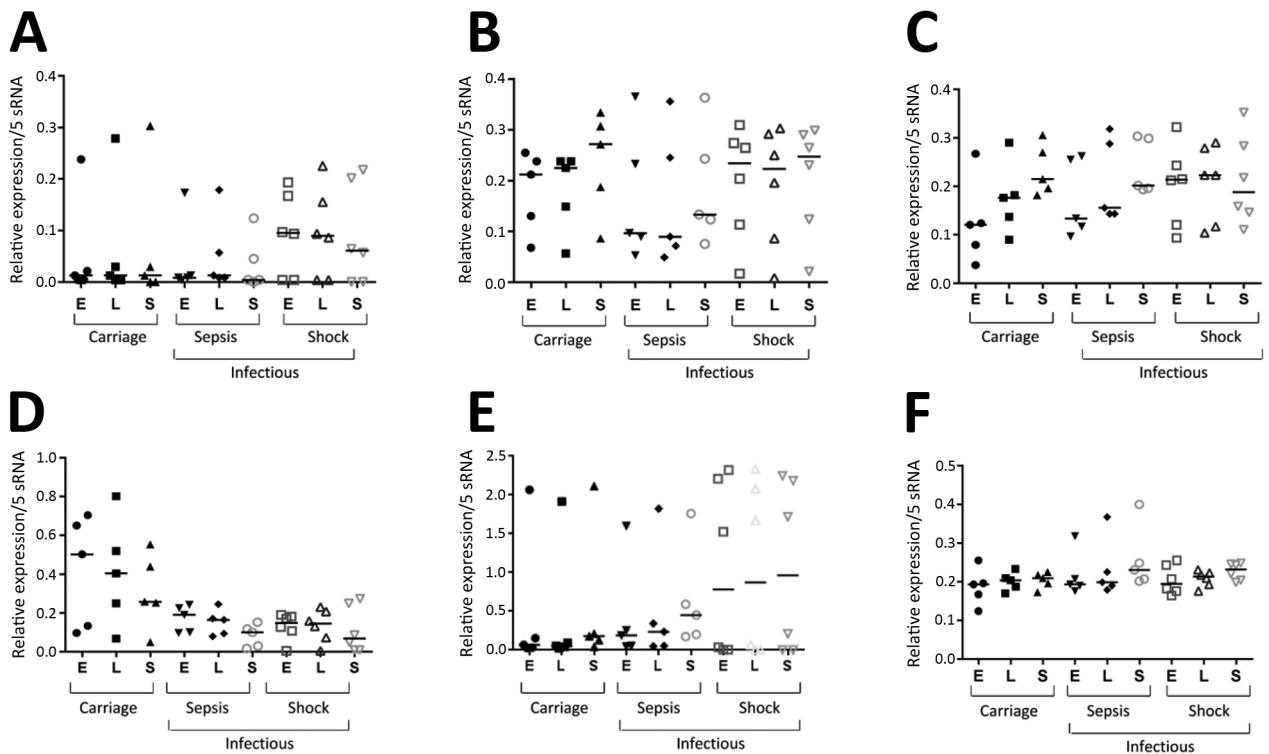
**PI-Encoded RNA Expression**

Because of low amounts (10–100 CFU/mL of blood) of bacteria isolated from patients with bloodstream infections (20), *S. aureus* isolates must be cultured before assessing sRNA expression. We selected 16 strains for subsequent analyses: 5 from nasal carriers, 6 from patients with sepsis, and 5 from patients with septic shock. Each group of samples contained strains from the same sequence types (ST5, ST8, and ST25) (Table 2). We intentionally included strains belonging to the same ST (ST8), with strains from nasal carriers, from patients with sepsis, and from patients with septic shock. Strain selection was also dictated by availability in our collection. For these 16 isolates, we assessed sRNA expression levels at OD<sub>600 nm</sub> = 2 (early exponential), OD<sub>600 nm</sub> = 4 (late exponential), and OD<sub>600 nm</sub> = 8 (stationary)

growth phases (Figure 2; online Technical Appendix Figure). Growth curves for all isolates were superimposable (Figure 3, panel A).

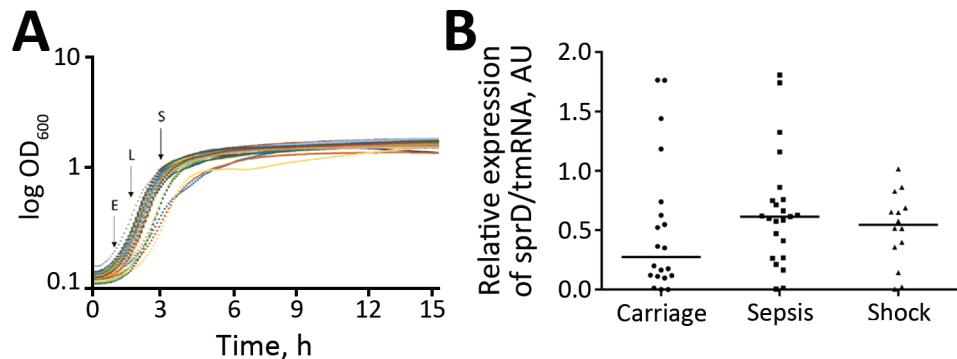
We compared overall sRNA expression levels among the infectious subgroups. For all strains, tmRNA was constitutively expressed, and there were no differences in expression among strains (Figure 2). This finding is consistent with the status of the tmRNA gene as a housekeeping gene involved in ribosome rescue (21). The 6S RNA was also constitutively expressed, and there were no differences in expression among strains. Expression of the 5 Spr RNAs varied widely among the strains (Figure 2). We found that sRNA showed different expression profiles within the same ST (online Technical Appendix Figure), which indicated the complexity and variability of sRNA-driven gene regulation in *S. aureus*.

Expression of sprD was heterogeneous in strains from asymptomatic carriers but more homogeneous in infected patients (Figure 2). These results were inferred



**Figure 2.** Expression of 5 sRNAs and tmRNA in 16 *Staphylococcus aureus* isolates from patients with bloodstream infections (nonsevere sepsis or septic shock) and asymptomatic colonized carriers (carriage), Rennes, France. A) sprA; B) sprB; C) sprC; D) sprD; E) sprX; F) tmRNA. Isolates were derived from ST5, ST8, and ST25. Expression was measured by using Northern blotting after strain isolation and culture. Total RNAs were obtained during early exponential (E), late exponential (L), and stationary (S) growth phases. Horizontal lines indicate medians of expression for each growth phase and sRNA. tmRNA was used as reference sRNA for subsequent quantitative PCR analyses that monitored expression levels of other sRNAs because its expression is stable for all isolates at each growth phase. Black dots indicate carriage strains at E growth phase; black squares indicate carriage strains at L growth phase; black triangles indicate carriage strains at S growth phase; inverted black triangles indicate strains causing sepsis at E growth phase; black diamonds indicate strains causing sepsis at L growth phase; circles indicate strains causing sepsis at S growth phase; squares indicate strains causing shock at E growth phase; triangles indicate strains causing shock at L growth phase; inverted triangles indicate strains causing shock at S growth phase. ST, sequence type; sRNA, small RNA; spr, small pathogenicity island RNA; tmRNA, transfer–messenger RNA.

**Figure 3.** Expression of *sprD* in 61 *Staphylococcus aureus* isolates, Rennes, France. A) Representative growth curves. Each curve indicates 1 strain. Arrows indicate times at which the total RNAs were collected. E, early exponential growth phase; L, late exponential growth phase; S, stationary growth phase. B) Isolates analyzed for *sprD* (*sm\_3800*) expression levels at E growth phase: 21 from asymptomatic carriers, 23 from patients with nonsevere sepsis, and 17 from patients with septic shock. For normalization, quantitative PCR was used to determine expression of tmRNA for each isolate as internal loading controls. Horizontal lines indicate medians. Using the comparative cycle threshold method, we normalized the amount of *sprD* against that of tmRNA relative to that of control strain L102 (methicillin-susceptible *S. aureus* colonization strain). Each symbol indicates mean for 3 independent experiments.  $p = 0.09$  for isolates from asymptomatic carriers versus isolates from patients with sepsis. AU, arbitrary units; log OD<sub>600</sub>, log optical density at 600 nm; ST, sequence type; sRNA, small RNA; spr, small pathogenicity island RNA; tmRNA, transfer-messenger RNA.



from Northern blotting performed for 3 independent RNA extractions. Afterwards, the set of analyzed strains was nearly quadrupled to 61 isolates, with 21 from carriers, 23 from nonsevere sepsis patients, and 17 from septic shock patients. Because Northern blotting showed variations in *SprD* expression levels between the clinical strain sets (Figure 2), we monitored *SprD* expression by using quantitative PCR at OD<sub>600nm</sub> = 2 (Figure 3, panel A). Strains from asymptomatic carriers and sepsis patients expressed *SprD* heterogeneously, although *SprD* was expressed at low levels in all strains isolated from patients with septic shock (Figure 3, panel B).

#### Discrimination of Isolates by Expression Levels of RNAIII and RNAIII/*SprD*

Another RNA implicated in *S. aureus* virulence is RNAIII (9), which is an archetype of RNA-mediated regulation of virulence genes. Therefore, we used quantitative PCR to monitor RNAIII expression levels for 61 isolates (Figure 4) during E growth phase (Figure 3, panel A). We detected significantly lower RNAIII levels in strains isolated from patients with bloodstream infections than in those from nasal carriers ( $p = 0.035$ ) (Figure 4, panel A). When we compared strains from patients with septic shock with commensal isolates, strains from patients with septic shock had significantly lower levels of RNAIII ( $p = 0.017$ ) (Figure 4, panel B). Calculated RNAIII expression levels in infectious and asymptomatic persons showed a progressive decrease from carriage to nonsevere sepsis to septic shock. When we combined the effects of *SprD* with RNAIII, this combination substantially discriminated carriage isolates from infectious isolates ( $p = 0.0065$ ) (Figure 4, panel C), carriage isolates from sepsis isolates ( $p = 0.018$ ), and carriage isolates from septic shock isolates ( $p = 0.025$ ) (Figure

4, panel D). We conducted receiver operating characteristic analyses to determine the capacity of differential expression of RNAIII and *SprD* in predicting disease outcome. Our findings indicate differences in RNAIII/*SprD* expression levels between colonizing strains and infectious strains (Figure 4, panel C, inset).

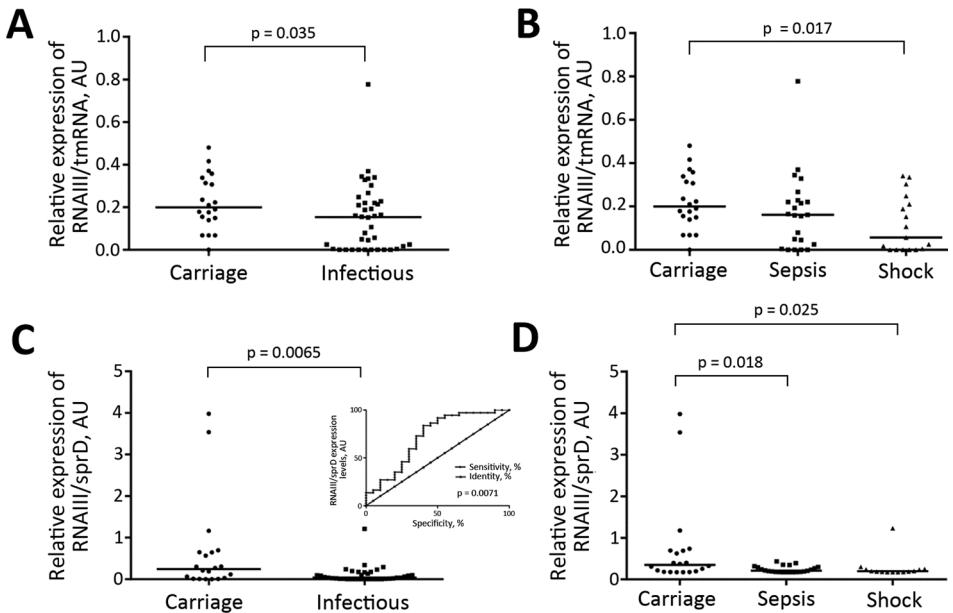
#### Discrimination of Isolates by Expression Levels of Sbi

*SprD* and RNAIII negatively regulate expression of Sbi by blocking translation through binding with the Sbi mRNA (6,18). We performed Western blotting for 61 isolates in growth phase E by using polyclonal antibodies against intracellular and membrane proteins to monitor Sbi levels (Figure 5, panel A). As reported by Smith et al. (22), we found that molecular weights of Sbi detected in isolates were variable (mean  $\approx 50$  kDa) and the amount of Sbi varied among isolates (Figure 5, panel A). Individual assessments showed significantly lower Sbi levels in isolates from patients with bloodstream infections than in patients with nasal colonization ( $p = 0.04$ ).

#### Discussion

A set of 83 *S. aureus* strains with known genotypes was collected from asymptomatic carriers and patients with nonsevere sepsis or septic shock. We used this collection for a prospective study of the presence and expression of certain sRNAs in the core and accessory genome. We also monitored expression of Sbi, an immune evasion protein whose expression is negatively controlled by the sRNAs *SprD* and RNAIII (14) and *SaeR*, a positive regulator of Sbi. In clinical and carriage staphylococcal strains, the presence of  $\geq 2$  PI-encoded sRNAs, *sprB* (*sm\_3600*) and *sprC* (*sm\_3610*), was indicative of the presence of PIs and prophages. These PI-encoded sRNAs, particularly *sprB*, could

**Figure 4.** Discrimination of colonizing strains of *Staphylococcus aureus* from patients with bloodstream infections, Rennes, France. A) RNAIII analysis of strains from carriers and infected persons; B) RNAIII analysis of strains from carriers and persons with nonsevere sepsis or septic shock; C) RNAIII/sprD analysis of strains from carriers and infected persons; D) RNAIII/sprD analysis of strains from carriers and persons with nonsevere sepsis or septic shock. RNAIII and sprD levels were calculated relative to those for tmRNA. RNAIII expression was monitored by quantitative PCR during early exponential growth phase for 61 strains. p values (by Mann-Whitney U test) for significant differences are shown. Panel C inset shows receiver operating characteristic analysis showing discrimination of carriage strains from infectious strains. Horizontal lines indicate medians. Using the comparative cycle threshold method, we normalized the amount of RNAIII against that of tmRNA relative to that for control strain L102 (methicillin-susceptible *S. aureus* colonization strain). Each symbol indicates mean for 3 independent experiments. AU, arbitrary units; spr, small pathogenicity island RNA; tmRNA, transfer-messenger RNA.



be used as probes to improve genotyping studies. These sRNAs probably appear during the transition between ST22 and ST25 (Figure 1, panel A). The sRNA sprB is absent in most group 1 isolates. In some strains, we cannot rule out that sequence variations among the sRNA genes might hamper their amplification.

Molecular typing can identify genetic diversity of strains, which is required for epidemiologic surveillance of infections. Bacterial strain typing methods include generation of DNA banding patterns, sequencing, and hybridization. Bacterial genotyping has benefited from identification of novel locus-specific typing markers. The sRNAs might be useful probes for genotyping bacteria because their overall content varies considerably, even among closely related strains. Furthermore, because several sRNAs are encoded by mobile genetic elements, these sRNAs reflect the acquisition/loss of virulence factors encoded by these elements and molecules that confer antimicrobial drug resistance. In phylogenetic studies, selected sRNAs located in accessory genomes might shed light on genomic diversity.

The immunologic ability of patients to eradicate pathogens is a major determinant of infection outcome, and patients with septic shock are often immunocompromised (23). Our data suggests that, for staphylococcal bloodstream infections, one must also consider attributes of infecting strains, including at least an immune evasion molecule and sRNAs RNAIII and sprD. The effector of the

*agr* quorum-sensing system was expressed at lower levels in strains isolated from patients with bloodstream infections, especially those with septic shock, than in asymptomatic carriers. Therefore, low RNAIII levels might identify *S. aureus* isolates that are responsible for bloodstream infections, even after isolation and culture. *agr*-defective *S. aureus* is a frequent cause of bloodstream infections (24). Our observations concur with the previous identification of inactivating mutations in the *S. aureus agr* virulence regulator, which have been associated with poor outcomes in for patients with bloodstream infections (3). Coupling the expression levels of RNAIII and SprD could discriminate colonization from infection and also provide useful information about severity of bloodstream infections. However, this possibility must be confirmed with a larger set of clinical isolates.

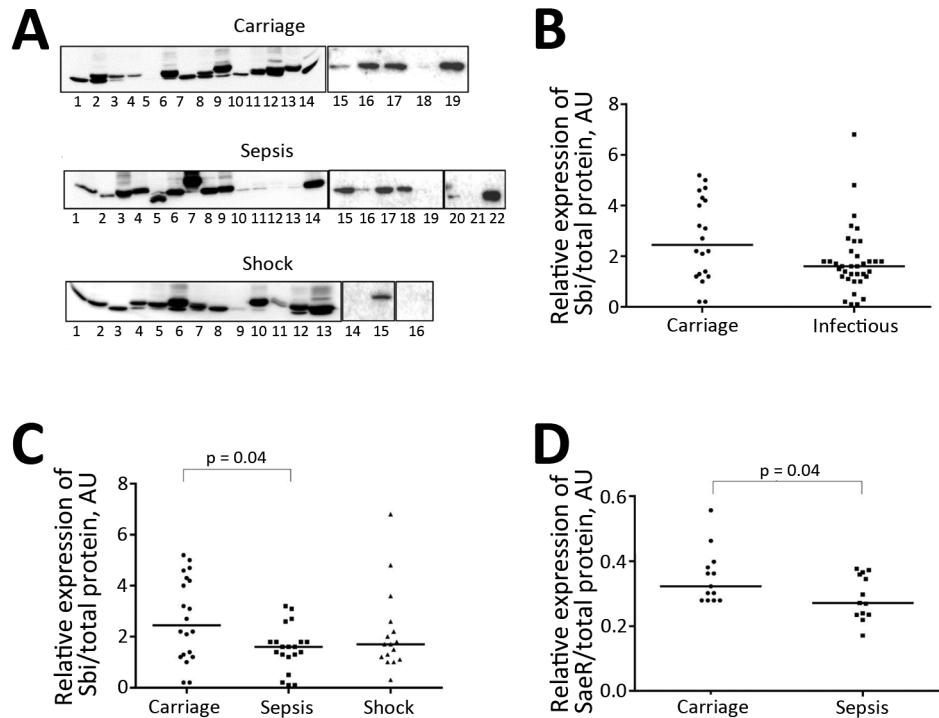
Because sprD and RNAIII negatively control the expression of the Sbi immune evasion protein (14), we also monitored Sbi expression in the isolates. Sbi is an immune evasion factor (25) involved in the *S. aureus*-induced inflammatory response (26). Cell wall-anchored Sbi proteins are essential components for *S. aureus* survival in the commensal state (27). Detection of additional Sbi proteins in strains isolated from asymptomatic carriers than in those from patients with septic shock is consistent with their role during colonization and in immune tolerance. Expression of Sbi at the transcriptional level is positively regulated by SaeRS, which is composed of the histidine kinase SaeS

**Figure 5.** Expression levels of Sbi and SaeR proteins of *Staphylococcus aureus* isolated from patients with bloodstream infections and asymptomatic carriers, Rennes, France. A)

Western blot of Sbi protein for 61 strains during early exponential growth phase. A protein sample from strain 19 was loaded on each gel and used as an internal control to prevent intensity variations of the bands between each experiment.

Carriage: lane 1, 1; lane 2, 10; lane 3, 15; lane 4, 19; lane 5, L102; lane 6, 1911; lane 7, 0026; lane 8, 0310; lane 9, 2203; lane 10, 2155; lane 11, 2752; lane 12, 2167; lane 13, 1954; lane 14, 1955; lane 15, 21; lane 16, 11; lane 17, L45; lane 18, 8; lane 19, 23. Sepsis: lane 1, 49; lane 2, 18; lane 3, 88; lane 4, 82; lane 5, 46; lane 6, 71; lane 7, 59; lane 8, 75; lane 9, 91; lane 10, 86; lane 11, 9; lane 12, 43; lane 13, 20; lane 14, 52; lane 15, 85; lane 16, 104; lane 17, 54; lane 18, 58; lane 19, 61; lane 20, 101; lane 21, 62; lane 22, 74. Shock: lane 1, 26;

lane 2, 44; lane 3, 17; lane 4, 14; lane 5, 42; lane 6, 30; lane 7, 34; lane 8, 45; lane 9, 57; lane 10, 89; lane 11, 27; lane 12, 11; lane 13, 28; lane 14, 430; lane 15, 069; lane 16, 886. B) and C) Sbi/total protein. D) SaeR/total protein. SaeR is a positive regulator of Sbi protein. p values (by Mann-Whitney U test) for significant differences are shown. Experiments were performed in triplicate. Horizontal lines indicate medians. To exclude loading variations between samples, values were normalized against total protein levels. AU, arbitrary units; Sbi, second immunoglobulin-binding.



and the response regulator SaeR (28). Expression of Sbi is controlled by  $\geq 3$  regulators: negatively by 2 sRNAs, and positively by a 2-component system. This system indicates why RNAIII and sprD levels are not inversely correlated with Sbi levels in isolates tested. Carriage strains have higher SaeR levels than the sepsis strains, which is consistent with higher Sbi levels in the carriage strains than sepsis strains (Figure 5).

The transition from commensalism to infection in *S. aureus* is an essential but complex process. From a clinical standpoint, most *S. aureus* infections are derived from previous colonizers (29). When those strains switch to invasiveness, the transition may be related to regulatory network expression changes, including within sRNAs. Sequencing has identified changes in regulatory functions of strains isolated from a person who initially was a carrier and then showed development of a fatal bloodstream infection (30), which suggested that molecular evolution might play a key role in this process. Our results suggest that certain sRNAs from the gene regulatory network in a human pathogen will provide insights into commensal-to-pathogen transitions. These sRNAs could be used as surrogate markers for severity

of staphylococcal infections and as biomarkers for prophylaxis and monitoring of *S. aureus* infection. Comparison of frequency and expression of selected sRNAs in *S. aureus* isolates that colonize or become infectious might be a way to identify associations between sRNA expression and disease patterns.

In vitro expression levels of some *S. aureus* sRNAs might not reflect their in vivo levels (31). Nevertheless, direct analysis of expression levels of bacterial sRNAs directly in blood from patients with bloodstream infections is technically difficult because of low levels of bacteria collected. We compared sRNA expression in fresh isolates from patients with the same isolates after they were thawed after be frozen for 3 weeks. We found no differences in RNAIII and sprD expression between isolates obtained directly from patients and isolates that had been frozen. Subsequent investigations will address the functional and clinical relevance of expression patterns of RNAIII and sprD. Broadening our investigations to include additional sRNAs might identify biomarkers that predict staphylococcal disease severity in infected patients. In addition to their roles as biomarkers, sRNAs could also be targets for innovative therapeutic approaches.

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A patent for the new RNA biomarkers reported in this article has been filed by the Institut National de la Santé et de la Recherche Médicale Transfert (Paris, France).

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Dr. Bordeau is a research engineer at University of Rennes 1, Rennes, France. Her primary research interests are functions and structures of sRNAs.

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# Ebola Virus Disease, Democratic Republic of the Congo, 2014

Carolina Nanclares, Jimmy Kapetshi, Fanshen Lionetto, Olimpia de la Rosa, Jean-Jacques Muyembe Tamfun, Miriam Alia, Gary Kobinger, Andrea Bernasconi

During July–November 2014, the Democratic Republic of the Congo underwent its seventh Ebola virus disease (EVD) outbreak. The etiologic agent was Zaire Ebola virus; 66 cases were reported (overall case-fatality rate 74.2%). Through a retrospective observational study of confirmed EVD in 25 patients admitted to either of 2 Ebola treatment centers, we described clinical features and investigated correlates associated with death. Clinical features were mainly generic. At admission, 76% of patients had  $\geq 1$  gastrointestinal symptom and 28%  $\geq 1$  hemorrhagic symptom. The case-fatality rate in this group was 48% and was higher for female patients (67%). Cox regression analysis correlated death with initial low cycle threshold, indicating high viral load. Cycle threshold was a robust predictor of death, as were fever, hiccups, diarrhea, dyspnea, dehydration, disorientation, hematemesis, bloody feces during hospitalization, and anorexia in recent medical history. Differences from other outbreaks could suggest guidance for optimizing clinical management and disease control.

Ebola virus disease (EVD), a severe, often fatal illness in humans, has remained a major public health concern in many parts of sub-Saharan Africa since it appeared in 1976 in Zaire (now the Democratic Republic of the Congo [DRC]). The severity of disease caused by Ebola virus (family *Filoviridae*) varies considerably among the 5 known species. Three species (*Zaire ebolavirus* [ZEBOV], *Bundibugyo ebolavirus*, and *Sudan ebolavirus*) have caused outbreaks affecting dozens, and sometimes hundreds, of persons in Africa. Case-fatality rates (CFRs) vary by species and are highest for ZEBOV (up to 90%) (1). The largest EVD epidemic occurred during 2014–2016 in West Africa; it resulted in 28,646 cases and 11,323 deaths and affected 10 countries in Africa, Europe, and North America (2).

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Simultaneously, in 2014, another EVD outbreak occurred in Equateur Province in northwestern DRC. The first case, reported on July 26, 2014, in a pregnant woman married to a bush meat hunter, came from the village of Inkanamongo (3,4), close to the town of Boende; she was believed to be the index case-patient. This area is  $\approx 700$  km from the capital city, Kinshasa. No paved roads connect the 2 settlements, and communications depend mainly on travelers (3). The World Health Organization (WHO) declared the outbreak on August 24, 2014 (4).

During the outbreak, July 26–October 4, 2014, a total of 66 EVD cases were reported (28 probable, 38 confirmed); 49 deaths were reported, for a CFR of 74%, including deaths in the community of persons with suspected EVD (5,6). One third of these case-patients were documented to have had direct contact with the index case-patient. A basic reproduction number of 0.84 (3) was estimated, which was lower than in any previous EVD epidemic (7). In comparison, West Africa had a reproduction number of 1.7–2.0 for the first 9 months of the outbreak (8). WHO declared the outbreak in DRC over on November 21, 2014, forty-two days after the last EVD case-patient tested negative and was discharged from the hospital (5).

Genome sequencing identified ZEBOV as the outbreak's causative agent. However, genetic characterization of the virus pointed to a local variant because it has 99.2% of the genome in common with the strain isolated from the 1995 outbreak in Kikwit, DRC, and  $>96.8\%$  identified in common with the ZEBOV simultaneously circulating in West Africa (3,9).

To respond to this epidemic, in collaboration with the local authorities, Médecins Sans Frontières (MSF) opened an Ebola treatment center (ETC) to manage persons with suspected or confirmed EVD in Lokolia; this ETC was fully functional on September 10, 2014. A second ETC was set up inside the regional hospital in Boende on August 28. The activities in both ETCs were conducted until week 44. We describe the clinical features of EVD and predictors of death among patients treated in these ETCs during the 2014 outbreak in DRC.

## Methods

We conducted a retrospective observational study on the basis of medical records of all patients with confirmed

EVD who were admitted for care at the MSF-supported ETCs in Lokolia and Boende during August 28–November 8, 2014. Patients were identified through the standard case definition established by the Ministry of Health in collaboration with WHO (3).

EVD was confirmed by quantitative reverse transcription PCR (RT-PCR), which is considered the most sensitive method and can detect virus from early acute disease through early recovery (10). Initially, samples were sent in dry tubes and EDTA to the Institut National de Recherche Biomédicale (Kinshasa, DRC) to be tested; later the Institut National de Recherche Biomédicale set up a field laboratory in the Lokolia ETC. To confirm EVD, samples were sent to the WHO reference center at the Centre International de Recherche Médicales de Franceville in Gabon (3). Patients were treated in accordance with then-current protocols established for viral hemorrhagic fever by MSF and WHO urgent interim guidance for case management, endorsed by the Ministry of Health of DRC (11,12). Patients with confirmed EVD were discharged after a negative RT-PCR result, 3 days without any major symptoms, and general clinical improvement. For a patient suspected to have EVD, discharge required 2 negative PCR results 48 hours apart, of which 1 was performed at least 72 hours after symptom onset.

#### Data Collection

We collected epidemiologic and demographic data (age, sex), history of exposure, dates of onset, and symptoms and signs before and during hospitalization on an anonymized encrypted electronic database. Each day, as part of their routine clinical duties in the ETCs, health staff recorded the health condition of patients. At discharge, clinicians reviewed the medical files of each patient and double-checked data at the moment of encoding into Microsoft Excel version 2007 (Microsoft Corp., Redmond, WA, USA) before analysis with Stata version 12 (Stata-Corp LP, College Station, TX, USA). Fever was defined as an axillary body temperature  $\geq 37.5^{\circ}\text{C}$  ( $\geq 99.5^{\circ}\text{F}$ ). We considered hemorrhagic signs as the following: bloody feces; hematemesis; epistaxis; gingival or oral bleeding; nonmenstrual vaginal bleeding; bleeding after an intramuscular injection or venipuncture; and red eyes/subconjunctival hemorrhage, even though conjunctivitis also can cause this sign (3). We grouped as gastrointestinal symptoms the following: nausea, vomiting, diarrhea (bloody and watery diarrhea), and abdominal pain. We measured, in days, the duration of symptoms and signs only for patients who survived. Laboratory data were recorded in a separate register and subsequently were matched with clinical data by using MSF identification. The cycle threshold ( $C_t$ ) result from the first EVD PCR whole-blood test at admission was used as a proxy indicator of ZEBOV viral load (i.e., the lower the  $C_t$ ,

the higher the viral load) (13). An EVD  $C_t$  of 25 is considered equivalent to a viral load of  $1.28 \times 10^7$  copies/mL of blood (14).  $C_t < 20$  suggest a very high viral load.

#### Statistical Analysis

We restricted our dataset to RT-PCR–positive patients. Results were reported as proportions, means, or medians for the descriptive analysis. We used Fisher exact test to test the hypothesis involving dichotomous variables, Student  $t$  test to compare 2 parametric continuous variables, and Mood's median test to compare nonparametric continuous variables. Variables were modeled in a Probit model to test correlation of continuous ( $C_t$ , incubation day, age) or categorical (age group, sex) variables toward the dichotomy outcome (survived/died). Variable normality, when doubtful, was tested with Shapiro-Wilk test to choose the appropriate additional test. The relative risk (RR) and its SE and 95% CI were calculated according to Altman (15). Finally, we tested predictors for death through Kaplan-Meier analysis using the log-rank test of equality for categorical variables and Cox proportional hazard regression for continuous variables to calculate the hazard rate. For survival analysis models, we modeled survival from the day of symptom onset with a censoring time of 30 days because all deaths occurred before 30 days after symptom onset. We included the predictor in the model if the test had a  $p$  value  $\leq 0.25$ . Hypotheses tested were 2-tailed, and we considered statistical significance only in the presence of  $p$  values  $< 0.05$ .

#### Results

Sixty-five persons whose illness met the case definition for EVD were admitted to 1 of the 2 MSF-supported ETCs in Boende and Lokolia during outbreak weeks 35–44. For 25 (38%) patients, EVD was confirmed by RT-PCR. The remaining patients with suspected EVD were determined to have malaria or other infections and were treated accordingly. Of the 25 EVD-confirmed patients, 13 (52%) were male. The 25 EVD patients treated at the ETCs represented 66% of the 38 confirmed EVD cases notified during this outbreak. Twelve (48%) of the 25 case-patients died.

The median age of patients with confirmed EVD was 32 years (range 1–77 years) (Table 1). Male patients were older than female patients ( $p = 0.03$ ): a median of 32 (25th percentile 12, 75th percentile 47) years versus 25 (25th percentile 6.5, 75th percentile 38.5) years, respectively. Case-patients 15–45 years of age were the most represented (11 [44%]); 9 (36%) case-patients were  $< 15$  years of age.

Time from symptom onset to admission, available for 24 patients, averaged 4.69 days (range 1–9 days). This interval was shorter for survivors (4.25 days) than for persons who died (5.21 days) and for female patients (4.82 days) than for male patients (4.61 days). We did not find any

**Table 1.** Characteristics of Ebola virus disease case-patients treated in 2 Ebola treatment centers, Lokolia and Boende, Democratic Republic of the Congo, July–November 2014\*

Characteristic	Total	Male	Female	p value
Sex, no. (%)	25 (100)	13 (52)	12 (48)	
Median age, y (IQR)	32 (10–39)	32 (12–47)	25 (6.5–38.5)	0.03
Age group, y, no. (%)				
≤5	4 (16.0)	1 (7.7)	3 (25.0)	
>5 to ≤15	5 (20.0)	3 (23.1)	2 (16.7)	
>15 to ≤45	11 (44.0)	5 (38.4)	6 (50.0)	
>45	5 (20.0)	4 (30.8)	1 (8.3)	
Patient cycle threshold at admission, no. (mean ± SD)	23 (29.22 ± 5.90)	12 (30.61 ± 5.39)	11 (27.7 ± 6.32)	
≤5 y	3 (22.07 ± 3.63)			<0.001
>5 to ≤15 y	5 (35.06 ± 4.64)			
>15 to ≤45 y	10 (28.17 ± 4.23)			<0.001
>45 y	5 (29.76 ± 6.25)			
Time from onset to admission, mean d ± SD	4.69 ± 2.63	4.61 ± 2.93	4.82 ± 2.34	
Survived	4.25 ± 3.13			
Died	5.21 ± 1.95			
Median hospital stay, d (IQR)	4 (3–12)			
Survived	10 (3–13)	3	24	0.09
Died	4 (2.5–5.5)	1	18	
Time from onset to outcome, d (IQR)				
Survived	11.5 (7.5–19.5)	4	30	0.28
Died	9 (7–12)	3	23	

\*Blank cells indicate the numbers were too small for meaningful calculation. IQR, interquartile range.

significant difference between the 2 ETCs in terms of delays of hospitalization and outcome. At admission, the most common symptoms reported were asthenia (84%); fever (80%); and anorexia, vomiting, and diarrhea (56% each). In general, gastrointestinal symptoms accounted for 28% of all reported symptoms, but no patients showed signs of dehydration at admission. Hemorrhagic signs, documented at admission for 7 (28%) patients, were mainly gastrointestinal bleedings. Anorexia, myalgia, and abdominal pain occurred significantly more often in adults (patients >15 years of age) than in children ( $p < 0.05$ ). Only anorexia correlated with death (odds ratio [OR] 6.99,  $p < 0.05$ ) (Table 2).

$C_t$  at admission was available for 23 (96%) patients. The average  $C_t$  was  $29.2 \pm 5.9$  (range 17.9–39.1).  $C_t$  for children <5 years of age was lower, indicating higher viral load, than for children 5–15 years ( $p < 0.01$ ). Two (9%) patients, 1 and 53 years of age, who died had a  $C_t < 20$ . Survivors spent a median of 10 days in the hospital (maximum stay 24 days). The others died a median of 4 days (range 1–18,  $p = 0.09$ ) after admission.

During hospitalization, asthenia (96%); anorexia and diarrhea (68% each); and vomiting, myalgia, and fever up to  $39.5^\circ\text{C}$  (60% each) were the most common symptoms (Table 3). The symptoms of longest duration were asthenia (4 days), red eyes (4 days), tender abdomen (4 days), anorexia (2.9 days), and arthralgia (2.4 days).

In addition to the 7 patients who had hemorrhagic signs at admission, hemorrhagic signs developed in 7 patients during hospitalization. In these patients, bloody feces (7 patients), bleeding from an injection site (6 patients), and red eyes (or subconjunctival hemorrhage) and hematemesi (5 patients) developed. Including red eyes, hemorrhagic

signs lasted an average of 3.5 days during hospitalization. Of these 7 patients, 5 died during hospitalization; the 2 survivors had subconjunctival hemorrhage, and 1 had bloody feces. Overall, 14 (56%) patients had hemorrhagic signs during illness.  $C_t$  at admission was significantly lower for patients with than without hemorrhagic signs ( $24.83 \pm 4.41$  vs.  $32.59 \pm 4.57$ ;  $p < 0.01$ ), and we found no correlation between development of hemorrhage and age or sex.

Nineteen (76%) patients reported at least 1 gastrointestinal symptom before admission. Diarrhea and vomiting were the most common gastrointestinal symptoms (56% each); 10 (40%) patients had both. Ten patients reported abdominal pain. During hospitalization, 2 additional patients had gastrointestinal symptoms, for a total of 21 (84%) patients with at least 1 of these symptoms during illness. Nine (36%) patients also were dehydrated during hospitalization.

Cough was noted for 1 patient, who also had dyspnea, at admission and for 2 patients during their illness. Additionally, at admission, only 1 patient reported dyspnea, and it developed in 5 additional patients during hospitalization (dyspnea developed in 3 patients the day before death). This symptom was suspected to be a pregonic acidosis complication rather than direct respiratory involvement. Moreover, only 1 patient had the characteristic sign of hiccups at admission and it developed in 3 (16%) additional patients during hospitalization. All patients who had hiccups died.

The CFR was the most important outcome analyzed in this study. Of 25 patients hospitalized with confirmed EVD, 12 died, for an overall CFR of 48%. The CFR was higher for female (67%) than for male (31%) patients (Figure 1). The CFR was highest for children <5 years of age (75%),

**Table 2.** Symptoms and signs, and their prediction for death, among patients admitted to 2 Ebola treatment centers during the Ebola virus disease outbreak, Lokolia and Boende, Democratic Republic of the Congo, July–November 2014

Symptom/sign	Survived, no. (%)	Died, no. (%)	Total, no. (%)	Case-fatality rate, %	Relative risk (95% CI)	p value
<b>Symptoms</b>						
Asthenia	10 (76.9)	11 (91.7)	21 (84.0)	52.4	2.09 (0.36–12.00)	0.59
Anorexia	4 (30.8)	10 (83.3)	14 (56.0)	71.4	3.93 (1.07–14.37)	0.01
Headache	5 (38.5)	7 (58.3)	12 (48.0)	58.3	1.52 (0.66–3.5)	0.44
Myalgia	5 (38.5)	6 (50.0)	11 (44.0)	54.5	1.27 (0.56–2.86)	0.69
Right upper quadrant abdominal pain	4 (30.8)	6 (50.0)	10 (40.0)	60.0	1.5 (0.67–3.33)	0.42
Difficulty swallowing	1 (7.7)	4 (33.3)	5 (20.0)	80.0	2 (1–3.9)	0.16
Nausea	2 (15.4)	3 (25.0)	5 (20.0)	60.0	1.33 (0.56–3.16)	0.64
Arthralgia	2 (15.4)	2 (16.6)	4 (16.0)	50.0	1.05 (0.35–3.08)	1
Dyspnea	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Cough	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Back pain	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Disorientation	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Stomach pain/cramps	1 (7.7)	0	1 (4.0)	0		
<b>Signs</b>						
Fever up to 39.5°C	9 (69.2)	10 (83.3)	19 (76.0)	52.6	1.58 (0.47–5.29)	0.64
Vomiting	5 (38.5)	9 (75.0)	14 (56.0)	64.3	2.35 (0.83–6.67)	0.11
Diarrhea	5 (38.5)	9 (75.0)	14 (56.0)	64.3	2.36 (0.83–6.67)	0.11
Fever >39.5°C	1 (7.7)	0	1 (4.0)	0		
Hiccups	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Nonhemorrhagic rash	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Dehydration	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
<b>Hemorrhagic signs</b>						
≥1 of the signs below	2 (15.4)	5 (41.7)	7 (28.0)	71.4	1.83 (0.87–3.87)	0.2
Bloody feces	1 (7.7)	4 (33.3)	5 (20.0)	80.0	2 (1–3.9)	0.16
Hematemesis	0	3 (25.0)	3 (12.0)	100.0	2.44 (1.48–4.04)	0.09
Red eyes/subconjunctival hemorrhage	1 (7.7)	1 (8.3)	2 (8.0)	50.0	1 (0.24–4.45)	1
Epistaxis	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Gingival/oral bleeding	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48
Nonmenstrual vaginal bleeding	0	1 (8.3)	1 (4.0)	100.0	2.18 (1.41–3.37)	0.48

followed by persons 15–45 years of age (64%). These age groups also had significantly lower average  $C_t$  (<0.01).

The median time from symptom onset to death was 9 days (25th percentile 7, 75th percentile 12, range 3–23 days). Nine (75%) patients who died had at least 1 hemorrhagic sign during illness, and all hemorrhagic signs except red eyes/subconjunctival hemorrhage were associated with higher CFR. All the 12 patients who died had also vomiting, diarrhea, or both at some point during their illness.

Clinical care for the most severely affected patients focused on maintaining circulatory volume and blood pressure, mainly through maintenance of hydration. Nine patients who died received intravenous treatment.

Symptoms such as fever, hiccups, diarrhea, dyspnea, dehydration, disorientation, hematemesis, and bloody feces that developed during hospitalization significantly correlated with an increased risk for death ( $p < 0.01$ ). Lower  $C_t$  at admission also correlated significantly with death.

A Probit regression model that included  $C_t$  at admission and sex indicated that an increase in  $C_t$  correlated with a decreased probability of death by  $-0.038$  (95% CI  $-0.059$  to  $-0.018$ ;  $p < 0.01$ ) (Figure 2). A Cox regression model that included  $C_t$  at admission, age, and sex confirmed that  $C_t$

was the best predictor of death (hazard ratio 0.81, 95% CI 0.68–0.96;  $p = 0.02$  (Cox, Table 4).

## Discussion

In 2014, in parallel with the large EVD outbreak in West Africa, the DRC faced its seventh outbreak of EVD since the first report of the virus in 1976 (Table 5). By the end of this lesser-known outbreak, 49 persons with EVD had died, for a CFR of 74% (3), a rate consistent with previous EVD outbreaks in the region (16) but higher than with the EVD outbreak caused by Bundibugyo virus in Isiro, DRC, in 2012 (CFR 46.7%) (17,18) and in Kasai Oriental province, DRC, in 2008 (43.7%) (19). Although this rate could be overestimated because of a lack of information about possible survivors in the community, most EVD patients died during the first phase of the outbreak, before the implementation of an adequate referral and treatment system. Following the model established in 1995 (20), care for a large number of patients occurred in hospital and the MSF-supported ETCs in Lokolia and Boende. However, during epidemiologic weeks 36–44, the CFR of 48% for the 25 persons with confirmed EVD treated in the 2 ETCs did not differ substantially from the 43% reported by Bah et al. in Conakry, Guinea (21), and from the 51% reported by Fitzpatrick et al. in Sierra Leone (14).

**Table 3.** Symptoms and signs developed during hospitalization during the Ebola virus disease outbreak in Ebola treatment centers, Lokolia and Boende, Democratic Republic of the Congo, July–November 2014\*

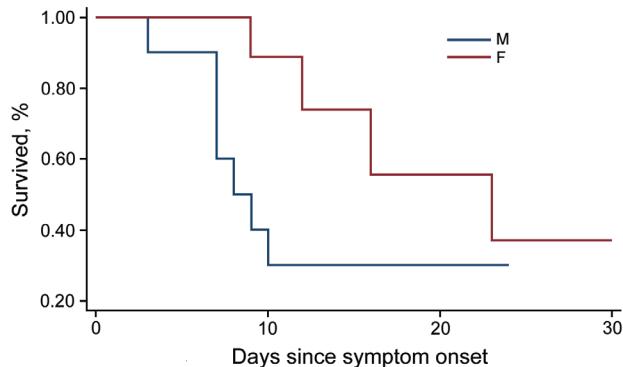
Symptom, sign	Survived, no. (%)	Died, no. (%)	Total, no. (%)	Average		Relative risk (95% CI)	p value	Patient age group, y, %		p value
				no. days	CFR			≤15	>15	
<b>Symptom</b>										
Asthenia	12 (92.3)	12 (100)	24 (96.0)	4	50.0	0		88.9	100.0	0.36
Anorexia	7 (53.8)	10 (83.3)	17 (68.0)	2.9	58.8	2.35 (0.66–8.33)	0.2	44.4	81.3	0.075
Myalgia	6 (46.1)	9 (75.0)	15 (60.0)	2.3	60.0	2 (0.71–5.62)	0.22	11.1	87.5	0
Arthralgia	5 (38.4)	6 (50.0)	11 (44.0)	2.4	54.5	1.27 (0.56–2.86)	0.69	11.1	62.5	0.017
Headache	6 (46.1)	5 (41.7)	11 (44.0)	2	45.5	0.90 (0.39–2.09)	10	22.2	56.3	0.11
RUQ abdominal pain	3 (23.1)	7 (58.3)	10 (40.0)	2.3	70.0	2.10 (0.92–4.78)	0.11	11.1	56.3	0.034
Difficulty swallowing	2 (15.4)	6 (50.0)	8 (32.0)	1	75.0	2.12 (0.99–4.53)	0.09	0	50.0	0.012
Nausea	3 (23.1)	5 (41.7)	8 (32.0)	2.3	62.5	1.52 (0.69–3.31)	0.41	0	50.0	0.012
Dyspnea	0 (0)	6 (50.0)	6 (24.0)		100.0	3.16 (1.63–6.13)	0.005	11.1	31.3	0.267
Disorientation	0 (0)	6 (50.0)	6 (24.0)		100.0	3.16 (1.63–6.14)	0.005	11.1	31.3	0.267
Cough	1 (7.7)	2 (16.6)	3 (12.0)	1	66.7	1.46 (0.58–3.69)	0.59	0	18.8	0.243
Chest pain	1 (7.7)	2 (16.6)	3 (12.0)	1	66.7	1.46 (0.58–3.69)	0.59	0	18.8	0.243
Back pain	1 (7.7)	1 (8.3)	2 (8.0)	2	50.0	1 (0.24–4.45)	10	0	12.5	0.4
Stomach pain/cramps	2 (15.4)	0	2 (8.0)	1	0	0	0.0	0	12.5	0.4
Diarrhea	6 (46.1)	11 (91.7)	17 (68.0)	2.3	64.7	5.17 (0.8–33.5)	0.03	44.4	81.3	0.075
Fever up to 39.5°C	4 (33.3)	11 (91.7)	15 (60.0)	1.5	73.3	7.33 (1.11–48.2)	0.003	44.4	68.8	0.222
Vomiting	7 (53.8)	8 (66.6)	15 (60.0)	1.3	53.3	1.33 (0.54–3.20)	0.68	100.0	100.0	0.053
Dehydration	1 (7.7)	8 (66.6)	9 (36.0)	1	88.9	3.55 (1.47–8.56)	0.003	22.2	43.8	0.264
Hiccups	0	4 (33.3)	4 (16.0)		100.0	2.62 (1.52–4.53)	0.04	11.1	18.8	0.54
Tender abdomen	1 (7.7)	2 (16.6)	3 (12.0)	4	66.7	1.46 (0.58–3.68)	0.59	0	18.8	0.243
Fever >39.5°C	1 (7.7)	1 (8.3)	2 (8.0)	1	50.0	1.04 (0.24–4.45)	10	11.1	6.3	0.6
Nonhemorrhagic rash	0	1 (8.3)	1 (4.0)	2	100.0	2.18 (1.41–3.37)	0.48	0	6.3	0.64
<b>Hemorrhagic sign</b>										
Hemorrhagic	4 (30.7)	9 (75.0)	13 (52.0)	3.5	69.20	2.77 (0.97–7.87)	0.04	44.4	56.3	0.44
Hemorrhagic, excluding red eyes/subconjunctival hemorrhage	2 (15.4)	9 (75.0)	11 (44.0)	1	85.70	3.81 (1.34–10.8)	0.03	33.3	50.0	0.3
Bloody feces	1 (7.7)	6 (50.0)	7 (28.0)	1	85.7	2.57 (1.25–5.28)	0.03	33.3	25.0	0.499
Bleeding from injection site	1 (7.7)	5 (41.7)	6 (24.0)	1	83.3	2.26 (1.13–4.50)	0.07	22.2	25.0	0.637
Red eyes/subconjunctival hemorrhage	3 (23.1)	3 (25.0)	6 (24.0)	4	50.0	1.05 (0.41–2.67)	10	11.1	31.3	0.267
Hematemesis	0	5 (41.7)	5 (20.0)		100.0	2.87 (1.57–5.19)	0.01	11.1	25.0	0.391
Hemoptysis	0	2 (16.6)	2 (8.0)		100.0	2.30 (1.44–3.66)	0.22	0	12.5	0.4
Epistaxis	0	1 (8.3)	1 (4.0)		100.0	2.18 (1.41–3.37)	0.48	0	6.3	0.64
Gingival/oral bleeding	0	1 (8.3)	1 (4.0)	1	100.0	2.18 (1.41–3.37)	0.48	11.1	0	0.36

\*RUQ, right upper quadrant.

Although possibly biased by the case definition, asthenia (84%) and fever (80%) were the most common symptoms at admission, followed by diarrhea, anorexia, and vomiting (56% each). Among these, only anorexia correlated with death ( $p = 0.01$ ). Gastrointestinal symptoms (mainly vomiting and diarrhea) were common at presentation, which numerous other groups also have reported (8,16,17,21–25). Among all patients in the cohort reported here, at admission at least 7.6 of every 10 patients had 1 gastrointestinal symptom or difficulty swallowing and 4 patients had diarrhea and vomiting, posing an increased risk for transmission, as was documented in West Africa (8,24,25). In contrast, no cholera-like diarrhea symptoms were observed in this outbreak, in contrast to several locations in West Africa during the 2014–2016 outbreak (26), and the DRC patients admitted to the MSF ETCs had no clinically relevant sign of dehydration. The high prevalence of gastrointestinal symptoms we noted

indicates that these symptoms should not be ignored during EVD screening and as part of transmission control efforts. Failing to recognize gastrointestinal symptoms early during the course of disease increases the potential for fatal misdiagnosis (16,24,27,28) and for delayed declaration of the outbreak (20,27,29).

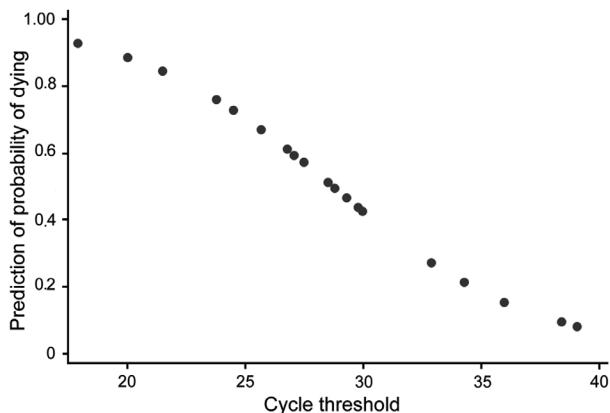
Although the ZEBOV isolated during this outbreak is 99% homologous to the Kikwit strain from 1995 (3), the high frequency of maculopapular rash, bilateral conjunctiva injection, and sore throat withodynophagia reported in many of the patients from the 1995 outbreak were not observed in this outbreak (16,30). Also, high frequencies of coughing and hemoptysis, which were common in the 1995 outbreak, were not observed. We did not find a high prevalence of other symptoms, such as difficulty swallowing, arthralgia, and nausea, among the patients in our study, as was described in Isiro (17,23) and in Kikwit (16). Hiccups, considered a characteristic and severe sign of EVD



**Figure 1.** Survival distribution (Kaplan-Meier) by sex of Ebola virus disease patients admitted to 2 Ebola treatment centers, Lokolia and Boende, Democratic Republic of the Congo, July–November 2014.

(31), occurred in only 1 patient at admission without the co-presence of any hemorrhagic sign, and hiccups developed in 3 (16%) additional patients during hospitalization, similar to what was described in Kikwit (16).

Guimard et al. reported that conjunctivitis was highly predictive of EVD and included it in an algorithm for EVD diagnosis (20) because it has been described as a relatively early sign of EVD, detectable in 45%–60% of patients (30). At Connaught Hospital (Freetown, Sierra Leone), conjunctivitis was much more prevalent in the early phase of the outbreak in 2014 than in the latter stages (25). In our study, the documented sign of red eyes/subconjunctival hemorrhage is mainly attributable to conjunctivitis, and its presence did not increase the risk for death (RR 1.05). By calculating the risk for death in correlation with at least 1 hemorrhagic sign, if we included red eyes/subconjunctival hemorrhage as a hemorrhagic sign, we assessed an RR of 3.81. However, if we excluded conjunctivitis, the RR is



**Figure 2.** Prediction of Ebola virus disease patients' probability of dying in relation to their *Zaire ebolavirus* viral load as determined by cycle threshold at admission to an Ebola treatment center, Lokolia and Boende, Democratic Republic of the Congo, July–November 2014.

**Table 4.** Symptoms and signs during hospitalization of Ebola virus disease patients admitted to 2 Ebola treatment centers, Lokolia and Boende, Democratic Republic of the Congo, 2014\*

Symptom/sign	Hazard ratio (95% CI)	p value
Fever up to 39.5°C	7.33 (1.11–48.2)	<0.01
Hiccups	2.62 (1.52–4.53)	0.04
Diarrhea	5.17 (0.8–33.5)	0.03
Dyspnea	3.16 (1.63–6.13)	<0.01
Dehydration	3.55 (1.47–8.56)	<0.01
Disorientation	3.16 (1.63–6.14)	<0.01
Hematemesis	2.85 (1.57–5.19)	0.01
Bloody feces	2.57 (1.25–5.28)	0.03
History of anorexia	3.93 (1.07–14.37)	0.01
$C_t$ at admission	0.79 (0.64–0.97)	0.03
After multivariate analysis†		
$C_t$ at admission	0.81 (0.68–0.96)	0.02
Age, y	1.01 (0.97–1.05)	0.62
Sex, F	4.47 (0.59–10.3)	0.22

\* $C_t$ , cycle threshold.

†Cox regression test.

2.77. Although conjunctivitis was considered a risk factor in the early phase of the EVD outbreak in West Africa (8) and can be common in EVD patients, our findings suggest its value should be reconsidered.

The prevalence of hemorrhagic signs (52%) among the patients treated during the 2014 outbreak in the DRC did not differ from that described in Uganda (Bundibugyo virus) (18,23) but was higher than the 41% observed in Kikwit and lower than the 78% in the first EVD outbreak in Zaire in 1976 (16,32). Recent reports documenting the West Africa epidemic reported lower prevalence: 0.9% (24); <1% to 5.7% (8); <5% (26); 6%, reporting in this case only melena and hematemesis (25); and 1.9% (22). Only Bah et al. (21) reported 51% of EVD patients with hemorrhages, but in this case also subconjunctival redness, which might have been conjunctivitis, was included.

We did not observe a significant correlation between older age and death ( $p = 0.16$ ) in a Probit model with  $C_t$  at admission and sex, as described previously (8,18,21,24,33). Female sex was the main risk factor associated with death (RR 2.16,  $p = 0.07$ ), but once adjusted for  $C_t$  and age in a Cox regression model, it was no longer significantly associated with death (hazard rate 4.47,  $p = 0.22$ ). Other groups have reported that male patients were more at risk than female patients for death in the context of EVD (33).

Overall,  $C_t$  at admission was the only statistically significant predictor of death ( $p < 0.01$ ). Average  $C_t$  at admission also was lower for case-patients who died than for those who survived (9.5 vs. 11.5,  $p < 0.01$ ). This association between high viral load and death was reported recently (10) in West Africa (14,24) and in Uganda in 2000 (29). In our study, low  $C_t$  also was associated with severe hemorrhagic signs during hospitalization.

We report a clinical dataset for the seventh EVD outbreak in the DRC. Although it comprises all the patients admitted in 2 ETCs, the relatively small number of

**Table 5.** Previous Ebola virus disease outbreaks notified in Democratic Republic of the Congo since 1976\*

Year	Region	No. cases	No. deaths	Case-fatality rate, %	Species
1976	Yambuku, Mongala district	318	280	88.0	ZEBOV
1977	Tandala, Equateur Province	1	1	100.0	ZEBOV
1995	Kikwit, Bundunu Province	315	254	80.6	ZEBOV
2007	Mweka and Leubo, Kasai Occidental Province	264	187	70.8	ZEBOV
2008–2009	Mweka and Luebo, Kasai Occidental Province	32	14	43.7	ZEBOV
2012	Isiro, Oriental Province	77	36	46.7	BDBV
2014	Boende, Equateur Province	66	49	74.2	ZEBOV

\*BDBV, *Bundibugyo ebolavirus*; ZEBOV, *Zaire ebolavirus*.

cases documented is a limitation of the study and should be considered in drawing conclusions from these data. Consequently, some level of selection bias cannot be excluded because some persons with minor symptoms or very serious EVD are likely not to have arrived at an ETC. Nevertheless, our analysis provides a better understanding of symptoms and viral loads in relation to clinical outcome.

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**EMERGING  
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# Use of Testing for West Nile Virus and Other Arboviruses

Jakapat Vanichanan, Lucrecia Salazar, Susan H. Wootton, Elizabeth Aguilera, Melissa N. Garcia, Kristy O. Murray, Rodrigo Hasbun

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**Release date: August 10, 2016; Expiration date: August 10, 2017**

### Learning Objectives

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

- Evaluate the epidemiology and clinical presentation of West Nile neuroinvasive disease (WNND)
- Distinguish the rate of testing for West Nile Virus (WNV) in potential cases of WNND in the current study
- Analyze factors associated with greater rates of testing for WNV in potential cases of WNND
- Identify factors associated with lower rates of testing for WNV in potential cases of WNND

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In the United States, the most commonly diagnosed arboviral disease is West Nile virus (WNV) infection. Diagnosis is made by detecting WNV IgG or viral genomic sequences in serum or cerebrospinal fluid. To determine frequency of this testing in WNV-endemic areas, we examined the proportion of tests ordered for patients with meningitis and encephalitis

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at 9 hospitals in Houston, Texas, USA. We identified 751 patients (567 adults, 184 children), among whom 390 (52%) experienced illness onset during WNV season (June–October). WNV testing was ordered for 281 (37%) of the 751; results indicated acute infection for 32 (11%). Characteristics associated with WNV testing were acute focal neurologic deficits; older age; magnetic resonance imaging; empirically prescribed antiviral therapy; worse clinical outcomes; and concomitant testing for mycobacterial, fungal, or other viral infections. Testing for WNV is underutilized, and testing of patients with more severe disease raises the possibility of diagnostic bias in epidemiologic studies.

Arboviruses (arthropodborne viruses) are viruses that can infect humans via arthropod vectors, including mosquitoes, ticks, and sand flies. In the United States, the most common arboviral disease is infection with West Nile virus (WNV), which is transmitted largely by mosquitoes of the genus *Culex*. Since the first detection of WNV in the United States in 1999, several outbreaks of WNV infection have occurred in cyclic patterns (1,2). Texas is considered to be an area where transmission of WNV is endemic and occasionally epidemic; to date, >4,000 clinical cases in Texas have been reported (3,4).

Most patients with WNV infection are asymptomatic, but uncomplicated West Nile fever develops in  $\approx 20\%$  (5). In contrast, West Nile neuroinvasive diseases (WNND) occurs in <1% of infected persons; however, a substantial proportion of illness and death occurs among these patients, who experience clinical manifestations such as long-term neuropsychiatric sequelae and chronic renal insufficiency (6–11). WNND is characterized by encephalitis and meningitis and sometimes (rarely) acute flaccid paralysis (7,8).

Acute WNV infection is diagnosed by detection of WNV-specific IgM in serum, cerebrospinal fluid (CSF), or both (1). The validation of commercial tests for serum WNV IgM demonstrated sensitivity of 86%–96% and specificity of up to 100% (12,13). Despite the availability of sensitive testing for WNV, serologic testing is often underutilized, probably because of lack of physician awareness. In a blood donor screening study, <50% of patients with symptomatic WNV infection sought medical care, and only 5% of them received a diagnosis of WNV infection (5). Moreover, in a small study of 60 patients with meningitis and encephalitis, only 40% were serologically tested for WNV (14). Underutilization of testing contributes to multiple biases (e.g., selection, misclassification) within epidemiologic studies, which are often used to drive public health policy and resources for mosquito control. Accurate data about patterns of WNV utilization in routine clinical practice is needed for enhancement and tailoring of future public health interventions.

## Methods

### Study Population and Case Definition

We performed a retrospective multicenter descriptive study of meningitis and encephalitis patients  $\geq 2$  months of age at any of 9 hospitals associated with Memorial Hermann Hospital in the greater metropolitan area of Houston, Texas, USA, from January 1, 2005, through December 31, 2010. The study was approved by the University of Texas Health Science Center in Houston Committee for the Protection of Human Subjects and the Memorial Hermann Hospital Research Review Committee.

Inclusion criteria for a case were a community-acquired illness with CSF pleocytosis (leukocytes  $>5$  cells/mm<sup>3</sup>) in a patient with meningitis (level 1 or 2 of diagnostic certainty for aseptic meningitis) (15); encephalitis (possible, probable, or confirmed) (16); or both. Patients with acute flaccid paralysis were included only if they had concomitant meningitis or encephalitis. Exclusion criteria were CSF positivity by Gram stain for bacteria or yeast from cytospin samples, past craniotomy, or current ventriculoperitoneal shunt.

### Data Collection and Parameter Definitions

Baseline clinical characteristics were recorded at the time the patient was seen in the emergency department and included demographic data, concurrent conditions (determined by Charlson Comorbidity Index), immunologic status, clinical features (including neurologic examination findings and Glasgow Coma Scale scores), laboratory test results, and case management. Lymphocytic pleocytosis was defined as a total CSF leukocyte composition of  $>50\%$  lymphocytes. Empirical treatment was defined as initiation of antibacterial or antiviral agents before the results of the CSF cultures or molecular diagnostic methods were available. Participant outcomes were assessed at the time of discharge from the hospital by using Glasgow Outcome Scale scores (17): a score of 1 indicates death; 2, a vegetative state (inability to interact with the environment); 3, severe disability (unable to live independently but follows commands); 4, moderate disability (able to live independently but unable to resume some previous activities, at work or in social life); and 5, mild or no disability (able to resume normal activities with minimal to no physical or mental deficits). An adverse clinical outcome was defined as a Glasgow Outcome Scale score of 1–4.

Etiologies of meningitis and encephalitis were divided into 6 categories (bacterial, viral, fungal, mycobacterial, noninfectious, and unknown), according to the final diagnosis when participants were discharged from the hospital. For the purposes of this study, we defined peak WNV season as June 1–October 31 of each year.

### Diagnosis of WNV Infection or Other Arboviral Disease

Testing for WNV in serum and CSF was performed by enzyme immunoassay in the Memorial Hermann Hospital laboratory. We considered a positive reaction to be IgM  $>1.1$  and IgG  $>1.5$  units. General arbovirus serology panels (for St. Louis encephalitis, Eastern equine encephalitis, and Western equine encephalitis viruses) were performed by indirect fluorescence antibody assay at the Associated Regional and University Pathologists laboratory (Houston, TX, USA). The cutoff for a positive reaction for each virus was  $\geq 1:16$ . A case was defined as acute WNV infection if viral genomic sequences

(by reverse transcription PCR [RT-PCR]), specific IgM, or both) were detected in serum or CSF. The diagnosis was acute infection from other arboviruses when samples were positive for specific arboviral IgM in the absence of evidence of WNV infection.

### Statistical Analyses

Data were analyzed by using SPSS version 21 (IBM, Austin, TX, USA). Baseline and clinical characteristics having a clinically plausible association with suspicion of WNV and other arboviral infections were examined by bivariate analysis. We used the Fisher exact,  $\chi^2$ , and Student *t* tests. To adjust for multiple comparisons, we applied the Bonferroni correction, and we considered  $p < 0.001$  to be statistically significant. We examined continuous data by using analysis of variance.

## Results

### Cohort

During the study period, 965 patients with a diagnosis of meningitis or encephalitis were screened for eligibility. We excluded 214 patients for the following reasons: positive Gram stain (113 patients), presence of shunt (84 patients), and postcraniotomy meningitis (17 patients). The other 751 patients were eligible: 567 were adults and 184 were children, 357 (48%) were male, and median age was 31 years (range 2 months–92 years) (Table 1). Among the 751 patients, onset of illness occurred during WNV season for 390 (52%), and 237 (32%) had encephalitis. Serum was submitted for arbovirus testing (WNV/St. Louis encephalitis virus and general arboviral panel) for 300 (40%) patients, WNV testing for 281 (37%) patients, general arboviral panel testing for 174 (23%) patients, and St. Louis encephalitis virus testing for 21 (3%) patients. A total of 725 (97%) patients were hospitalized, and adverse outcomes were seen in 85 (11%). Although the etiology was unknown for most (518 [69%]), among identifiable etiologies, the most common was viral (21%; 160 of 751 patients). Incidence of WNV infection was 4% (32 of 751 patients), which made it the third most common neuroinvasive virus causing infection during this period (following enterovirus and herpes simplex virus).

### Clinical Characteristics of Patients

Of the 281 patients tested for WNV, most were adult (234 [83%];  $p < 0.001$ ) and white (134 [48%];  $p = 0.004$ ) (Table 2). No difference between testing status (tested or not tested) groups was found in terms of Charlson Comorbidity Index scores or HIV status. The only clinical variable significantly associated with a trend toward more WNV testing was altered mental status (76 [28%] of 281 tested for WNV vs. 84 [18%] of 470 not tested for WNV;  $p = 0.01$ ).

Epidemiologically, the trend was toward more WNV testing of patients with meningitis/encephalitis who were hospitalized during June–October (170/281 [60%] vs. 220/470 [47%];  $p = 0.02$ ).

In term of physical findings, the presence of focal neurologic deficits was associated with ordering of WNV serologic testing (63 [22%] of 281 vs. 43 [9%] of 470;  $p < 0.001$ ). The trend was also toward more WNV testing among patients with a clinical diagnosis of encephalitis than of meningitis (115 [41%] of 281 vs. 122 [26%] of 470;  $p < 0.01$ ) and among those with an abnormal Glasgow Coma Scale score (45 [16%] of 281 vs. 43 [9%] of 470;  $p = 0.007$ ).

**Table 1.** Baseline characteristics and disease management, outcomes, and etiologies for 751 patients with meningitis or encephalitis, Houston, Texas, USA

Variable	No. (%)
Baseline characteristic	
Adult*	567 (75)
Male	357 (48)
White	306 (41)
Concurrent medical condition	
Charlson Comorbidity Index score $\geq 1$	70 (9)
HIV infection	42 (6)
Clinical features	
Encephalitis†	237 (32)
Illness onset during West Nile virus season‡	390 (52)
Testing performed	
West Nile virus serology	281 (37)
Other arbovirus serology	174 (23)
Magnetic resonance imaging of brain	290 (39)
Management and outcomes	
Admission	725 (97)
Received empirical antibiotic treatment	582 (77)
Received empirical antiviral treatment	193 (26)
Adverse outcome§	85 (11)
Etiologies	
Unknown	518 (69)
Viral	160 (21)
Enterovirus	63 (8)
Herpes simplex virus	48 (6)
West Nile virus	32 (4)
Other¶	17 (2)
Bacterial#	43 (6)
Fungal**	15 (2)
<i>Mycobacterium tuberculosis</i>	8 (1)
Noninfectious††	7 (1)

\* $>18$  y of age. Median age 31 (range 2 mo–92 y).

†Possible, probable or confirmed diagnosis of encephalitis (13).

‡Jun–Oct.

§Glasgow Outcome Scale score 1–4 (14).

¶Varicella zoster virus (n = 7), St. Louis encephalitis virus (n = 3), acute HIV infection (n = 3), cytomegalovirus (n = 2), Epstein-Barr virus (n = 1), influenza virus (n = 1).

#*Streptococcus pneumoniae* (n = 15), *Mycoplasma pneumoniae* (n = 5), *Staphylococcus aureus* (n = 3), *Streptococcus agalactiae* (n = 3), *Haemophilus influenzae* (n = 3), *Escherichia coli* (n = 3), *Listeria monocytogenes* (n = 2), *Enterococcus* spp. (n = 2), *Neisseria meningitidis* (n = 2),  $\alpha$ -hemolytic *Streptococcus* (n = 1), *Streptococcus pyogenes* (n = 1), coagulase-negative *Staphylococcus* (n = 1), *Brucella* sp. (n = 1), *Treponema pallidum* (n = 1).

\*\**Cryptococcus neoformans* (n = 14), *Histoplasma capsulatum* (n = 1).

††Malignancies (n = 3), systemic lupus erythematosus (n = 1), sarcoidosis (n = 1), acute disseminated encephalomyelitis (n = 1), cerebral aneurysm (n = 1).

**Table 2.** Baseline clinical characteristics among 751 patients with meningitis and encephalitis, by West Nile virus testing utilization, Houston, Texas, USA

Clinical characteristic	West Nile virus testing requested, no. (%), n = 281	West Nile virus testing not requested, no. (%), n = 470	p value*
<b>Demographic</b>			
Male	129 (46)	228 (49)	0.50
Adult†	234 (83)	332 (70)	<0.001
White	134 (48)	172 (37)	0.004
<b>Concurrent medical conditions</b>			
Charlson Comorbidity Index score $\geq 1$	32 (11)	38 (8)	0.15
HIV infection	16 (6)	26 (6)	1.0
<b>Clinical features</b>			
Altered mental status	76 (28)	84 (18)	0.01
Headache	232 (83)	378 (80)	0.50
Nausea/vomiting	179 (64)	311 (66)	0.52
Seizure	22 (8)	31 (7)	0.39
Illness onset during West Nile virus season‡	170 (60)	220 (47)	0.002
Fever $\geq 38^{\circ}\text{C}$	109 (38)	214 (46)	0.08
Glasgow Coma Scale score <15	45 (16)	43 (9)	0.007
Nuchal rigidity	71 (25)	112 (24)	0.66
Rash	4 (1)	17 (4)	0.11
Focal neurologic abnormalities	63 (22)	43 (9)	<0.001
Clinical diagnosis of encephalitis§	115 (41)	122 (26)	<0.001

\* $p < 0.001$  after Bonferroni correction considered statistically significant.

† $> 18$  y of age. Median age (range) of patients tested 35 (0.2–89), not tested 29 (0.1–92);  $p < 0.001$ .

‡Jun–Oct.

§Possible, probable, or confirmed diagnosis of encephalitis according to the definition in (13).

### Laboratory Results, Treatment, and Outcomes

Per inclusion criteria, all participants had undergone lumbar puncture and had evidence of CSF pleocytosis (Table 3). The trend was toward less testing among patients for whom CSF pleocytosis was higher ( $p = 0.017$ ) and for those with hypoglycorrhachia (CSF glucose  $< 45$  mg/dL;  $p = 0.005$ ). The ordering of serologic testing did not vary according to CSF lymphocytic pleocytosis and CSF protein level  $> 100$  mg/dL. Concomitant microbiological workups for mycobacterial, fungal, and other viral infections in CSF were performed more frequently for those in the group for whom WNV serologic testing had been ordered ( $p < 0.001$ ). Magnetic resonance imaging of the brain was performed for 290 (39%) of the 751 patients but was significantly more likely to be performed for patients for whom WNV serologic testing had been ordered (139 [49%] of 281 vs. 151 [32%] of 470;  $p < 0.001$ ).

No significant difference was found between groups with regard to empirical initiation of antibiotic therapy; however, WNV serologic testing was associated with a higher proportion of patients who were empirically prescribed antiviral therapy and for whom outcomes were worse ( $p < 0.001$ ). Among all causes of meningitis and encephalitis in this study, the trend was toward less WNV testing for those for whom the confirmed etiology was viral (46 [16%] of 281 vs. 114 [24%] of 470;  $p = 0.017$ ); no difference was found for other etiologic groups such as unknown, bacterial, mycobacterial, or fungal.

### WNV and Other Arbovirus Test Results

Of the 281 patients who were tested for WNV, results were positive for 32 (11%) and were compatible with acute

WNV infection. All positive results for these 32 patients were obtained during June–October, as demonstrated in the epidemiologic curve (Figure). Of those with a diagnosis of acute WNV infection, equal numbers had meningitis ( $n = 16$ ) and encephalitis ( $n = 16$ ). General arbovirus panel testing was ordered for 174 patients, and for 11 (6%) of these, results for IgM against St. Louis encephalitis virus were positive. Among these 11 patients, positive WNV serologic results were compatible with acute WNV infection for 7, indicating the possibility of cross-reaction. However, for 4 patients, WNV serologic results were negative, compatible with their having true acute infection with St. Louis encephalitis virus.

Serum was tested for WNV IgM and IgG for 168 (60%) of 281 patients; CSF was tested for IgM for 44 (16%) and serum was tested for IgM only for 40 (14%) patients (Table 4). For only 4 (1%) of the 281 patients were CSF IgM and serum IgM and IgG tested. Of the 32 patients with acute WNV infection, for 12 (38%), serum WNV IgM was positive and serum IgG was negative; and for 8 (25%), serum IgM and IgG were positive. For 4 (12%) of these 32 patients, serum and CSF IgM were both positive, and for 1 (3%), serum IgM was positive and CSF IgM was negative. RT-PCR of CSF was performed for 3 (1%) of the patients; no results were positive.

### Discussion

Our evaluation of the use of WNV diagnostics for patients with meningitis and encephalitis in routine clinical practice in a WNV-endemic area indicates that most cases were of unknown etiology. This finding is similar to that of the

**Table 3.** Laboratory results, treatment, and outcomes for 751 patients with meningitis and encephalitis, by West Nile virus testing, Houston, Texas, USA

Variable	West Nile virus testing requested, no. (%), n = 281	West Nile virus testing not requested, no. (%), n = 470	p value*
<b>Cerebrospinal fluid profile</b>			
Predominantly lymphocytes†	218 (78)	326 (71)	0.07
Protein >100 mg/dL	101 (36)	137 (29)	0.12
Glucose <45 mg/dL	27 (10)	79 (17)	0.005
<b>Cerebrospinal fluid microbiological testing</b>			
Bacteria, culture	272 (96)	447 (95)	0.35
Mycobacteria, culture or PCR	102 (36)	89 (19)	<0.001
Fungi, culture or antigen assay	91 (32)	71 (15)	<0.001
Herpes simplex virus, PCR	174 (62)	157 (33)	<0.001
Enterovirus, reverse transcription PCR	104 (37)	139 (30)	0.03
<b>Magnetic resonance imaging of brain</b>			
	139 (49)	151 (32)	<0.001
<b>Management and outcomes</b>			
Hospitalization	276 (98)	449 (96)	0.06
Empirical antibiotic treatment	215 (77)	367 (78)	0.65
Empirical antiviral treatment	98 (35)	95 (20)	<0.001
Adverse outcome‡	51 (18)	34 (7)	<0.001
<b>Etiology</b>			
Unknown	206 (73)	312 (66)	0.051
Viral	46 (16)	114 (24)	0.017
Bacterial	16 (6)	27 (6)	1.00
Fungal	6 (2)	9 (2)	0.80
Mycobacterial	5 (1)	3 (1)	0.43
Noninfectious	2 (1)	5 (1)	1.00

\*p<0.001 after Bonferroni correction considered statistically significant.

†Median leukocytes, cells/mL (range) 134 (1–4275) for samples tested and 143 (0–49000) for samples not tested for West Nile virus; p = 0.017.

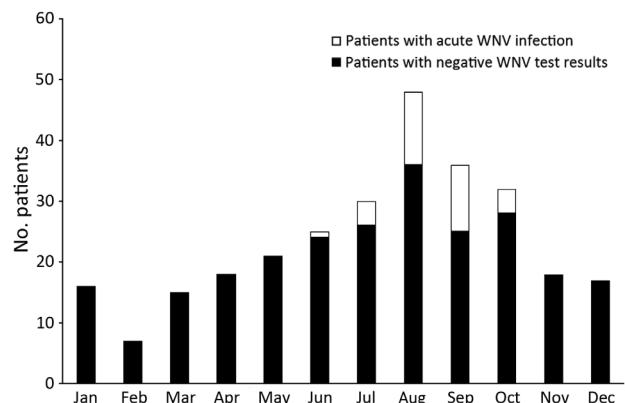
‡Glasgow Outcome Scale score 1–4 (14).

California Encephalitis Project (18). In our study, this finding can be explained by underutilization of testing in this patient population. The most common identifiable etiology in both studies was viral infection; however, our study detected WNV in 4% of patients and the California Encephalitis Project in only 1.2% (18). This difference could be explained by an epidemiologic difference (in the circulation of WNV) because more cases were reported from Texas than from California during the study periods (1). We found that only 37% of patients with clinically compatible illness in our study were tested for WNV, similar to 40% tested during a 2012 outbreak in Arizona, which reflects substantial underutilization of WNV testing in routine practice (14).

As previously reported, we found that arboviral infections were more commonly diagnosed for adults (19). Among children in the United States, WNV is the second most common arboviral disease, after La Crosse virus encephalitis (19). In our study, only 1 child received a diagnosis of WNND, but this number may be low because only 25% of children were tested for WNV. Furthermore, no patients in our study were tested for La Crosse encephalitis; such testing would have enabled a more specific comparison of accuracy. Patients with clinical features of encephalitis (altered mental status, abnormal Glasgow Coma Scale score, or focal neurologic abnormalities) were tested for WNV more frequently. However, meningitis can be found in 30%–50% of patients with WNND (7,8), similar to the 50% in our study. Therefore, meningitis should be

recognized as a common manifestation of WNND, and appropriate testing should be conducted.

All 32 cases of acute WNND occurred during June–October, similar to a US Centers for Disease Control and Prevention report that 94% of WNV cases occur during July–September (1). This information supports a decision to routinely send specimens collected during June–October for WNV testing. Of note, our study included all patients who had meningitis/encephalitis throughout the year; cases occurring outside WNV season might affect clinical characteristics. However, the occurrence of all WNND cases



**Figure.** Numbers of patients for whom West Nile virus serologic testing was performed, by month, combined over 5 years (January 1, 2005, through December 31, 2010). A total of 281 patients were tested.

**Table 4.** West Nile virus testing and results for patients with meningitis and encephalitis, Houston, Texas, USA

Variable	Patients, no. (%)
Testing requested, n = 281	
Serum IgM and IgG	168 (60)
Only cerebrospinal fluid IgM	44 (16)
Only serum IgM	40 (14)
Serum IgM and cerebrospinal fluid IgM	17 (6)
Serum WNV IgM, IgG and cerebrospinal fluid IgM	4 (1)
Only cerebrospinal fluid, by reverse transcription PCR	3 (1)*
Only serum IgG	2 (1)
Unknown	3 (1)
Results for patients with acute West Nile virus infection, n = 32	
Serum IgM + / serum IgG –	12 (38)
Serum IgM + / serum IgG +	8 (25)
Serum IgM + / cerebrospinal fluid IgM +	4 (12)
Only cerebrospinal fluid IgM +	4 (12)
Only serum IgM +	3 (9)
Serum IgM + / cerebrospinal fluid IgM –	1 (3)

\*All 3 samples had negative test results.

during June–October demonstrates the seasonal distribution of WNV infection and emphasizes the need to test for WNV during WNV season.

Patients with a higher level of CSF pleocytosis, hypoglycorrhachia, and lymphocytic pleocytosis >50% were less likely to get tested for WNV. Previous studies found that CSF in patients with WNND was more likely to contain <500 leukocytes/mL and to have protein and glucose levels within reference range. On the other hand, neutrophilic pleocytosis can be found in up to 40% of patients during acute infection (7,20). Thus, type of CSF pleocytosis should not influence the decision whether to submit samples for WNV testing. Patients tested for WNV infection were more likely to empirically be given antiviral therapy and to undergo evaluations for mycobacterial, fungal, and other viral infections (21). Moreover, patients for whom WNV serum testing was ordered were more likely to undergo brain magnetic resonance imaging and to experience adverse clinical outcomes; these factors are probably driven by a more severe clinical presentation because most patients had encephalitis. Of note, patients with a diagnosis of viral meningitis or encephalitis were less likely to be tested for WNV. This finding probably resulted from testing for WNV after receiving negative results for routine viral testing (including PCR for herpes simplex virus and enterovirus). Unfortunately, because of the design of this study, we are unable to go back and test those for whom samples were not submitted for WNV testing at the time of their illness to determine the number of cases missed because testing was not performed.

According to the Centers for Disease Control and Prevention, laboratory-confirmed acute WNV cases must meet specific diagnostic criteria; however, recent evidence of IgM persistence in WNV-positive patients has affected our ability to diagnosis true acute cases (1). After the initial outbreak of WNV in New York in 1999, a study found that serum WNV IgM could be detected up to 500 days after

acute WNV infection in >50% of patients (22). A separate study in Houston also found evidence of persistent IgM; 42% and 23% of study participants were positive for IgM at 1 and 8 years after infection, respectively (23). As a result, patients with an isolated positive WNV IgM result in serum are considered to have a probable case. In contrast, with CSF WNV IgM testing almost all CSF IgM-positive patients converted to a negative status when CSF antibody testing was repeated within 47 days of illness onset (24). RT-PCR for WNV is an alternative diagnostic tool for acute infection, but its application is limited because viremia is typically undetectable by the time symptoms appear. The usefulness of RT-PCR is further complicated by the fact that median time of symptom onset to actual testing is 13 days (25). Those findings indicate that samples to test for CSF IgM, serum IgM, and serum IgG should be sent to a laboratory for all patients suspected of having WNND, to be certain that disease onset is acute. However, our study demonstrated that samples for all 3 tests were sent for only 1% of patients. This finding reflects that, for most patients, inappropriate WNV testing was performed in clinical practice. Finally, arboviral panel diagnostics can help rule out infection with St. Louis encephalitis virus, a less common cause of neuroinvasive disease in this population.

Although supportive treatment remains the standard of care for patients with WNND, performing appropriate WNV testing may yield several benefits. An accurate diagnosis more precisely defines disease burden and epidemiology, an ongoing surveillance deficiency (26). Moreover, identifying WNV may lead to early detection of long-term neurologic and neurocognitive sequelae after WNND and thus enable earlier intervention (9).

In conclusion, WNND remains a frequent cause of acute meningitis and encephalitis in adult and child populations. The current disease burden may be underestimated because of underutilization and inaccurate choice of diagnostic tests in routine clinical practice. Samples should be

submitted for appropriate WNV testing (CSF IgM, serum IgM, and serum IgG) as soon as possible for patients with meningitis or encephalitis in WNV-endemic areas during the WNV season.

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# Fluoroquinolone-Resistant and Extended-Spectrum $\beta$ -Lactamase-Producing *Escherichia coli* Infections in Patients with Pyelonephritis, United States<sup>1</sup>

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**Release date: August 12, 2016; Expiration date: August 12, 2017**

## Learning Objectives

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

- Assess recommendations regarding the treatment of pyelonephritis
- Distinguish the rates of fluoroquinolone resistance and extended-spectrum  $\beta$ -lactamase (ESBL) production among patients with pyelonephritis in the current study
- Evaluate risk factors for fluoroquinolone resistance in the current study
- Evaluate risk factors for ESBL production in the current study

## CME Editor

**Rhonda Ray, PhD**, Copyeditor, *Emerging Infectious Diseases*. Disclosure: Rhonda Ray, PhD, has disclosed no relevant financial relationships.

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<sup>1</sup>Preliminary results of this research were presented at the 2014 IDWeek Meeting, Philadelphia, PA, USA, October 8–12, 2014.

For 2013–2014, we prospectively identified US adults with flank pain, temperature  $\geq 38.0^{\circ}\text{C}$ , and a diagnosis of acute pyelonephritis, confirmed by culture. Cultures from 453 (86.9%) of 521 patients grew *Escherichia coli*. Among *E. coli* isolates from 272 patients with uncomplicated pyelonephritis and 181 with complicated pyelonephritis, prevalence of fluoroquinolone resistance across study sites was 6.3% (range by site 0.0%–23.1%) and 19.9% (0.0%–50.0%), respectively; prevalence of extended-spectrum  $\beta$ -lactamase (ESBL) production was 2.6% (0.0%–8.3%) and 12.2% (0.0%–17.2%), respectively. Ten (34.5%) of 29 patients with ESBL infection reported no exposure to antimicrobial drugs, healthcare, or travel. Of the 29 patients with ESBL infection and 53 with fluoroquinolone-resistant infection, 22 (75.9%) and 24 (45.3%), respectively, were initially treated with in vitro inactive antimicrobial drugs. Prevalence of fluoroquinolone resistance exceeds treatment guideline thresholds for alternative antimicrobial drug strategies, and community-acquired ESBL-producing *E. coli* infection has emerged in some US communities.

*Escherichia coli*, the predominant cause of community-acquired urinary tract infection (UTI) worldwide, is increasingly resistant to available antimicrobial drugs. In the United States, in vitro resistance of *E. coli* to trimethoprim/sulfamethoxazole (TMP/SMX) became prevalent in the 1990s (1). Over the past decade, fluoroquinolone resistance rates have increased to  $>10\%$  in some surveys (2,3).

In many parts of the world, *E. coli* fluoroquinolone resistance rates are  $>20\%$  among patients with community-acquired uncomplicated UTI and  $>50\%$  among patients with complicated infections (4). In addition, infections resulting from extended-spectrum  $\beta$ -lactamase (ESBL)-producing *E. coli* and other *Enterobacteriaceae* are becoming increasingly common in these same areas and are associated with sequence type (ST) 131, a globally disseminated, multidrug-resistant clone that frequently produces CTX-M-15 ESBL. These *E. coli* isolates are generally resistant to cephalosporins and often to other antimicrobial drug classes. In North America, ESBL-producing *E. coli* infections have occurred predominantly in patients with healthcare exposure and have not become prevalent as a cause of community-acquired infections (5–8).

The 2010 international treatment guidelines of the Infectious Disease Society of America (IDSA) recommend for acute

uncomplicated pyelonephritis a fluoroquinolone and an initial dose of an agent from another antimicrobial drug class (e.g., ceftriaxone or gentamicin) if the fluoroquinolone resistance rate is  $>10\%$  (9). For uncomplicated cystitis, the guidelines discourage use of an antimicrobial drug if its resistance rate is  $>20\%$ . The guidelines do not address a scenario in which ESBL-producing uropathogens have become prevalent among patients with community-acquired infections. Use of antimicrobial drugs for which the uropathogen shows in vitro resistance has been associated with substantially reduced response rates (1,10,11), which can lead to serious consequences, particularly for patients with pyelonephritis. Given rapid changes in global resistance patterns and a lack of recent active and prospective surveillance of community-acquired UTI in the United States, the extent to which the prevalence of fluoroquinolone resistance has increased and multidrug-resistant ESBL-producing strains have emerged in the community is unknown.

We sought to determine the prevalence of *E. coli* antimicrobial resistance among patients with acute pyelonephritis who sought care at a US emergency department (ED)-based sentinel research network. We focused on fluoroquinolone-resistant and ESBL-producing isolates from these patients and examined risk factors for antimicrobial drug resistance.

## Methods

### Participants

We recruited adults seeking care in EMERGENCY ID NET, a network of 10 university-affiliated urban US EDs (12). All 10 study sites (online Technical Appendix, <http://www-wnc.cdc.gov/EID/article/22/9/16-0148-Techapp1.pdf>) provided institutional review board approval.

We enrolled patients  $\geq 18$  years of age who sought care during July 2013–December 2014 and had flank pain or costovertebral tenderness; temperature  $\geq 38.0^{\circ}\text{C}$  ( $100.4^{\circ}\text{F}$ ) measured by any method (i.e., oral, rectal, or axillary); and a presumptive diagnosis of acute pyelonephritis (i.e., patient received treatment for this infection during ED visit or was prescribed treatment at discharge). All sites conducted an audit to compare characteristics of enrolled and nonenrolled eligible patients to estimate case-finding sensitivity and detect enrollment biases (online Technical Appendix).

### Design and Measurements

We conducted a cross-sectional study by using a convenience sample of prospectively identified patients. ED physicians or study coordinators who used standardized forms at the time of care collected the following: demographic characteristics (i.e., age, sex, race, ethnicity); symptom duration; urinary tract abnormalities; UTI within the previous year; concurrent and immunocompromising conditions; antimicrobial drug use within the previous 2 and 60 days; a fluoroquinolone- or

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ceftriaxone-resistant UTI within the previous 90 days and 1 year; long-term care residence; hospitalization; travel outside North America within the previous 90 days; illness severity; disposition; and treatments provided. The study population consisted of patients with urine specimens that grew a single uropathogen at  $\geq 10^4$  CFU/mL. We defined a uropathogen as an organism known to be associated with UTI; among possible pathogens, we found *E. coli*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Citrobacter*, *Enterobacter*, and *Salmonella* species; *Staphylococcus aureus* or *S. saprophyticus*; and *Enterococcus* and *Aerococcus* species. If a urine specimen grew  $>1$  organism, we considered it to be contaminated and excluded it. We also excluded specimens that grew *Lactobacillus*, non-*saprophyticus* coagulase-negative *Staphylococcus*, or *Corynebacteria* species; or  $\alpha$ - or  $\beta$ -hemolytic streptococci.

Patients' urine specimens were collected in sterile containers by mid-stream clean-catch technique (91.4%), urethral catheterization (5.8%), and other techniques (e.g., sample from collection bag [2.7%] or suprapubic aspirate [0.2%]). Laboratories determined MICs by using automated susceptibility testing with VITEK commercial panels (bioMérieux, Marcy l'Etoile, France) at 7 sites; Microscan (Dade Behring, Inc., Sacramento, CA, USA) at 2 sites; and Phoenix Instrument System (Becton Dickinson, Franklin Lakes, NJ, USA) at 1 site, according to manufacturer instructions.

Each site used *E. coli* antimicrobial drug resistance breakpoints based on MIC breakpoints ( $\mu\text{g/mL}$ ) of the Clinical and Laboratory Standards Institute (Wayne, PA, USA) as follows: ampicillin  $\geq 32$ ; TMP/SMX  $\geq 4/76$ ; ceftazidime  $\geq 8$ ; ceftriaxone  $\geq 4$ ; cefotaxime  $\geq 4$ ; ciprofloxacin  $\geq 4$ ; levofloxacin  $\geq 8$ ; ertapenem  $\geq 2$ ; and imipenem  $\geq 4$  (13). We report resistance rates for antimicrobial drugs included in the study site laboratories' standard susceptibility testing panel. In 2010, the Clinical and Laboratory Standards Institute changed breakpoints for cephalosporins, aztreonam, and carbapenems. We provided Etests (bioMérieux) for ceftriaxone and imipenem to sites that had not yet updated automated susceptibility testing systems to enable these sites to use the new breakpoints. We considered *Enterobacteriaceae* isolates that were nonsusceptible to ceftriaxone (i.e., MIC  $>1$   $\mu\text{g/mL}$ ) to be potentially ESBL producing (13). The sites shipped these isolates to a reference laboratory to confirm speciation, ESBL production, and molecular characterization (online Technical Appendix).

We classified patients as having complicated pyelonephritis if they were pregnant or male or had a current or pre-existing functional or anatomic urinary tract abnormality or current immunocompromising condition. Possible preexisting urinary tract abnormalities were history of kidney stones, genitourinary procedures within the past 30 days, prostatic pathology, bladder catheter within the past 30 days, neurogenic urinary retention, ureteral stricture, duplicated collecting system, renal or bladder cancer, renal transplant, ureteral

diversion, vesico-ureteral reflux, single kidney, and nephrostomy tubes. Immunocompromising conditions were diabetes, active cancer, systemic corticosteroid use, current use of other immunosuppressants, chronic debilitating condition, chronic renal insufficiency or failure, and HIV infection. We identified current complicating features on the basis of clinical findings or laboratory studies in the ED; possible complications were pregnancy, diabetes, bladder catheter, ureteral stent, percutaneous nephrostomy tube, prostatitis, nephrolithiasis, renal or perirenal abscess, and urinary retention. We recorded history of chronic debilitating illness, such as chronic obstructive pulmonary disease or heart or hepatic failure, but did not assign such illness as a criterion for complicated infection. We classified patients without criteria for complicated pyelonephritis as having uncomplicated pyelonephritis. We defined healthcare-associated infections as those in patients hospitalized or residing in a long-term care facility within the previous 90 days; other patients were classified as having community-acquired infections.

### Statistical Analysis

To manage the study data, we used REDCap electronic-data capture tools hosted by the University of California, Los Angeles, CA, USA (14). We used SAS Version 9.3 (Cary, NC, USA) and Microsoft Excel 2013 (Redmond, WA, USA) to analyze data and used descriptive statistics to summarize patient characteristics and resistance prevalence. We calculated relative risks and 95% CIs to determine associations between epidemiologic and clinical characteristics and presence or absence of fluoroquinolone-resistant and ESBL-producing *E. coli* infections.

### Results

Of 817 enrolled patients with acute pyelonephritis, 793 (97.1%) submitted a urine culture. Of those 793 patients, 272 (34.3%) were excluded from analysis: 149 (18.8%) had no culture growth; 74 (9.3%) grew  $\geq 1$  contaminant; 17 (2.1%) grew  $\geq 1$  isolate at  $<10^4$  CFU/mL; 25 (3.2%) grew  $>1$  organism at  $10^4$  CFU/mL; and 7 (0.9%) had no fever. The study population consisted of 521 patients who grew 1 uropathogen at  $\geq 10^4$  CFU/mL. The case finding audit revealed a 66% enrollment of eligible patients. Enrolled and nonenrolled patients were similar for most characteristics, including *E. coli* susceptibility rates to TMP/SMX, ceftriaxone, and fluoroquinolones (online Technical Appendix).

Among the 521 study patients, median age was 37 (range 18–88, interquartile range 26–52) years; 455 (87.3%) were female (Table 1). Most (446 [85.6%]) patients had a community-acquired infection; 74 (14.2%) had a healthcare-associated infection (70 with hospitalization and 9 with nursing home residence in the previous 90 days). A total of 286 (54.9%) patients had uncomplicated pyelonephritis; 235 (45.1%) had complicated pyelonephritis.

**Table 1.** Epidemiologic, clinical, and laboratory characteristics of 521 US emergency department patients with acute uncomplicated and complicated pyelonephritis, July 2013–December 2014\*

	Value		
	Total patients, N = 521	Uncomplicated, n = 286	Complicated, n = 235
Age, median y (IQR; range)	37 (26–52; 18–88)	30 (23–41; 18–79)	50 (36–58; 19–88)
Symptom duration, median d (IQR; range)	3.0 (2–5; 0–30)	3.0 (2–5; 0–30)	3.0 (2–5; 0–30)
Initial ED temperature, °C (IQR; range)	38.9 (38.4–39.4; 38.0–43.0)	38.9 (38.4–39.4; 38.0–40.3)	39.0 (38.4–39.4; 38.0–43.0)
Sex			
F	455 (87.3)	286 (100.0)	169 (71.9)
M	66 (12.7)	0 (0)	66 (28.1)
Race/ethnicity			
White/Hispanic	372 (71.4)	191 (66.8)	181 (77.0)
Black	119 (22.8)	76 (26.6)	43 (18.3)
Asian/Pacific Islander	22 (4.2)	15 (5.2)	7 (3.0)
Other	18 (3.5)	11 (3.9)	7 (3.0)
Unknown	5 (1.0)	3 (1.0)	2 (0.9)
Hispanic ethnicity			
Yes	281 (53.9)	155 (54.2)	126 (53.6)
No	233 (44.7)	126 (44.1)	107 (45.5)
Unknown	7 (1.3)	5 (1.7)	2 (0.9)
Antimicrobial drugs taken			
Within past 60 d	125 (24.0)	49 (17.1)	76 (32.3)
Within past 2 d	36 (6.9)	15 (5.2)	21 (8.9)
Healthcare-associated illness†	74 (14.2)	18 (6.3)	56 (23.8)
Complicating feature			
Concurrent condition	131 (25.1)	0 (0)	131 (55.7)
History of UTA	116 (22.3)	0 (0)	116 (49.4)
Current feature	61 (11.7)	0 (0)	61 (26.0)
UTIs within past year‡			
0	334 (64.5)	196 (68.8)	138 (59.2)
1	86 (16.6)	46 (16.1)	40 (17.2)
2	428 (8.1)	23 (8.1)	19 (8.2)
≥3	56 (10.8)	20 (7.0)	36 (15.5)
Travel outside North America within past 90 d§	17/520 (3.3)	8/286 (2.8)	9/234 (3.8)
Prior UTI caused by fluoroquinolone-resistant <i>E. coli</i>			
Within past year	16 (3.1)	4 (1.4)	12 (5.1)
Within past 90 d	14 (2.7)	2 (0.7)	12 (5.1)
Prior UTI caused by ceftriaxone-resistant <i>E. coli</i>			
Within past year	9 (1.7)	2 (0.7)	7 (3.0)
Within past 90 d	6 (1.2)	1 (0.3)	5 (2.1)
Severity of illness¶			
Mild	66 (12.7)	34 (11.9)	32 (13.6)
Moderate	267 (51.2)	156 (54.5)	111 (47.2)
Severe	188 (36.1)	96 (33.6)	92 (39.1)
Disposition			
Ward	240 (46.1)	100 (35.0)	140 (59.6)
MCA	40 (7.7)	13 (4.5)	27 (11.5)
Home	239 (45.9)	172 (60.1)	67 (28.5)
AMA	2 (0.4)	1 (0.3)	1 (0.4)

\*Values are given as no. (%) except as indicated. AMA, left against medical advice; *E. coli*, *Escherichia coli*; ED, emergency department; IQR, interquartile range; MCA, monitored care admission; UTA, urinary tract abnormality; UTI, urinary tract infection.

†Hospitalized or residing in a long-term care facility within past 90 days.

‡Percentages were calculated with UTI information available for 518 patients, 285 of whom had uncomplicated cases and 233 had complicated cases.

§Percentages were calculated with information available for 520 patients, 286 of whom had uncomplicated cases and 234 had complicated cases.

¶Mild indicates illness that does not affect patient's normal activities; moderate partially affects normal activities but does not confine patient to house or bed; severe affects activities considerably, such as confining patient to house or bed.

Of types of uropathogens in patients with uncomplicated and complicated pyelonephritis (Table 2), *E. coli* accounted for infections in 453 (86.9%) patients; 272 (60.0%) of the infections were uncomplicated, and 181 (40.0%) were complicated. Among the 286 patients with uncomplicated infections, *E. coli* accounted for 95.1%; among the 235 patients with complicated infections, *E. coli* accounted for 77.0% (Table 2).

*E. coli* antimicrobial drug resistance rates among patients with complicated pyelonephritis tended to be higher than rates for patients with uncomplicated cases, except for TMP/SMX (Table 3). Among all patients, *E. coli* resistance rates varied by drug: ampicillin, 57.2% (259/453); TMP/SMX, 36.4% (165/453); gentamicin, 9.9% (43/436); cefazolin, 14.2% (52/367); ceftriaxone, 7.7% (35/453); levofloxacin, 10.2% (33/325); and ciprofloxacin, 12.1% (48/397). Among

**Table 2.** Uropathogens identified among US emergency department patients with acute uncomplicated and complicated pyelonephritis, July 2013–December 2014

Uropathogen	No. (%)		
	Total, N = 521	Uncomplicated cases, n = 286	Complicated cases, n = 235
<i>Escherichia coli</i>	453 (86.9)	272 (95.1)	181 (77.0)
<i>Staphylococcus saprophyticus</i>	2 (0.4)	2 (0.7)	0 (0)
<i>Staphylococcus aureus</i>	4 (0.8)	0 (0)	4 (1.7)
<i>Proteus</i> sp.	4 (0.8)	3 (1.0)	1 (0.4)
<i>Enterobacter</i> sp.	5 (1.0)	1 (0.3)	4 (1.7)
<i>Klebsiella pneumoniae</i>	25 (4.8)	4 (1.4)	21 (8.9)
<i>Enterococcus</i> sp.	12 (2.3)	0 (0)	12 (5.1)
<i>Pseudomonas</i> sp.	7 (1.3)	0 (0)	7 (3.0)
Group B streptococcus	2 (0.4)	1 (0.3)	1 (0.4)
Other*	5 (1.0)	2 (0.7)	3 (1.3)

\*Other pathogens were 2 *Aerococcus urinae*, 2 *Citrobacter koseri*, and 1 *Salmonella* species.

53 fluoroquinolone-resistant isolates, 8 (15.1%) were susceptible to ampicillin, 34 (64.2%) to gentamicin, and 27 (50.9%) to ceftriaxone. Of 46 isolates tested for susceptibility to cefazolin, 19 (41.3%) were susceptible; all 48 (100%) isolates tested were susceptible to a carbapenem. *E. coli* antimicrobial drug resistance rates for patients with uncomplicated and complicated pyelonephritis varied by study site (online Technical Appendix Tables 2, 3).

Among patients with uncomplicated pyelonephritis, 17 (6.3%) of 272 *E. coli* isolates were resistant to fluoroquinolone. The range of prevalence by site was 0.0%–23.1%; for 2 sites, prevalence was >10% (Figure 1; online Technical Appendix Tables 2, 3). Among patients with complicated pyelonephritis, 36 (19.9%) of 181 *E. coli* isolates showed fluoroquinolone resistance. The range of prevalence by site was 0.0%–50.0%; 8 sites had a prevalence >10%, 4 of which had a prevalence ≥20%. We found fluoroquinolone resistance associated with complicated *E. coli* infection, prior use of antimicrobial drugs and fluoroquinolone, hospital admission, and prior UTI resulting from a fluoroquinolone- or ceftriaxone-resistant organism (Table 4).

Among patients with uncomplicated pyelonephritis, ESBL production was found in 7 (2.6%) of 272 *E. coli* isolates; range by study site was 0.0%–8.3% (Figure 2; online Technical Appendix Tables 2, 3). Among patients with complicated pyelonephritis, ESBL production was found in

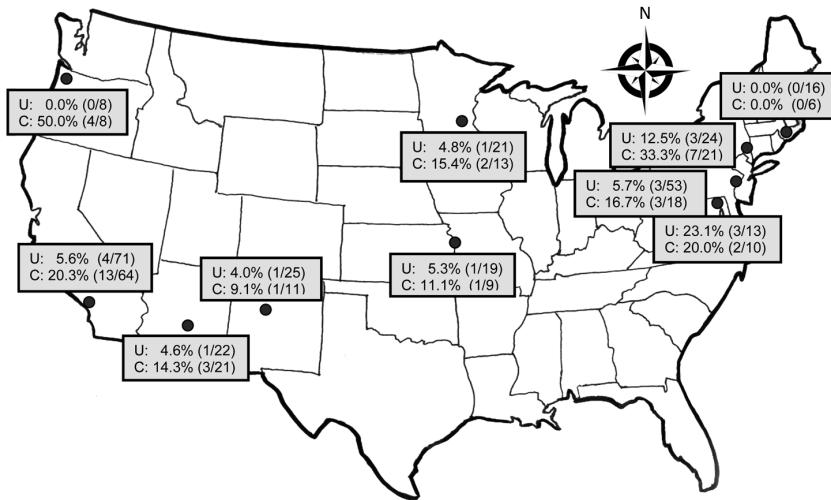
22 (12.2%) of 181 *E. coli* isolates; range by site was 0.0%–17.2% (Figure 2; online Technical Appendix Tables 2, 3). Frequencies of ESBL-producing *E. coli* isolates were higher among patients with antimicrobial drug resistance risk factors than among those without these factors (Table 5). Nineteen (65.5%) of 29 patients with ESBL-producing *E. coli* infection had a recognized risk factor for antimicrobial drug resistance (Figure 2; online Technical Appendix Tables 2, 3). Sixteen (55.2%) had antimicrobial drug exposure within the previous 60 days. During the previous 90 days, 6 (20.7%) had healthcare-setting exposure and 4 (13.8%) had travel outside North America. We found ESBL-producing *E. coli* infection associated with complicated infection, prior antimicrobial drug use, travel outside North America, and prior UTI resulting from a fluoroquinolone- or ceftriaxone-resistant organism. Among 37 isolates that grew other *Enterobacteriaceae*, including *Klebsiella pneumoniae*, 1 (2.7%) was ESBL producing. Among 29 ESBL-producing *E. coli* isolates, susceptibility rates to other antimicrobial drugs were 41.4% to TMP/SMX, 18.5% to ciprofloxacin, 21.7% to levofloxacin, 41.4% to gentamicin, and 100% to carbapenem. The prevalence of *E. coli* fluoroquinolone resistance correlated with the prevalence of ESBL-producing *E. coli* by site (Figure 3).

We further characterized 26 ESBL-producing *E. coli* isolates and 1 *K. pneumoniae* isolate. Among *E. coli* isolates,

**Table 3.** Antimicrobial drug resistance rates for *Escherichia coli* isolates from US emergency department patients with acute uncomplicated and complicated pyelonephritis, July 2013–December 2014\*

Antimicrobial drug	Patients with antimicrobial drug-resistant isolates, no./no. tested (%)		
	Total, N = 453	Uncomplicated cases, n = 272	Complicated cases, n = 181
Trimethoprim/sulfamethoxazole	165/453 (36.4)	111/272 (40.8)	54/181 (29.8)
Ampicillin	259/453 (57.2)	152/272 (55.9)	107/181 (59.1)
Cefazolin	52/367 (14.2)	18/219 (8.2)	34/148 (23.0)
Ceftriaxone	35/453 (7.7)	7/272 (2.6)	28/181 (15.5)
Ciprofloxacin	48/397 (12.1)	15/237 (6.3)	33/160 (20.6)
Levofloxacin	33/325 (10.2)	10/195 (5.1)	23/130 (17.7)
Gentamicin	43/436 (9.9)	19/261 (7.3)	24/175 (13.7)
Imipenem	0/135 (0)	0/90 (0)	0/45 (0)
Ertapenem	0/201 (0)	0/111 (0)	0/90 (0)
Meropenem	0/161 (0)	0/96 (0)	0/65 (0)
Doripenem	0/139 (0)	0/74 (0)	0/65 (0)

\*Denominators indicate number of isolates tested against a specific antimicrobial drug; the composition of testing panels varied by site.



**Figure 1.** Prevalence of fluoroquinolone-resistant *Escherichia coli* infection among emergency department patients with uncomplicated (U) and complicated (C) pyelonephritis by study site, United States, July 2013–December 2014. Study sites are listed in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/22/9/16-0148-Techapp1.pdf>); online Technical Appendix Tables 2 and 3 provide additional results on antimicrobial resistance rates. In vitro resistance to ciprofloxacin and/or levofloxacin is shown as % (no. of patients with a resistant isolate/total no. of patients tested)

PCR identified multiple ESBL types: 22 (84.6%) produced CTX-M-15 (16 [61.5%] produced only CTX-M-15; 6 [23.1%] produced CTX-M-15 and TEM-1); 2 (7.7%) produced CTX-M-27; 1 (3.8%) produced CTX-M-14; and 1 (3.8%) produced CTX-M-14 and TEM-1. The *K. pneumoniae* isolate produced SHV-1 and CTX-M-15 ESBL types. Sixteen (61.5%) of the *E. coli* isolates were clonal type O25b-ST131.

Among patients with an *E. coli* infection, 223 (49.2%) were discharged from the ED and 229 (50.6%) were admitted to the hospital; of these, 13 (5.8%) and 18 (7.9%), respectively, were treated with an antimicrobial drug that lacked in vitro activity against their infection. Of 53 patients with a fluoroquinolone-resistant and 29 with an ESBL-producing infection, 24 (45.3%) and 22 (75.0%), respectively, were initially treated with in vitro–inactive antimicrobial drugs. Among 29 patients with an ESBL infection, 9 (31.0%) were

discharged from the ED; an in vitro–inactive antimicrobial drug was initially prescribed to 7 (77.8%). Among 20 (69.0%) hospitalized patients with an ESBL infection, 15 (75.0%) were initially given an in vitro–inactive antimicrobial drug; 1 of those 15 patients was given gentamicin, 13 were given cephalosporin, and 1 was given both.

**Discussion**

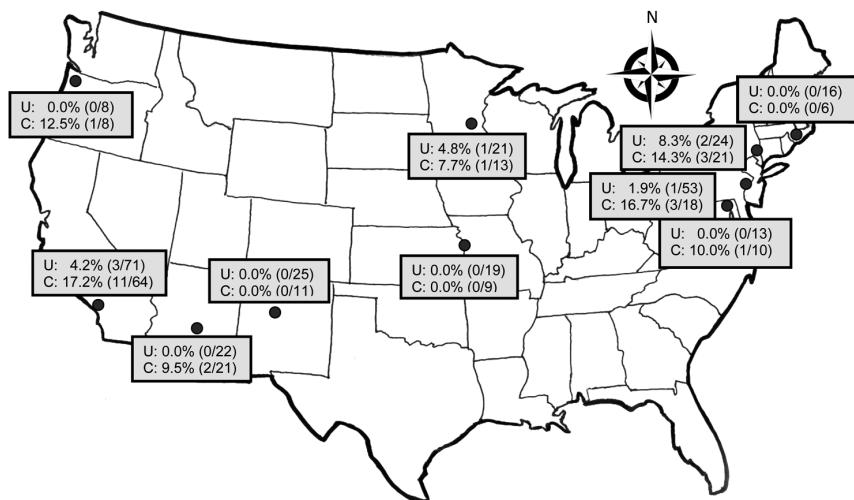
For 2013–2014, we found that prevalence of *E. coli* fluoroquinolone resistance was >10% for patients with uncomplicated pyelonephritis at 2 of 10 sites and ≥20% for patients with complicated infections at 4 of 10 sentinel sites surveyed in the United States. *E. coli* fluoroquinolone resistance was particularly prevalent in groups with antimicrobial drug resistance risk. These rates exceed thresholds for the 2010 IDSA treatment guidelines, which recommend consideration of an

**Table 4.** Factors associated with fluoroquinolone resistance among 453 US emergency department patients with pyelonephritis caused by *Escherichia coli*, July 2013–December 2014\*

Factor	Fluoroquinolone-resistance rate		Relative risk (95% CI)
	Factor present, no./total (%)	Factor absent, no./total (%)	
Complicated infection	36/181 (19.9)	17/272 (6.3)	3.2 (1.8–5.8)
Prior antimicrobial drugs taken			
Within past 60 d	24/94 (25.5)	29/359 (8.1)	3.2 (1.9–5.3)
Within past 2 d	9/27 (33.3)	44/426 (10.3)	3.2 (1.6–5.8)
Prior fluoroquinolone use			
Within past 60 d	12/19 (63.2)	41/434 (9.4)	6.7 (3.8–9.6)
Within past 2 d	6/8 (75.0)	47/445 (10.6)	7.1 (3.2–9.4)
IV antimicrobial drugs within past 30 d	6/26 (23.1)	47/425 (11.1)	2.1 (0.8–4.3)
LTC within past 90 d	1/3 (33.3)	52/450 (11.6)	2.9 (0.2–7.8)
Admitted to hospital within 90 d	11/42 (26.2)	42/411 (10.2)	2.6 (1.3–4.6)
Travel outside United States within past 90 d	5/17 (29.4)†	48/436 (11.0)	2.7 (1.0–5.5)
UTI resulting from fluoroquinolone-resistant <i>E. coli</i>			
Within past year	7/9 (77.8)	46/444 (10.4)	7.5 (3.7–9.6)
Within past 90 d	7/8 (87.5)	46/445 (10.3)	8.5 (4.3–9.8)
UTI resulting from ceftriaxone-resistant <i>E. coli</i>			
Within past year	5/6 (83.3)	48/447 (10.7)	7.8 (3.3–9.4)
Within past 90 d	5/5 (100.0)	48/448 (10.7)	9.3 (4.1–9.3)

\*Denominators differ because factors have a different distribution among the patient population. IV, intravenous; LTC, residence in a long-term care facility; UTI, urinary tract infection.

†Of the 5 patients that traveled outside the United States, 3 traveled to Mexico or Central America and 2 traveled to Asia.



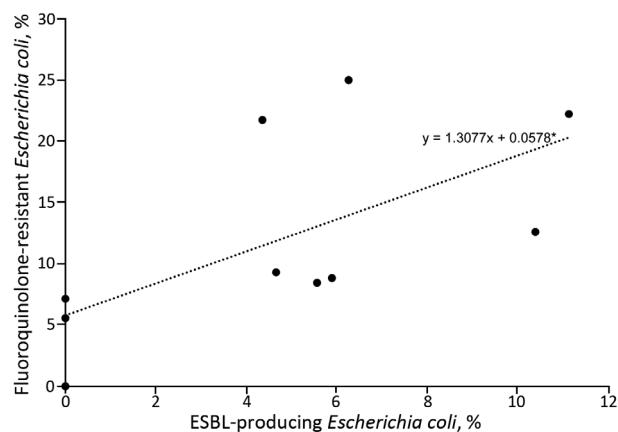
**Figure 2.** Prevalence of extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* infection among patients with uncomplicated (U) and complicated (C) pyelonephritis, by study site, United States, July 2013–December 2014. Study sites are listed in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/22/9/16-0148-Techapp1.pdf>); online Technical Appendix Tables 2 and 3 provide additional results on antimicrobial resistance rates.

additional antimicrobial drug of a different class and other agents (9). Data from a similar study we conducted during 2000–2004 (15) indicate that, among all healthcare-seeking ED patients with acute pyelonephritis, the prevalence of fluoroquinolone-resistant *E. coli* increased from 3.9% during 2000–2004 to 11.7% during 2013–2014. During 2000–2004, we found no infections caused by ESBL-producing bacteria. As in other parts of the world, ESBL-producing *Enterobacteriaceae* are emerging among patients with community-acquired UTI in the United States. For patients with uncomplicated and complicated pyelonephritis caused by *E. coli*, we found that 2.6% and 12.2%, respectively, had infection caused by an ESBL-producing organism; rates were even higher for patients with risk factors. The globally disseminated, multidrug-resistant clone ST131, which produces CTX-M-15

$\beta$ -lactamase, accounted for 85.2% of these infections. Of ESBL-infected patients, about one third lacked traditional antimicrobial resistance risk factors (i.e., antimicrobial drug or healthcare-setting exposure or international travel), suggesting that these isolates are now endemic in some US communities. Among ESBL-infected patients, about three quarters were initially treated with an antimicrobial drug lacking in vitro activity, including the sickest patients who required hospitalization. We did not collect outcome data, but lack of in vitro activity of the antimicrobial drug used for treatment has been associated with relatively poor response rates among patients with pyelonephritis (1,10,11).

Previous surveys have suggested that *E. coli* fluoroquinolone resistance rates are increasing in the United States. Among outpatients seeking care at 30 US centers during 2003–2004, 59 (6.8%) of 862 *E. coli* isolates were resistant to ciprofloxacin (2). Another analysis of >12 million urine specimens from US outpatient centers found that the *E. coli* fluoroquinolone resistance rate increased from 3.0% in 2000 to 17.1% in 2010 (3). Such laboratory-based resistance surveillance data may exaggerate the prevalence of resistance because patients for whom cultures are performed would be expected to have received prior therapy and to have had healthcare exposure more frequently than patients without cultures. Isolate-driven studies require retrospective review of records, which have missing and inaccurate data; also, uncertainty may exist regarding whether a specimen is from a patient with an actual clinical infection, rather than being a colonized or contaminated specimen. In routine practice, providers typically estimate local resistance rates on the basis of an antibiogram published by the local hospital laboratory. Resistance rates determined from antibiograms are prone to bias and indicate only whether the specimen was obtained from an outpatient or inpatient location.

In contrast, we conducted syndromic surveillance of patients who sought care at a geographically diverse network



**Figure 3.** Prevalence of fluoroquinolone-resistant and ESBL-producing *Escherichia coli* infections among patients with uncomplicated and complicated pyelonephritis, by study site, United States, July 2013–December 2014. Each dot indicates a study site; the line to show the general trend between fluoroquinolone resistance and ESBL-producing *E. coli* was generated by using simple linear regression. ESBL, extended spectrum  $\beta$ -lactamase.

**Table 5.** Factors associated with ESBL production among 453 US emergency department patients with pyelonephritis caused by *Escherichia coli*, July 2013–December 2014\*

Factor	ESBL-producing <i>E. coli</i> rate		Relative risk (95% CI)
	Factor present, no./total (%)	Factor absent, no./total (%)	
Age >65 y	4/22 (18.2)	25/431 (5.8)	3.1 (0.96–8.1)
Complicated infection	22/181 (12.2)	7/272 (2.6)	4.7 (2.0–12.0)
Prior antimicrobial drugs			
Within past 60 d	16/94 (17.0)	13/359 (3.6)	4.7 (2.2–10.0)
Within past 2 d	4/27 (14.8)	25/426 (5.9)	2.5 (0.8–6.7)
IV antimicrobial drugs taken within past 30 d	4/26 (15.4)	25/425 (5.9)	2.6 (0.8–6.9)
LTC within past 90 d	1/3 (33.3)	28/450 (6.2)	5.4 (0.3–14.9)
Hospital admittance within past 90 d	5/42 (11.9)	24/411 (5.8)	2.0 (0.7–5.2)
Travel outside United States within past 90 d	4/17 (23.5)†	25/436 (5.7)	4.1 (1.3–10.1)
UTI caused by fluoroquinolone-resistant <i>E. coli</i>			
Within past year	5/9 (55.6)	24/444 (5.4)	10.3 (3.8–17.5)
Within past 90 d	5/8 (62.5)	24/445 (5.4)	11.6 (4.4–18.3)
UTI caused by ceftriaxone-resistant <i>E. coli</i>			
Within past year	5/6 (83.3)	24/447 (5.4)	15.5 (6.2–19.2)
Within past 90 d	5/5 (100.0)	24/448 (5.4)	18.7 (8.0–18.7)
Fluoroquinolone resistance	24/53 (45.3)	5/400 (1.3)	36.2 (14.2–104.7)

\*Denominators differ because factors have a different distribution among the patient population. ESBL, extended spectrum β-lactamase; IV, intravenous; LTC, residence in a long-term care facility; UTI, urinary tract infection.  
†Of these 4 patients who traveled to other regions, 3 traveled to Mexico or Central America and 1 traveled to Asia.

of US EDs. We studied acute pyelonephritis because it is a distinct clinical syndrome for which cultures are routinely obtained and because isolates grown are less likely to be contaminants or colonizers, compared with those from patients with suspected cystitis. Historical data were obtained by real-time patient interviews, enabling accurate classification of complicated and uncomplicated pyelonephritis and ascertainment of antimicrobial drug resistance risk factors. To identify biases, we compared characteristics of enrolled and nonenrolled qualifying patients and found their characteristics to be similar, suggesting the validity of our sampling. Consequently, our data and that of other studies indicate that, in some parts of the United States, the rate of *E. coli* fluoroquinolone resistance among uropathogens is >10% among patients with uncomplicated pyelonephritis and >20% among those with complicated infections. However, variability exists; among patients with uncomplicated pyelonephritis, 3 of 10 sites had fluoroquinolone resistance rates <5%. Consistent with findings of previous investigations, we found *E. coli* fluoroquinolone resistance associated with complicated infection, prior use of antimicrobial drugs and fluoroquinolone, hospitalization, and prior UTI caused by a fluoroquinolone- or ceftriaxone-resistant organism (16,17).

By using active, prospective surveillance, we found that ESBL-producing *E. coli* infections have now emerged to a considerable degree among patients with clinically confirmed community-acquired infections in parts of the United States, including among persons lacking commonly recognized antimicrobial drug resistance risk factors. This new observation is not unexpected, given the reported epidemiology of ESBL-producing *Enterobacteriaceae* infections in communities outside North America. CTX-M enzymes are currently the most prevalent ESBL types worldwide. The ST131 clone is largely responsible for the international

epidemic caused by CTX-M-15–producing *E. coli*, including infections seen in the United States (7,18,19).

A few US laboratory-based surveillance studies have reported community-acquired ESBL infections. Peirano et al. (19) described ESBL-producing *E. coli* isolates from 30 community-dwelling patients at 5 Chicago-area hospitals during 2008. These ESBL-producing strains represented 2%–8% of *E. coli* isolates at each hospital. Khawcharoenporn et al. (20) reported that ≈5% of *Enterobacteriaceae* isolates from ED patients with presumed UTI during 2008–2009 were ESBL-producing, although specific risk data were not provided. Doi et al. (7) reviewed records of patients with cultures that grew ESBL-producing *E. coli* isolates at 1 hospital in each of 5 US cities during 2009–2010. Among 13,270 *E. coli* isolates, 523 (3.9%) were ESBL producing. Of the 291 patients infected or colonized with ESBL-producing *E. coli* as outpatients, infections of 107 (36.8%) were thought to be community associated. Community ESBL-producing *E. coli* isolates were resistant to multiple agents: 87.5% to ciprofloxacin or levofloxacin and 39.4% to gentamicin. All isolates from that study were susceptible to a carbapenem; we also found that all ESBL-producing isolates in our investigation were susceptible to a carbapenem.

Studies from outside North America have identified several characteristics associated with ESBL infection, such as recurrent and complicated UTI; advanced age; recent hospitalization; use of a β-lactam or fluoroquinolone; travel to Asia, Middle East, or Africa; and fresh water swimming (5,21–23). Banerjee et al. (23) conducted a case-control study among adults with *E. coli* clinical isolates cultured in the Chicago area and found that ESBL infection was associated with travel to India, ciprofloxacin use, and age. We found ESBL-producing *E. coli* infection associated with complicated infection, prior antimicrobial drug use, travel

outside North America, and prior UTI resulting from fluoroquinolone- or ceftriaxone-resistant infection. The importance of investigating past susceptibility data when considering empirical treatment is highlighted by our observation that both fluoroquinolone resistance and ESBL-production were associated with previous resistant infections.

Our study has limitations. We were unsuccessful in enrolling all consecutive patients, which may have introduced bias in our selection of patients. However, our audit of eligible case-patients showed similarity of enrolled and non-enrolled patients, including their *E. coli* antimicrobial drug susceptibility rates; furthermore, most (>97%) enrolled patients had urine cultures collected, reducing potential bias. The prevalence of ESBL-producing strains may have been underestimated because we did not use ESBL-selective media and used only ceftriaxone instead of several advanced-generation cephalosporins to screen for presence of ESBL-producing strains. However, this method enabled us to have greater site participation; furthermore, screening isolates with a ceftriaxone MIC >1 µg/mL has been reported to have a sensitivity >98% on the basis of phenotypic testing (24). In addition, some patients may not have had pyelonephritis if a contaminated specimen was misinterpreted as noncontaminated, although >97% of patients had urine collected by a technique that minimizes contamination (i.e., clean catch, urethral catheterization, or suprapubic aspiration), and the diagnosis of pyelonephritis was further supported by clinical assessment. Our definition of confirmed infection as growth of 1 uropathogen at >10<sup>4</sup> CFU/mL may have missed some cases of pyelonephritis, although only ≈5% grew a single uropathogen at <10<sup>4</sup> CFU/mL or grew >1 uropathogen at >10<sup>4</sup> CFU/mL. Our hospitals were large US urban centers and may not represent patients in other settings, emphasizing the importance of local surveillance.

IDSA treatment guidelines for acute uncomplicated pyelonephritis recommend that, if the fluoroquinolone-resistance rate is >10%, then in addition to a fluoroquinolone, an agent of another class (i.e., ceftriaxone or gentamicin) should be administered (9). Our findings indicate that fluoroquinolone resistance rates for *E. coli* are approaching or exceed this threshold for patients with uncomplicated pyelonephritis in many parts of the United States. For uncomplicated cystitis, the guidelines recommend alternative agents if the resistance rate is >20%, which is the current situation for fluoroquinolones in many settings for patients with complicated pyelonephritis. Unfortunately, we found that only one half to two thirds of fluoroquinolone-resistant *E. coli* isolates were susceptible to ceftriaxone or gentamicin. Rates of fluoroquinolone-resistant and ESBL-producing *E. coli* infections correlate to geographic location. Prior exposure to antimicrobial drugs or a healthcare setting, travel outside the United States, and a history of an antimicrobial drug-resistant infection substantially increases the chance that a person will have a

current fluoroquinolone-resistant or ESBL-producing *E. coli* infection. Therefore, in settings with high fluoroquinolone resistance rates, in settings where ESBL-producing *Enterobacteriaceae* infections have emerged, or among persons with antimicrobial drug resistance risk factors (especially patients with or at risk for severe sepsis), healthcare providers should consider empirical treatment with a carbapenem or another agent found to be consistently active on the basis of the local antibiogram. In this study, ≈50% of patients with pyelonephritis were managed as outpatients. Currently, no oral antimicrobial drugs with consistent in vitro activity are available for empirical treatment of pyelonephritis caused by ESBL-producing *E. coli* uropathogens. Our findings, including the variability in the prevalence of resistance by site, show that increased local efforts to enhance surveillance for antimicrobial drug resistance are necessary to best inform treatment decisions. Furthermore, availability of new antimicrobial drugs must be expedited.

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# Enterohemorrhagic *Escherichia coli* Hybrid Pathotype O80:H2 as a New Therapeutic Challenge

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We describe the epidemiology, clinical features, and molecular characterization of enterohemorrhagic *Escherichia coli* (EHEC) infections caused by the singular hybrid pathotype O80:H2, and we examine the influence of antibiotics on Shiga toxin production. In France, during 2005–2014, a total of 54 patients were infected with EHEC O80:H2; 91% had hemolytic uremic syndrome. Two patients had invasive infections, and 2 died. All strains carried *stx2* (variants *stx2a*, *2c*, or *2d*); the rare intimin gene (*eae-ξ*); and at least 4 genes characteristic of pS88, a plasmid associated with extraintestinal virulence. Similar strains were found in Spain. All isolates belonged to the same clonal group. At subinhibitory concentrations, azithromycin decreased Shiga toxin production significantly, ciprofloxacin increased it substantially, and ceftriaxone had no major effect. Antibiotic combinations that included azithromycin also were tested. EHEC O80:H2, which can induce hemolytic uremic syndrome complicated by bacteremia, is emerging in France. However, azithromycin might effectively combat these infections.

Enterohemorrhagic *Escherichia coli* (EHEC) are a subset of Shiga toxin-producing *E. coli* (STEC) that cause diarrhea and hemorrhagic colitis; illness can progress to hemolytic uremic syndrome (HUS) in 5%–10% of cases (1,2). HUS is the most frequent etiology of pediatric acute renal failure, and its lethality is 3%–5% worldwide (1,3) and 1% in France (4). Long-term renal injuries occur in 20%–30% of HUS patients (1,3,5).

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EHEC serotype O157:H7 accounts for ≈60% of HUS cases worldwide (6,7). Other well-known serogroups associated with HUS include O26, O111, O145, O55, O103, O121, and O91. EHEC O80:H2 strains are rarely reported in the literature but have been detected in France. In 2013, an EHEC O80:H2 strain was responsible for a severe case of HUS with relapse associated with bacteremia (8). We identified several genetic traits in this isolate, such as a rare variant of the intimin gene (*eae-ξ*) and genetic determinants related to the pS88 plasmid associated with extraintestinal-virulence pathogenic *E. coli* (ExPEC) (9). This plasmid, mainly found in avian pathogenic *E. coli* and *E. coli* strains that cause neonatal meningitis, may partly explain the bacteremia observed, which has been reported in patients with HUS.

The occurrence of bacteremia during EHEC infections warrants antibiotic treatment for those infections. However, antibiotics usually are not recommended for EHEC infection because of the risk for worsening HUS, notably by induction of synthesis or secretion of Shiga toxin (Stx) (1,10–12). Therefore, bacteremia during EHEC infection represents a new therapeutic challenge. However, a 2011 outbreak in Germany linked to EHEC O104:H4 (13) underscored the potential benefit of certain antibiotics when HUS occurs (14,15). Thus, the use of antibiotics during EHEC infections remains a source of debate (2,16).

Our study aimed to determine the incidence rate of HUS cases associated with the singular EHEC O80:H2, describe their clinical features, and examine the molecular characteristics of the strains. In addition, we assessed the effects of different antibiotics on Stx production in representative strains.

## Materials and Methods

### Clinical Data

For this study, we considered all *E. coli* O80:H2 isolates received during January 2005–October 2014 by the Centre National de Référence Associé *Escherichia coli* (Paris, France). We then collected demographic and clinical data from patients' medical records (e.g., age, sex, location); presence of diarrhea (with or without blood); possible

source of infection; presence of neurologic or other complications (including pancreatitis, hepatitis, myocarditis, and bacteremia); whether the patient had HUS; and outcome at time of follow-up (e.g., relapse, residual renal injuries [including proteinuria and renal failure], arterial hypertension, or death). HUS was defined as anemia (hemoglobin <10 g/dL), thrombopenia (platelets <150,000/mm<sup>3</sup>), and renal failure (creatinine above reference for age, weight, and sex, or  $\geq 0.2$  protein/creatinine ratio).

### Bacteria Strains

We recovered isolates 35344 and 35431 from stool and blood cultures, respectively, of a HUS patient who was the subject of a recent case report (8). These strains belonged to sequence type 301 and harbored 4 intestinal virulence genes (*stx2c*, *stx2d*, *hlyA*, and *eae-ξ*) and most of the extraintestinal virulence genes carried by plasmid pS88 (8). The Laboratoire National de Référence pour les *Escherichia coli* (Marcy l'Étoile, France) and reference laboratories from Spain, Italy, and Germany were associated with this study and provided us with their EHEC O80 strains when available. The reference strain EDL933 (O157:H7, *stx1a*, *stx2a*, and *eae-γ*) served as the control in the study of Stx production (17). We stored all EHEC strains at  $-80^{\circ}\text{C}$  in 5% glycerol.

### Serotyping

The Centre National de Référence des *Escherichia coli*, *Shigella*, et *Salmonella* at the Institute Pasteur (Paris, France) initially determined the O80 serogroup by using a method based on the analysis of the O antigen genes cluster *rfb* restriction fragments length polymorphism (18); this result was recently confirmed by O80-specific PCR with primers targeting O80 polymerase gene *wzy* (GenBank accession no. AB812032). This new PCR was included in our previously described O-serogroup multiplex PCR (19). We assessed specificity of the new multiplex PCR on template DNA extracted from 130 O reference strains as previously described (20). Primers used for the EHEC O-serogrouping multiplex PCR are provided (online Technical Appendix Tables 1, 2, <http://wwwnc.cdc.gov/EID/article/22/9/16-0304-Techapp1.xlsx>). We determined the H serogroup by using PCR targeting the *fliC* genes (21).

### Molecular Characterization

Among EHEC O80 strains, we screened several genetic determinants by multiplex PCR as previously described (22), including intestinal virulence genes (*stx1*, *stx2*, *eae*, and *hlyA*) and extraintestinal virulence genes associated with plasmid pS88 (*sitA*, *eitB*, *cia*, *iss*, *iucC*, *iroN*, *hlyF*, *etsC*, *cvaA*, and *ompT<sub>p</sub>*) (9,23). We also determined the variants of *stx2* (*stx2a*, *stx2b*, *stx2c*, and *stx2d*) and the variant of *eae* by using PCR-based methods (24,25).

To investigate the genetic diversity of the O80:H2 strains studied, we used the DiversiLab genotyping method (bioMérieux, Marcy-l'Étoile, France), which is based on PCR amplification of repeat sequences of DNA (rep-PCR) as previously described (26). We then compared these genotypes with representative strains of serogroups (O157, O104, O121, and O111) previously typed and recorded in our DiversiLab database (S. Bonacorsi, unpub. data).

### Effect of Various Antibiotics on Stx Production

We prepared inocula to assess Stx production in the presence or absence of antibiotics as previously described (27). We obtained log-phase growth of strains in brain–heart infusion broth by using overnight incubation at  $37^{\circ}\text{C}$  and then diluted the result with Luria-Bertani broth for an inoculum of  $10^6$  CFU/mL. We added 3 antimicrobial agents (azithromycin, ciprofloxacin, and ceftriaxone) at final MICs of 0.5 and 0.25 for a single assay. We also tested combinations of antibiotics at a MIC of 0.5. For each strain, we also performed an antibiotic-free assay. We collected the bacterial cultures after 18 h of incubation at  $37^{\circ}\text{C}$  and centrifuged at  $4^{\circ}\text{C}$  at 2,000 rpm for 10 min. We filtered supernatants through a 0.22- $\mu\text{m}$  pore-size filter (Millipore, Bedford, MA, USA) and stored at  $-20^{\circ}\text{C}$  until needed.

We quantified Stx1 and Stx2 by using a chemiluminescent immunoassay for EHEC toxins (Liaison, DiaSorin, Spain). We expressed the results in relative light units, and converted each result to a concentration of Stx (ng/mL) by using a standard curve obtained by serial dilution of a highly positive sample and the manufacturer-provided positive control (70 ng/mL Stx concentration). We performed each measure 3 times.

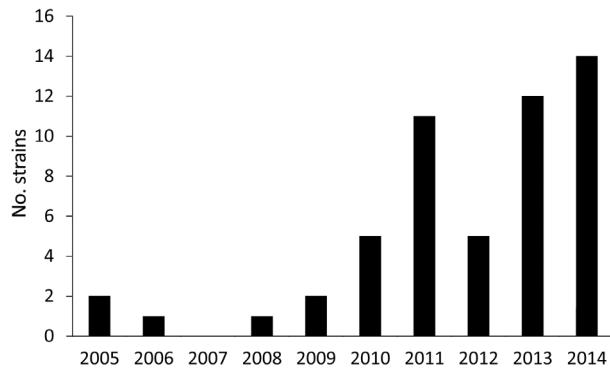
### Statistical Analysis

We calculated means, medians, and SDs in Excel (Microsoft Corp., Redmond, WA, USA). Student paired *t*-test was used to compare means of Stx concentrations; *p* values <0.05 were considered statistically significant. Quantitative variables are presented as median and range or quartile range.

### Results

During January 2005–October 2014, the Centre National de Référence Associé *Escherichia coli* collected 57 strains of EHEC O80:H2 in France. These strains were isolated, mostly from stool specimens, from 54 patients; 2 and 3 isolates each were recovered from 2 patients. Clinical data were available for all but 1 patient.

The spatiotemporal distribution of the O80:H2 infections clearly indicates an increased number of infections during the past 5 years (Figure 1). In 2014, the EHEC O80 serogroup was the second-leading cause of pediatric HUS in France (4). Most of the cases occurred during summer and the beginning of autumn. Geographic distribution of



**Figure 1.** Number of enterohemorrhagic *Escherichia coli* O80:H2 strains detected annually, France, January 2005–October 2014.

O80:H2 EHEC infections in France revealed high 10-year cumulative incidences (>1/100,000) in Franche-Comté (2.83/100,000 children) and in Rhône-Alpes (1.19/100,000 children), contrasting with the distribution of O157 infections, which are rarely detected in these areas (Figure 2).

Among the 53 patients for whom clinical data were available, 48 (91%) had HUS; 27 (51%) were male. Median age for these 48 patients was 1.2 years (range 0.2–39 years, interquartile range [IQR] 0.7–1.6 years). Only 1 adult HUS patient (a 39-year-old) was reported. The 5 (9%) non-HUS patients were largely older (1, 2, 6, 21, and 40 years old). Among HUS patients, fever was present in 45%; median leukocyte count was 13,000 cells/mm<sup>3</sup> (data were not available for 14 patients), and 56% had leukocytosis (>11,500 leukocytes/mm<sup>3</sup>) (online Technical Appendix Table 3). Diarrheal illness was reported for 83% of HUS patients (bloody diarrhea for 30%); median time from onset of diarrhea to diagnosis of HUS was 6 days (data available for 37 patients). Diarrheal illness in family members was recorded in only 2 HUS cases. One patient had a relapse complicated by bacteremia (8), 1 patient died after myocardial complication with pancreatic abscess from which an O80:H2 EHEC strain was isolated, and 1 patient died from septic shock with intestinal necrosis and peritonitis. Eight (17%) cases of neurologic complication (17%) were reported. All the neurologic complications were seizures (documented in 6 cases by imaging) with ischemic strokes. Among the 8 patients with neurologic complications, 2 died, and 1 has difficulty concentrating (19 months after HUS); for 1 patient, the neurologic state could not be assessed, and for 4 others, the neurologic outcome was favorable.

Of the 48 HUS patients, 27% needed acute dialysis support (median duration 10 days, range 3–21 days, IQR 7.4–13.5 days), and 28% had long-term renal sequelae, including proteinuria for all cases, hypertension in 3 cases, and chronic renal failure in 1 case (median follow-up duration 8 months, range 1–108 months, IQR 2–41

months). Finally, only 21% of medical records mentioned the possible source of infection, and no hypothesis could be formulated.

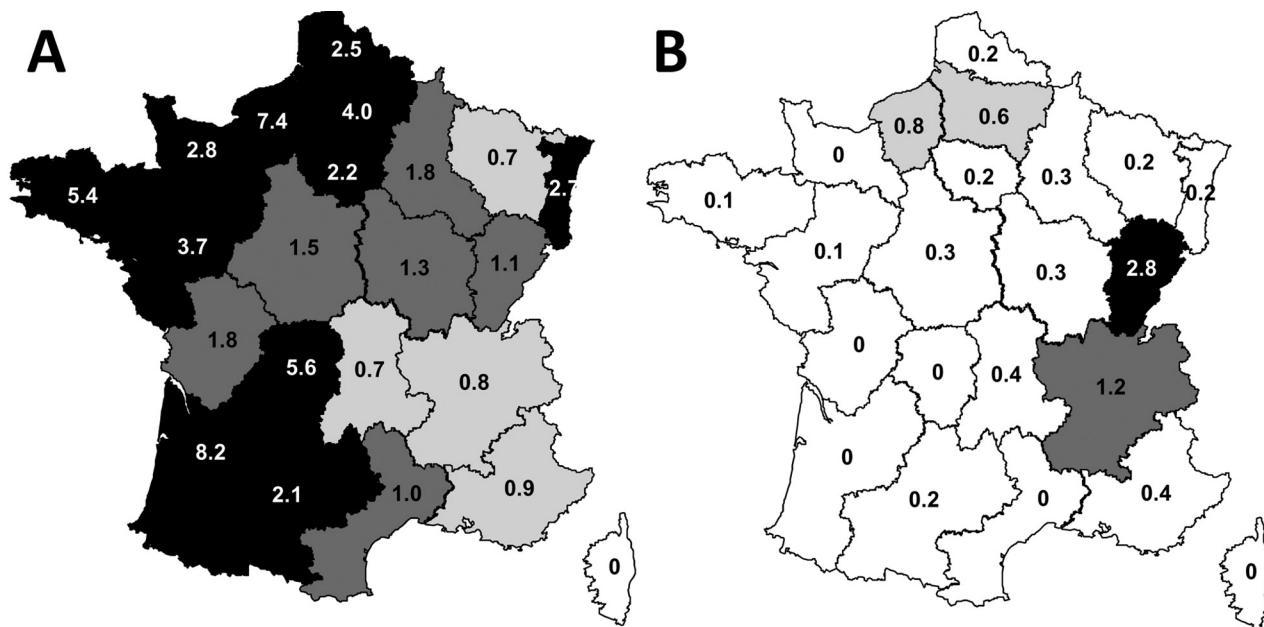
Genetic characterization showed that all O80:H2 strains of human origin collected in France carried the *stx2* genes and no *stx1* genes (online Technical Appendix Table 3). The *stx2* subtype could not be determined for the 2 strains (isolated in 2006 and in 2010) because they had lost their *stx2* gene despite preservation at –80°C. Among the remaining strains, 69% had a combination of *stx2* variants, *stx2c/2d* (62%) and *stx2a/2d* (7%); 31% harbored unique variants, *stx2a* (22%) and *stx2d* (9%). All strains had the intimin encoding gene *eae* and its variant *eae-ξ*, and 87% carried the enterohemolysin *ehxA* gene. All 57 strains shared ≥4 characteristic genes of the pS88 plasmid, *sitA*, *cia*, *hlyF*, and *ompT<sub>p</sub>*; 98% had the *iss* and *iroN* genes; 96% had the *cvaA* gene; and 61% had the *iucC* and *etsC* genes (online Technical Appendix Table 3).

Antimicrobial drug susceptibility testing revealed that most strains were multidrug resistant; rates of resistance were 91% for amoxicillin, 89% for nalidixic acid, 82% for cotrimoxazole, and 71% for kanamycin (online Technical Appendix Table 3). Overall, 52% of the strains were resistant to all 4 antibiotics.

To examine whether an animal could be the potential source of EHEC O80:H2, we solicited the Laboratoire National de Référence pour les *Escherichia coli*. Only 1 strain from an animal source (LNR-511-4, isolated from raw cow milk cheese) was available. This strain carried *eae-ξ*, *ehxA*, and *stx2a* genes and 7 genes associated with the pS88 plasmid related to ExPEC (*sitA*, *cia*, *iss*, *iroN*, *hlyF*, *cvaA*, and *ompT<sub>p</sub>*). This strain also was resistant to amoxicillin, kanamycin, and nalidixic acid.

To investigate the distribution in Europe of this emerging EHEC serogroup, several national reference laboratories in Europe were associated with this study and were asked to send us their available EHEC O80 strains. We obtained 5 strains from Spain, whereas Italy and Germany had no such strain in their collections. Among the 5 strains from Spain, 3 were of human origin (IH42632/03a, IH33264/07a, and IH102878/12a) and 2 were of animal origin (VTB-262 from a cow and FV4476 from a pig [the pig-origin strain was actually isolated in Slovakia]) (28). All 5 strains shared *eae-ξ*, but *stx2* (*stx2a*) was found in only 2 strains (both of human origin), and none had *ehxA*. Only the 3 human strains harbored most of the investigated genes associated with pS88. The contrast between animal strains and human strains from the national reference laboratory in Spain was also evident in their antibiotic-resistance profiles; only the human strains were multidrug resistant (online Technical Appendix Table 3).

To analyze the genetic diversity and genetic relatedness with other EHEC serogroups, all the O80:H2 strains



**Figure 2.** Regional 10-year cumulative incidence rates of hemolytic uremic syndrome cases caused by enterohemorrhagic *Escherichia coli* serotypes O157:H7 and O80:H2, France, January 2005–October 2014. A) Serotype O157:H7. B) Serotype O80:H2. White, <0.5 cases/100,000 children; light gray shading, 0.5–0.7 cases/100,000 children; medium gray shading, 0.8–0.9 cases/100,000 children; dark gray shading, 1–2 cases/100,000 children; black, >2 cases/100,000 children.

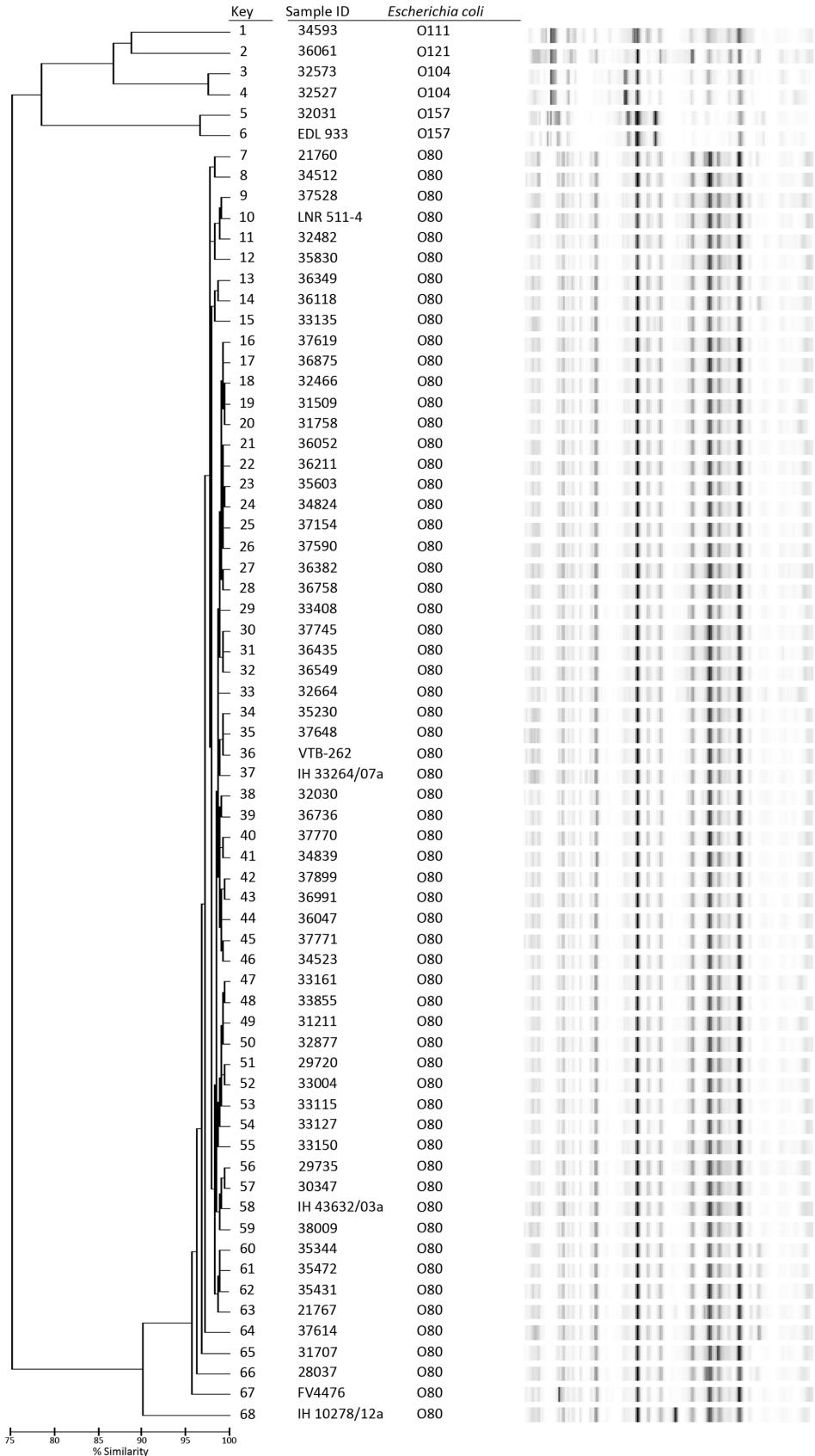
were analyzed by using rep-PCR and compared with some other serogroups (O157, O104, O111, and O121) (Figure 3). All EHEC O80:H2 strains shared 95% similarity except strain IH102878/12a from Spain (90% similarity). These data suggest that almost all EHEC O80 strains belong to a unique clonal group, regardless of their geographic origin and source (human or animal). EHEC O80:H2 strains were genetically distant to representative strains of serogroups O157, O104, O111, and O121.

To study the effect of antibiotics on Stx production in O80:H2 EHEC strains, we selected 4 representative strains (33115, 35344, 35431, and 36047) based on their genotypic and clinical characteristics (online Technical Appendix Table 3). Strain EDL933 (O157:H7) served concomitantly as the control. We examined susceptibility to the 3 antibiotics (Table). First, we estimated the basal production of Stx in different strains after 18 h of growth without antibiotics (Figure 4). The basal production among the different O80:H2 strains were comparable but significantly lower compared with that of strain EDL933, which produces  $\approx$ 100-fold more Stx than certain O80:H2 strains (35344 and 36047). We could not separately estimate the respective rates of Stx1 and Stx2.

For each strain, we examined the influence of the 3 antibiotics at concentrations below the MICs expressed as relative secretion of Stx compared with basal secretion (without antibiotics) after 18 hours' incubation period (Figure 5, panels A–E). Overall, azithromycin was responsible

for  $\geq$ 5-fold decreases of Stx production in all O80:H2 strains at a MIC of 0.5. As expected, the same effect was observed with the EDL933 strain. At all concentrations tested, ciprofloxacin significantly induced a major increase of Stx secretion for all strains except strain 35344. The increase was particularly marked for O80:H2 strains compared with O157 strains; a 100-fold increase was observed for 33115 and 35431 (Figure 5, panels B and C), compared with a 6-fold increase for EDL933 (Figure 5, panel E). Ceftriaxone, in contrast with other antibiotics, did not significantly alter Stx production except for 1 O80:H2 strain (Figure 5, panel C).

Finally, we wanted to determine whether the beneficial effect of azithromycin on Stx production would persist in the presence of 2 other antibiotics, a combination which might be used in cases of bacteremia. The combinations of antibiotics were tested on 2 O80:H2 strains (Figure 6). Azithromycin paired with ciprofloxacin significantly reduces Stx production compared with ciprofloxacin alone. However, this production was higher than that observed with azithromycin alone, and for both strains, Stx levels were higher for azithromycin/ciprofloxacin compared with no antibiotic. These data indicate that the macrolide might only partially inhibit the noxious effect of ciprofloxacin. The effect of azithromycin in combination with ceftriaxone depended on the strain tested. For strain 35344, the association slightly increased Stx production compared with ceftriaxone, whereas for strain 33115, the opposite was



**Figure 3.** Dendrogram obtained after DiversiLab genotyping analysis (based on PCR amplification of repeat sequences of DNA) of 56 enterohemorrhagic *Escherichia coli* (EHEC) O80 strains from humans in France compared with other isolates detected in France, Germany, and Spain, January 2005–October 2014. Other isolates include 1 animal-origin strain from France (LNR511-4, bovine, 2012); 5 animal- and human-origin isolates from Spain (FV4476, porcine; VTB-262, bovine; IH43632/03a, IH33264/07a, and IH 102878/12a, human); and 6 comparison strains from other serogroups (EDL933 and 32031, O157; 32527 and 32573, O104, isolated during a 2011 outbreak in Germany; 36061, O121; and 34593, O111). ID, identification.

**Table.** Antibiotic susceptibility of enterohemorrhagic *Escherichia coli* O80:H2 and O157:H7 isolates used in an antibiotics assay, France, January 2005–October 2014

Isolate (source)	Serotype	MIC, µg/mL		
		Azithromycin	Ciprofloxacin	Ceftriaxone
35344 (stool)	O80:H2	1	0.5	0.25
35431 (blood)	O80:H2	16	0.5	0.06
33115 (pancreas)	O80:H2	16	0.25	0.06
36047 (stool)	O80:H2	16	0.06	0.06
EDL933 (ground beef)	O157:H7	32	0.12	0.12

observed. However, in this experiment, the observed Stx rate was lower in ceftriaxone and azithromycin/ceftriaxone assays compared with basal secretion.

## Discussion

In this study, we described the emergence in France of a new virulent EHEC serotype, O80:H2, that harbors singular genetic characteristics of a hybrid STEC/ExPEC pathotype. Several important conclusions can be drawn.

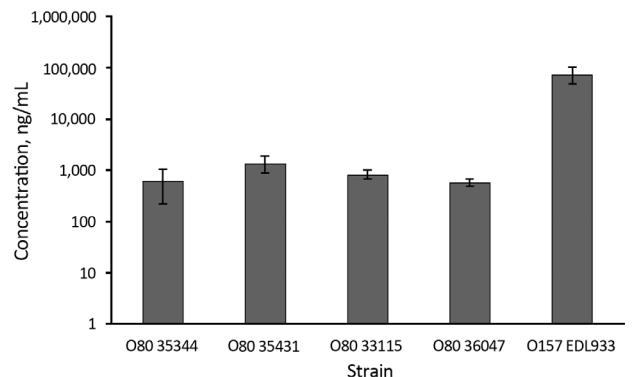
First, the EHEC O80:H2 strain appears to be at least as virulent as the EHEC O157:H7 strains present in France. Indeed, the rate of HUS-associated complications, such as renal injuries (28%) and death (4%), of the O80:H2 strain were comparable with that of O157:H7 (1,4). Moreover, EHEC O80:H2 isolates have the particular properties to induce invasive infections;  $\geq 2$  of the 53 patients affected had bacteremia or deep abscess. Extraintestinal infections very rarely are associated with EHEC; to our knowledge, only 6 cases of bacteremia have been previously described in the context of HUS (8). None of these 6 reports extensively searched for extraintestinal virulence factors. Whether the extraintestinal virulence of EHEC O80:H2 is related to the presence of genetic traits characteristic of an extraintestinal virulence-associated plasmid remains to be determined. Identification of the salmochelin-encoding genes (*iroN*) in 98% of the EHEC O80:H2 from France is of particular interest. This gene is clearly involved in the pathophysiology of *E. coli* bacteremia and meningitis (9,29).

Because the surveillance system for STEC is voluntary in France and because *stx*-specific PCR is performed only in cases involving diarrhea with HUS suspicion, accurate data are not available on the rates of diarrhea without HUS or of bloodstream infections caused by this serotype and others. Only cases of diarrhea occurring among HUS patient contacts are systematically investigated. Therefore, many cases of O80:H2-related diarrhea are probably undiagnosed, and we cannot draw any conclusion concerning the risk for HUS in cases of diarrhea associated with STEC O80:H2. Moreover, non-HUS cases of bacteremia caused by O80:H2 strains will escape detection.

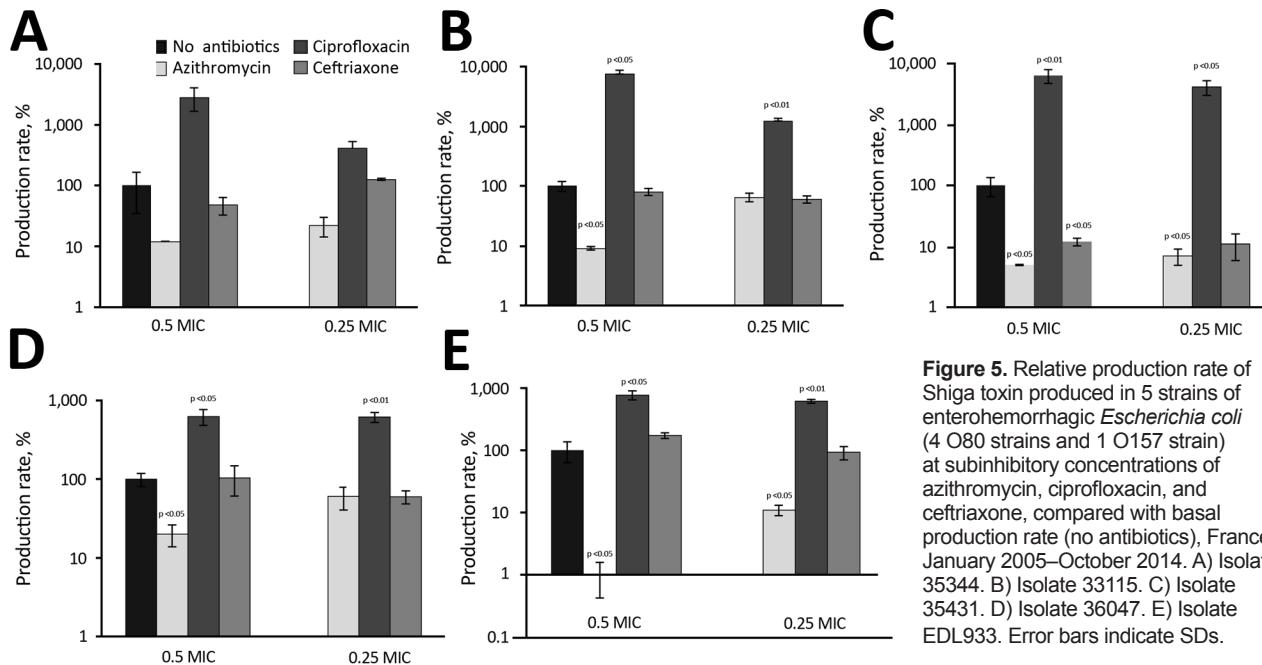
We were not able to identify the potential source of this emerging EHEC pathotype. All reported cases have been sporadic, and the possibility of foodborne infection remains speculative, even though O80:H2 strains were isolated from few animals. The highest incidence of O80:H2 infection was

observed in regions of France where EHEC O157 infections are not predominant, suggesting a possible atypical route or source of infection. Although Spain does not share borders with the high-incidence regions of France, it was the only country where a significant number of EHEC O80:H2 strains were found. Two O80:H2 EHEC strains from Spain (IH102878/12a and IH33264/07a) were very similar to French strains belonging to the same clonal group with a similar virulence genotype, suggesting a direct lineage between the isolates in Spain and France. As is usually observed for other serotypes, most of the infections with these strains occurred during summer and the beginning of autumn (4,7).

The molecular characterization of the EHEC O80:H2 strains was of particular interest. The presence in all strains from Spain and France of the very rare *eae*- $\xi$  gene combined with results of the genetic diversity study using rep-PCR strongly indicate that these strains, whatever their origin, have a common ancestor combining the O80:H2 serotype and an *eae*- $\xi$  gene containing LEE. On this unique genetic background, several genetic events occurred: the acquisition of  $\geq 1$  prophage encoding Stx, the presence of a plasmid similar to that found in ExPEC, and the presence of a plasmid encoding enterohemolysin. The chronology of these events remains speculative. However, acquisition of Stx2-encoding genes seems to remain particularly active because 3 different variants in  $\geq 4$  different modes (in combination or alone) were observed. The diversity of the extraintestinal virulence plasmidic genes



**Figure 4.** Mean concentrations (logarithmic scale) of Shiga toxin produced in the absence of antibiotics by selected strains of enterohemorrhagic *Escherichia coli* serotypes O80, France, January 2005–October 2014. O157 reference strain (EDL933) was used as control. Error bars indicate SDs.



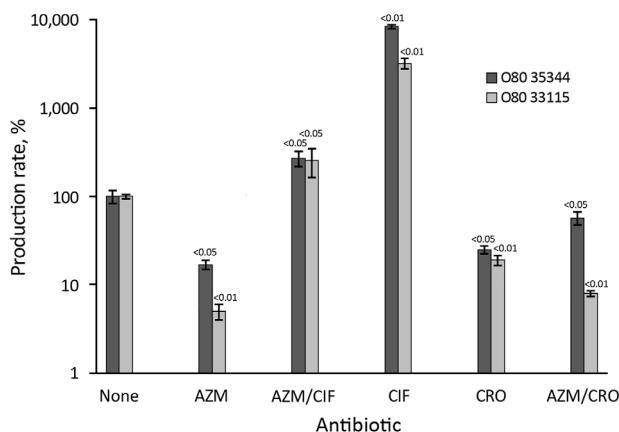
**Figure 5.** Relative production rate of Shiga toxin produced in 5 strains of enterohemorrhagic *Escherichia coli* (4 O80 strains and 1 O157 strain) at subinhibitory concentrations of azithromycin, ciprofloxacin, and ceftriaxone, compared with basal production rate (no antibiotics), France, January 2005–October 2014. A) Isolate 35344. B) Isolate 33115. C) Isolate 35431. D) Isolate 36047. E) Isolate EDL933. Error bars indicate SDs.

combination suggests the interplay of certain plasmidic determinants and intestinal pathogenic traits. The minimal combination of plasmidic genes common to all strains was the association of *ompT<sub>p</sub>* and *hlyF*, which might represent a beneficial influence on the intestinal pathogenic virulence of *E. coli* O80:H2. Chromosomal *ompT* and *hlyF* are involved in the secretion of outer membrane vesicles, which might serve as transporters of toxins and thus might boost the virulence of Stx-producing *E. coli* (30,31). Whether *ompT<sub>p</sub>* has a similar role remains to be further investigated.

Finally, clinical features and molecular characterization indicating the potential invasive pathogenicity of EHEC O80:H2 raise the question of which antibiotic should be used in such infections. Several clinical studies have suggested a deleterious effect of antibiotics during EHEC infection, leading to recommendations to not use such treatment (2). The major explanation of such an adverse effect is the induction of Stx secretion through the SOS response, which is stimulated by antibiotics such as fluoroquinolones in vitro and in experimental models (32,33). Several studies demonstrated that the effect of antibiotics on HUS depends on their class (27,33–37). Ciprofloxacin raises the production and release of Stx in vitro and is associated with a higher mortality rate in pigs (33). Other studies have shown that some antibiotics such as azithromycin might be associated with a decrease of the production and release of Stx in vitro (37) and with favorable outcomes in in vivo studies in piglets and mice (33,38). Finally, during a 2011 outbreak of EHEC O104 infection in Germany, a patient treated by ciprofloxacin plus imipenem unexpectedly had a better prognosis than all others (14). This result

suggests that response to antibiotics might also differ depending of the strain involved (34).

Our analysis of Stx production in the presence of antibiotics has clearly indicated that ciprofloxacin should not be used in cases of EHEC O80 infection. In contrast, azithromycin provided a beneficial in vitro effect and might be useful in cases of EHEC O80–associated diarrhea. These results are consistent with the systematic review by Agger et al. (16), who concluded that a protein synthesis inhibitor can be considered during EHEC infections when specific criteria are met. To our knowledge, only a few studies have



**Figure 6.** Relative production rate of Shiga toxin produced in 2 strains of enterohemorrhagic *Escherichia coli* O80 (isolates 35344 and 33115) at subinhibitory concentrations of azithromycin, ciprofloxacin, ceftriaxone (alone and in combination), compared to basal production rate (no antibiotics), France, January 2005–October 2014. AZM, azithromycin; AZM/CIF, azithromycin/ciprofloxacin; AZM/CRO, azithromycin/ceftriaxone; CIF, ciprofloxacin; CRO, ceftriaxone. Error bars indicate SDs.

tested the effect of antibiotics in combination (14,39). Our combination assay results would suggest that azithromycin plus ceftriaxone might be a reasonable choice in cases of systemic infection.

These findings should be regarded as preliminary and require confirmation. However, despite these promising *in vitro* results and because our assays were performed without measuring cytotoxicity, we cannot yet advocate the use of these antibiotics for treatment of patients infected with EHEC O80:H2. However, a planned national clinical trial in France (NCT02336516) to test the efficacy of azithromycin in children with postdiarrheal HUS might soon provide some answers.

In conclusion, a clonal group of EHEC O80:H2 strains of unknown origin and with the ability to induce invasive infections and lethality is emerging in France and represents a new therapeutic challenge. The interplay between intestinal and extraintestinal virulence factors in this new hybrid STEC/ExPEC pathotype remains to be elucidated. Azithromycin might be a possible option to prevent invasive infections caused by EHEC O80:H2, whereas azithromycin/ceftriaxone might be useful in treating such infections.

### Acknowledgments

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Dr. Soysal is a pediatrician working in Assistance Publique–Hôpitaux de Paris (AP-HP). Her research domain is pediatric infections, particularly pathogenicity and therapeutic management of *E. coli* intestinal infections.

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# Elevated Risk for Antimicrobial Drug-Resistant *Shigella* Infection among Men Who Have Sex with Men, United States, 2011–2015

Anna Bowen, Julian Grass, Amelia Bicknese, Davina Campbell, Jacqueline Hurd, Robert D. Kirkcaldy

*Shigella* spp. cause ≈500,000 illnesses in the United States annually, and resistance to ciprofloxacin, ceftriaxone, and azithromycin is emerging. We investigated associations between transmission route and antimicrobial resistance among US shigellosis clusters reported during 2011–2015. Of 32 clusters, 9 were caused by shigellae resistant to ciprofloxacin (3 clusters), ceftriaxone (2 clusters), or azithromycin (7 clusters); 3 clusters were resistant to >1 of these drugs. We observed resistance to any of these drugs in all 7 clusters among men who have sex with men (MSM) but in only 2 of the other 25 clusters ( $p<0.001$ ). Azithromycin resistance was more common among MSM-associated clusters than other clusters (86% vs. 4% of clusters;  $p<0.001$ ). For adults with suspected shigellosis, clinicians should culture feces; obtain sex histories; discuss shigellosis prevention; and choose treatment, when needed, according to antimicrobial drug susceptibility. Public health interviews for enteric illnesses should encompass sex practices; health messaging for MSM must include shigellosis prevention.

Shigellosis, the third most common human bacterial enteric infection in the United States, causes ≈500,000 illnesses each year (1). Because as few as 10 bacteria can cause infection, shigellosis outbreaks typically are large; control can be difficult unless interventions are implemented early in an outbreak (2). Although bloodstream infection is uncommon among immunocompetent hosts, shigellosis patients frequently are treated with antimicrobial medications to reduce illness duration and possibly transmission (3). High rates of resistance to ampicillin and trimethoprim/sulfamethoxazole have made ciprofloxacin, ceftriaxone, and azithromycin the preferred antimicrobial agents for adults and children with shigellosis; ceftriaxone is also the preferred treatment for invasive shigellosis (4–6). However, shigellae resistant to these drugs have emerged in the United States and abroad (7–17). Although shigellosis rates are highest for young children, most reports document

ciprofloxacin- or azithromycin-resistant shigellosis largely among men who have sex with men (MSM) (7,8,10,12–17). Estimates of risk for antimicrobial drug-resistant shigellae among different populations or by transmission route have not been reported. We investigated the associations between transmission route and antimicrobial resistance among US shigellosis clusters reported during 2011–2015.

## Methods

Because sexual transmission data are not captured in the national enteric disease surveillance systems of the Centers for Disease Control and Prevention (CDC), we queried the enteric disease cluster management database of the CDC Outbreak Response and Prevention Branch (ORPB; Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Disease) for all US shigellosis clusters during January 2011–December 2015 (18–21). This database is not part of a surveillance system; instead, it is used in real time to track and guide response to clusters of enteric infections, including shigellosis, that CDC investigates. It contains unpublished summaries of patient demographics, suspected or confirmed transmission route and vehicles, cluster onset date and duration, and pulsed-field gel electrophoresis (PFGE) patterns associated with the cluster. ORPB identifies shigellosis clusters for inclusion in this database in 3 ways: 1) when CDC's PulseNet observes a *Shigella* PFGE pattern approximately twice as often as baseline and in >1 US state or territory during a 60-day period, PulseNet assigns a cluster code to that pattern; 2) state health departments can choose to contact CDC about ongoing single-state or multistate clusters; 3) since 2014, CDC also has used data from the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS), which strives to test every 20th *Shigella* isolate nationally and 3 representative isolates from every shigellosis outbreak, to identify isolates harboring resistance to clinically important antimicrobials (22,23). CDC then queries PulseNet for other isolates with PFGE patterns indistinguishable from the resistant isolate. For *Shigella*, all identified clusters are added to the ORPB database.

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Our analysis comprised clusters with both antimicrobial drug susceptibility data in NARMS and documentation of transmission route or affected population. We linked clusters to NARMS by PulseNet cluster code or local outbreak code to obtain information about the antimicrobial resistance phenotype associated with the cluster. We used standard definitions for ciprofloxacin and ceftriaxone resistance and defined azithromycin resistance as an azithromycin MIC greater than that for wild-type *Shigella* (24). Among isolates with phenotypic azithromycin resistance, we used PCR to screen for the macrolide resistance genes *mphA* and *ermB*. We classified a cluster as resistant to  $\geq 1$  of these antimicrobial drugs if any isolate within the cluster harbored resistance to  $\geq 1$  of these drugs.

Few public health jurisdictions routinely collect the MSM status of shigellosis patients, but they might do so through telephone interviews, in-person interviews, medical record reviews, or links to sexual health databases. Public health jurisdictions did not use standardized definitions for MSM-associated transmission; however, we categorized transmission as MSM-associated if any MSM-associated transmission was recorded for a cluster. If subclusters with distinct transmission routes were reported within a single cluster, we analyzed the subclusters as separate clusters. Using Fisher exact test, we compared proportions of clusters with antimicrobial resistance by transmission category.

## Results

Of 49 clusters reported during 2011–2015, we excluded 19 (39%) because of insufficient data (transmission data but no antimicrobial resistance data, 5 clusters; neither transmission data nor antimicrobial resistance data, 14 clusters). Of the 30 (61%) remaining clusters, 2 encompassing subclusters with MSM-associated and other person-to-person transmission were further divided by transmission category, for a total of 32 clusters eligible for analysis (Table 1, <http://wwwnc.cdc.gov/EID/article/22/9/16-0624-T1.htm>). Suspected or confirmed transmission was reported to involve child care centers, camps, or schools (10 clusters); MSM (7 clusters); other person-to-person transmission (7 clusters); food (6 clusters); or recreational water (2 clusters). Nine clusters were caused by shigellae resistant to at

least 1 of the following: ciprofloxacin (3 clusters), ceftriaxone (2 clusters), or azithromycin (7 clusters). Three of these clusters were resistant to  $>1$  of these drugs. At least 1 isolate from each azithromycin-resistant cluster was confirmed to harbor the macrolide resistance genes *mphA* or *ermB*. Of the 9 antimicrobial drug-resistant clusters, 8 were caused by *S. sonnei* and 1 by *S. flexneri*. Isolates from all 7 MSM-associated clusters harbored resistance to  $\geq 1$  of these drugs (Table 1). The prevalence of resistance among MSM-associated and other clusters significantly differed for ceftriaxone ( $p = 0.04$ ), azithromycin ( $p < 0.001$ ), azithromycin and either ciprofloxacin or ceftriaxone ( $p = 0.007$ ), and any of these 3 drugs ( $p < 0.001$ ) (Table 2). The proportion of MSM-associated clusters with resistance to any of these 3 drugs (i.e., ciprofloxacin, ceftriaxone, or azithromycin) was 3–77 times greater, and the proportion with resistance to azithromycin was 3–500 times greater, than the proportion of other clusters with such resistance phenotypes. None of 10 clusters associated with child care, camps, or schools was caused by ciprofloxacin-, ceftriaxone-, or azithromycin-resistant strains.

## Discussion

All shigellosis clusters that we identified among MSM in the United States during 2011–2015 were caused by strains resistant to  $\geq 1$  of the preferred antimicrobial agents for shigellosis. Although our sample was small, the estimated prevalence of resistance to preferred antimicrobial drugs for MSM-associated shigellosis clusters was 3–77 times the prevalence for clusters with nonsexual transmission routes. Although shigellosis with these antimicrobial drug resistance phenotypes has been documented among MSM internationally, the reasons for this association are unknown (7,8,12–14,16,17). Additional studies are needed to elucidate these findings; identify specific risk factors; understand clinical outcomes for patients infected with these resistant strains and in the setting of HIV infection; and develop effective interventions to prevent infection of MSM with shigellae, particularly drug-resistant shigellae.

Although our results suggest that shigellae with resistance to ciprofloxacin, ceftriaxone, or azithromycin circulate predominantly among MSM in the United States, these

**Table 2.** Differences in antimicrobial resistance phenotype by transmission route among clusters of *Shigella* infection, United States, January 2011–December 2015\*

Antimicrobial resistance phenotype	MSM-associated transmission, no. (% , 95% CI†), n = 7	Transmission other than MSM-associated, no. (% , 95% CI†), n = 25	p value‡
CIP	2 (29, 5–67)	1 (4, 0.2–18)	0.1
CRO	2 (29, 5–67)	0 (0, 0–11)	0.04
AZM	6 (86, 47–99)	1 (4, 0.2–18)	<0.001
AZM, CIP, or CRO	7 (100, 65–100)	2 (8, 1.3–24)	<0.001
AZM and either CIP or CRO	3 (43, 12–78)	0 (0, 0–11)	0.007

\*AZM, azithromycin; CIP, ciprofloxacin; CRO, ceftriaxone; MSM, men who have sex with men.

†Mid-p exact 95% CI of the percentage resistant.

‡By 2-tailed Fisher exact test.

strains are likely to emerge among other populations. Efforts to facilitate improved hygiene practices among persons at high risk for shigellosis or at high risk for transmitting shigellosis to others (e.g., child care attendees, staff, and parents; marginally housed persons; international travelers; and food handlers) are needed now to limit transmission when multidrug-resistant *Shigella* strains inevitably begin circulating among these populations.

Although the associations we found between antimicrobial drug-resistant shigellosis and transmission route (i.e., MSM-associated transmission vs. other transmission) are striking, this analysis has several limitations. Because the ORPB cluster management database is used to guide response rather than as a formal reporting system, it is likely to contain only a small fraction of shigellosis clusters, and the details about each cluster are not always complete. Furthermore, many public health jurisdictions do not routinely perform PFGE on shigellae, making it difficult to detect clusters and, when a cluster is detected, to locate all cases associated with the cluster. Therefore, clusters included in this analysis might not be representative of all US shigellosis clusters, and the number of cases reported per cluster is likely to be smaller than reality. Additionally, nearly 40% of clusters were not analyzed because of missing data. However, the selection of clusters in this analysis is unlikely to have biased the association between antimicrobial drug resistance phenotype and transmission route. Next, both antimicrobial drug resistance phenotypes and transmission routes are likely to be heterogeneous within each cluster. We also lacked information about patients' antecedent exposure to antimicrobial drugs, HIV infection, and other factors that might enhance our findings, and we did not have access to clinical treatment or outcome data. Finally, because of the small sample size, we cannot reliably compare the prevalence of resistance among MSM-associated clusters and other clusters. Nonetheless, the markedly higher prevalence of such resistance observed in our study is concerning and warrants future study.

The increasing use of PCR-based, culture-independent diagnostic tests for *Shigella* will make it difficult to identify cases and clusters of multidrug-resistant shigellosis and to provide laboratory-informed antimicrobial treatment (25). When shigellosis is suspected or when a culture-independent diagnostic test suggests *Shigella* infection, clinicians should culture fecal specimens and test *Shigella* isolates for antimicrobial drug susceptibility, including susceptibility to azithromycin (24). Patients who do not improve with treatment should be re-cultured. Further characterizing isolates by PFGE or whole-genome sequencing will assist health departments with cluster detection and control.

Alternative treatment options are limited for persons infected with ciprofloxacin-, ceftriaxone-, or azithromycin-resistant shigellae, making shigellosis prevention critical.

Clinicians should counsel all patients with suspected shigellosis about meticulous handwashing and hygiene (26). Among adult patients, sexual histories focused on sex of recent partners and specific sex practices can inform empiric treatment and indicate a need for further testing and counseling to prevent sexual transmission of shigellosis (27). If treatment is required, drug choice should be informed by the results of antimicrobial susceptibility testing. During public health interviews for enteric illnesses, routinely collecting sex of sex partners and, among those with multiple recent partners, where patients find partners would improve understanding of risk groups and permit more targeted interventions. Surveillance and interventions for shigellosis among MSM would be strengthened by more intensive collaboration between enteric disease and sexually transmitted disease programs within public health departments. Health messaging for MSM should include information about risk for and prevention of shigellosis and drug-resistant shigellosis (26,27).

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A.B. had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, study concept and design, drafting of the manuscript, statistical analysis, and study supervision. All authors contributed to data acquisition, analysis, or interpretation.

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# *Borrelia miyamotoi*–Associated Neuroborreliosis in Immunocompromised Person

Katharina Boden, Sabine Lobenstein,  
Beate Hermann, Gabriele Margos, Volker Fingerle

*Borrelia miyamotoi* is a newly recognized human pathogen in the relapsing fever group of spirochetes. We investigated a case of *B. miyamotoi* infection of the central nervous system resembling *B. burgdorferi*–induced Lyme neuroborreliosis and determined that this emergent agent of central nervous system infection can be diagnosed with existing methods.

The tickborne relapsing fever spirochete *Borrelia miyamotoi* was described in 1994 but only recently was recognized as a human pathogen (1). This spirochete is present in *Ixodes* ticks across the Northern Hemisphere, comparable to the distribution of *B. burgdorferi* sensu lato, but reported cases are still rare (2,3). Thus far, only 2 cases of central nervous system infection have been reported, both with a chronic course and both in immunocompromised persons (4,5). We investigated a case of *B. miyamotoi* infection that was initially diagnosed as seronegative early Lyme neuroborreliosis (LNB) that could be attributed to *B. miyamotoi* only by molecular–biological methods.

## The Case

On July 31, 2015, a 74-year-old woman with a history of non-Hodgkin lymphoma (follicular type, stage IV) came to the Burgenlandkreis Hospital (Naumburg, Germany). She reported dizziness, vomiting, and a headache of 5 days' duration. She had been treated for her lymphoma with cyclophosphamide, doxorubicin, vincristine, and prednisone with rituximab during May–August 2012. Thereafter, she received rituximab maintenance therapy (375 mg/m<sup>2</sup> every 8 weeks) until November 2014. The monoclonal antibody rituximab that targets the CD20 antigen expressed by B cells leads to anti-CD20–mediated B-cell depletion. This treatment, together with the underlying hematologic disease, resulted in an immunocompromised condition. The patient reported 2 tick bites while in

Elsteraue municipality (Saxony-Anhalt, Germany) during June and July 2015 but had no recent travel history. On examination, she had slight neck stiffness but no other notable findings.

A lumbar puncture was performed to assess the patient for viral or lymphomatous meningitis. Cerebrospinal fluid (CSF) showed a pleocytosis of 70 leukocytes/mL (reference 0–5 leukocytes/mL) and elevated protein at 1,718 mg/L (reference 150–400 mg/L); albumin quotient (Qalb) 34.8% (reference <9%), lactate 5.58 mmol/L (1.2–2.1 mmol/L), and glucose quotient 0.45 (>0.5); and an intrathecal IgM synthesis of 18%. The greatly elevated lactate and highly increased Qalb and protein made a viral cause unlikely. Pappenheim staining of a cytopspin preparation of CSF revealed a mixed cell population (32% polymorphonuclear leukocytes, 61% lymphocytes, and 7% monocytes) with heterogeneous morphology of lymphocytes that did not suggest lymphomatous meningitis. Serum and CSF were negative for *B. burgdorferi*–specific antibodies by the chemiluminescent immunoassay LIASON (DiaSorin, Vercelli, Italy). However, because the CSF constellation was typical for LNB, including the slight intrathecal IgM synthesis (6), the patient was treated with 2 intravenous ceftriaxone (2,000 mg 1×/d for 3 wks).

To exclude early tuberculous meningitis, we examined the CSF for mycobacteria by PCR, culture, and staining. To further substantiate the LNB diagnosis, we determined the B-cell attractant chemokine CXCL13 from CSF. The measured value of 1,155 pg/mL was clearly higher than the minimum concentration of 250 pg/mL, indicating neuroborreliosis and thus supporting our working diagnosis of LNB (7). After 5 days of treatment, the patient recovered fully. A second lumbar puncture after 7 days of treatment revealed a stable count of 76 monomorphic lymphocytes/μL. In addition, protein, Qalb, and CXCL13 had decreased (Table). However, we remained unable to detect *B. burgdorferi*–specific antibodies in serum or CSF, and *B. burgdorferi*–specific PCR of the first CSF specimen showed no amplification product.

To conclusively identify the pathogen, we tested the first CSF and serum samples by PCR for panbacterial 16S rRNA, with a positive result from CSF only. Using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) in GenBank, we found, to our surprise, that the sequence was identical to that for *B. miyamotoi*. Moreover,

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**Table.** CSF findings of *Borrelia miyamotoi* meningitis cases and patients with Lyme neuroborrelioses\*

Finding (reference)	Case (reference)			Lyme neuroborreliosis(6)†
	New Jersey, USA (4)	Netherlands (5)	Germany (this study)	
Leukocytes/ $\mu$ L (0–5 cells/ $\mu$ L)	65	388	70	170.5 [57.0–369]
Differential count	23% PMNC, 70% lymphocytes, 6% monocytes, 1% diverse	60% mononuclear cells	32% PMNC, 61% lymphocytes, 7% monocytes	
Protein level, mg/dL (150–400 mg/dL)	>300		1718	1,232 [697–1,926]
Qalb, $\times 10^3$ (<9)			34.8	17.2 [9.7–28.4]
Quantitative IgM, $\times 10^3$			18.1	Elevated in 70%
Glucose, mmol/L (2.2–4.2 mmol/L)	1.8	1.6	2.41	
Glucose ratio (>0.5)			0.45	
Lactate, mmol/L (1.2–2.1 mmol/L)			5.58	$\geq 3.5$ in 4%‡
Routine microscopy		Cellular CSF with high nos. of granulocytes and plasma cells	Cellular CSF with heterogeneous morphology	
CXCL13, pg/mL (100 to <250 pg/mL, borderline)			1,150; 8 d after start of therapy: 186	>415 (7)
Spirochetes visible in CSF by	Gram staining, Giemsa staining	Darkfield microscopy	Acridinorange staining, Pappenheim cytopsin	Typically negative

\*Blank cells indicate value not determined or was within reference range. CSF, cerebrospinal fluid; PMNC, polymorphonuclear leukocytes; Qalb, albumin quotient.

†n = 118 patients. Values are median [interquartile range].

‡Associated with headache.

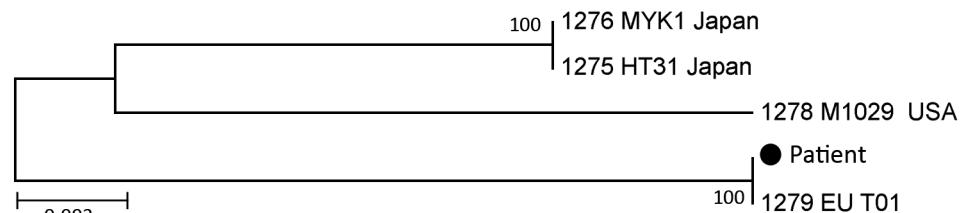
sequences of 16S rRNA, *flaB*, and 6 housekeeping genes revealed the European type of *B. miyamotoi* (Figure 1; details of molecular phylogenetic analysis and GenBank accession numbers in online Technical Appendix, <http://www.wnc.cdc.gov/EID/article/22/9/15-2034-Techapp1.pdf>).

*B. miyamotoi* is one of the *Borrelia* species that causes relapsing fever. The spirochetes of the relapsing fever *Borrelia* group are more easily detectable than *B. burgdorferi* spirochetes from blood and CSF by microscopy and PCR. We therefore reexamined the first Pappenheim-stained cytopsin preparation from CSF, but no spirochetes were recognizable. To increase the sensitivity, we restained the preparation with acridine orange. A few spirochetes were then microscopically visible in the preparation using an Axioskop (Zeiss, Germany) (Figure 2).

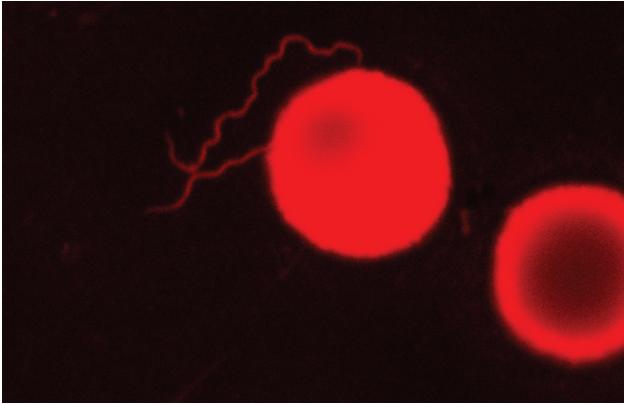
## Conclusions

The genus *Borrelia* is divided into 2 groups: *B. burgdorferi* sensu lato, which causes Lyme disease, and a group of species that cause relapsing fever. *B. miyamotoi* belongs to the second group and was first described in 2011 as a human pathogen in several patients from Russia who had an unspecific febrile illness where few patients experienced febrile relapses, which is the typical sign for relapsing fever (1). *B. miyamotoi* was also reported to have caused meningoencephalitis in a patient from New Jersey, USA (4), and a patient from the Netherlands (5). As with the patient we report, these patients had a history of non-Hodgkin lymphoma with recent rituximab treatment. Because of the increasing indications for rituximab treatment (10) and a prevalence of *B. miyamotoi* in *Ixodes ricinus* ticks in

**Figure 1.** Molecular phylogenetic analysis of *Borrelia* strain from cerebrospinal fluid of a 74-year-old immunocompromised woman in Germany (black dot) conducted by using 6 multilocus sequence typing genes (*clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *rplB*) of *Borrelia miyamotoi*. The



sequences obtained from the patient sample clustered with *B. miyamotoi* strain EU\_T01 from Europe, retrieved from the PubMLST *Borrelia* database (<http://pubmlst.org/borrelia/>). The phylogenetic relationship of the sample analyzed was inferred by using DNA sequences of chromosomal housekeeping genes. The maximum-likelihood method based on the general time reversible model (8) was applied. The tree with the highest log likelihood (–5531.9051) is shown. The percentage of trees in which the associated taxa clustered is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms (<http://bionj.org/about-bionj/>) to a matrix of pairwise distances estimated by using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale; branch lengths are measured in the number of substitutions per site. The analysis involved 5-nt sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The final dataset contained 3,642 positions. Evolutionary analyses were conducted in MEGA5 (9).



**Figure 2.** *Borrelia miyamotoi* in cerebrospinal fluid stained by acridine orange (LSM Exciter 5, Zeiss, Germany). The cerebrospinal fluid was from a 74-year-old woman with non-Hodgkin lymphoma. Original magnification  $\times 1,000$ .

Europe of up to 3.2% (2), clinicians should be aware that cases of *B. miyamotoi* neuroborreliosis may increase (4).

Detection of specific antibodies is a useful complementary diagnostic method. Only recently it was shown that assays based on the GlpQ protein, which is present in relapsing fever *Borrelia* but absent in *B. burgdorferi* sensu lato, might be useful in diagnosing *B. miyamotoi* infection (2,3). Moreover, up to 90% of PCR-confirmed *B. miyamotoi* infections were positive in a *B. burgdorferi* enzyme immunoassay, although only a few by 2-tiered testing (reviewed in 3). For the case we report, we had no access to a GlpQ assay, and the standard serologic tests for Lyme borreliosis were negative, as it was for the 2 published reports (4,5). However, a clinician relies on combining history, clinical examination, and routine CSF analyses. Clinicians should therefore be aware of possible *B. miyamotoi* neuroborreliosis, especially in patients who have a history of non-Hodgkin lymphoma and recent rituximab treatment. The clinical picture seems to be unspecific. Although the patient we report had acute symptoms (dizziness, vomiting, and headache) of short duration, the other published cases had decline in mental status (slow cognitive processing, memory deficits), and disturbed gait developing gradually over several months. All 3 *B. miyamotoi* cases had CSF pleocytosis with elevated CSF protein concordant with CSF changes found in LNB (Table). We even found slight intrathecal IgM synthesis, as is seen in 70% of patients with LNB (6). However, a viral cause must be excluded, especially when CSF protein is only slightly elevated.

In recent years, CXCL13 has been identified as a potentially sensitive and specific biomarker for diagnosing acute LNB. It was a useful indicator in our investigation and might be a suitable indicator for diseases caused by spirochetes in general, as was already shown for *B. burgdorferi* and *Treponema pallidum* (7). Furthermore, the

amounts of this chemokine might be used to assess the success of therapy because it declined rapidly in the patient we report after therapy began (Table). In contrast to the lack of sensitivity of culture and PCR for detecting *B. burgdorferi* in CSF, *B. miyamotoi*, as a member of the relapsing fever spirochete group, was detectable by microscopy and by PCR in the CSF in this patient, as well as in the 2 previously reported (4,5). Darkfield microscopy or staining with acridine orange might be required to increase sensitivity. In all 3 cases, PCR targeting the panbacterial 16S rRNA followed by sequencing showed the causative species. Our report of *B. miyamotoi* infection in a patient from Germany indicates that this emergent agent of central nervous system infection can be diagnosed by using existing methods if clinicians are aware of it.

### Acknowledgments

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S.L. managed the patient. K.B., B.H., and V.F. were involved in the diagnostic testing. G.M. performed the molecular biological investigations. All authors contributed to writing the article.

Dr. Boden is a physician at the University Hospital Jena and specializes in clinical chemistry, laboratory medicine, and medical microbiology. Her research interests include infectious diseases of the central nervous system and zoonotic pathogens.

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## May 2015: Vectorborne Infections Including:

- Transmission of Hepatitis C Virus among Prisoners, Australia, 2005–2012
- Recent US Case of Variant Creutzfeldt-Jakob Disease—Global Implications
- Novel Thogotovirus Associated with Febrile Illness and Death, United States, 2014
- Detecting Spread of Avian Influenza A(H7N9) Virus Beyond China
- Pathologic Changes in Wild Birds Infected with Highly Pathogenic Avian Influenza (H5N8) Viruses, South Korea, 2014
- Itaya Virus, a Novel Orthobunyavirus Associated with Human Febrile Illness, Peru
- Isolation of *Onchocerca lupi* in Dogs and Black Flies, California, USA
- Molecular Epidemiology of *Plasmodium falciparum* Malaria Outbreak, Tumbes, Peru, 2010–2012
- Delayed-Onset Hemolytic Anemia in Patients with Travel-Associated Severe Malaria Treated with Artesunate, France, 2011–2013
- Protective Antibodies against Placental Malaria and Poor Outcomes during Pregnancy, Benin
- The Fourth Case of Variant Creutzfeldt-Jakob Disease in the United States: Global Implications
- Canine Distemper in Endangered Ethiopian Wolves
- Comparative Sequence Analyses of La Crosse Virus Strain Isolated from Patient with Fatal Encephalitis, Tennessee, USA
- Low-level Circulation of Enterovirus D68–associated Acute Respiratory Infections, Germany, 2014
- Rapid Emergence of Highly Pathogenic Avian Influenza Subtypes from a Subtype H5N1 Hemagglutinin Variant
- Postmortem Stability of Ebola Virus
- Malaria Imported from Ghana by Returning Gold Miners, China, 2013

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**EMERGING  
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# Persistent *Bacillus cereus* Bacteremia in 3 Persons Who Inject Drugs, San Diego, California, USA

Gabrielle Schaefer, Wesley Campbell, Jeffrey Jenks, Cari Beesley, Theodoros Katsivas, Alex Hoffmaster, Sanjay R. Mehta, Sharon Reed

*Bacillus cereus* is typically considered a blood culture contaminant; however, its presence in blood cultures can indicate true bacteremia. We report 4 episodes of *B. cereus* bacteremia in 3 persons who inject drugs. Multilocus sequence typing showed that the temporally associated infections were caused by unrelated clones.

*Bacillus* species are typically considered blood culture contaminants, and distinguishing true versus pseudobacteremia requires recognition of the clinical context. Risk factors for infection include prosthetic heart valves, pacemakers, injection drug use, and immunosuppression (1). In 2013 in San Diego, California, USA, 3 persons who inject drugs (PWIDs) were diagnosed with persistent *B. cereus* bacteremia. To determine if there was a common source of infection, we performed multilocus sequence typing (MLST) of *B. cereus* from these patients.

## The Study

In September 2013, a 19-year-old woman (patient 1) sought care in the emergency department (ED) of the UC San Diego Medical Center, reporting a 2-day history of headache, fever, myalgia, nausea, vomiting, diarrhea, and right upper quadrant pain. Two days earlier she had injected heroin, using a clean needle and a previously used cotton filter. At admission, she had a fever (100°F), increased heart rate (112 beats/min), and right upper quadrant tenderness but no evidence of endocarditis. Test results showed leukocytosis ( $26.0 \times 10^9$  cells/L) and elevated levels of aspartate aminotransferase (107 U/L), alanine aminotransferase (112 U/L), and C-reactive protein (CRP; 6.5 mg/dL). Results for rapid HIV antibody test, acute hepatitis panel, and urine pregnancy test were negative. Intravenous (IV) fluids and

empiric IV vancomycin and piperacillin/tazobactam were administered; symptoms and clinical status were improved the next day. Cultures (4/4) of blood samples collected at admission grew *B. cereus* sensitive to clindamycin, gentamicin, and vancomycin. Transthoracic echocardiography showed no abnormalities.

Over the next 2 days, she remained afebrile and symptoms resolved, but bacteremia persisted. On hospital day 5, given the persistent bacteremia, we stopped piperacillin/tazobactam and started IV ciprofloxacin. Gentamicin was added on days 8–12 for its potential synergistic effect; bacteremia resolved on day 9. IV vancomycin and ciprofloxacin were continued for 4 weeks.

Fifteen days after patient 1 was admitted, a 43-year-old man (patient 2) sought care at the same institution for low back pain. He had used heroin 2 days earlier; injection technique details are unknown. At admission, he was afebrile and had vital signs and blood cell counts within the normal range, no focal neurologic findings, and unrevealing magnetic resonance imaging results. The patient was discharged with a diagnosis of musculoskeletal back pain. After *B. cereus* grew in 1 of 2 blood cultures, he was asked to return. Although asymptomatic and lacking any stigmata of endocarditis, he was admitted for further observation and started empirically on IV vancomycin. Blood cultures continued to grow clindamycin- and vancomycin-sensitive *B. cereus* until hospital day 4. Transesophageal echocardiography showed no evidence of endocarditis. IV vancomycin was continued for 2 weeks.

In October 2013, a 58-year-old man (patient 3) sought care in the ED of an affiliated hospital, the VA San Diego Healthcare System, for debilitating abdominal pain. He had a prosthetic right hip and history of chronic polyarticular pain. He admitted previous injection of crushed oxycodone but denied recent injections. Laboratory results showed leukocytosis ( $21.2 \times 10^9$  cells/L) and a high CRP level (36 mg/dL). Abdominal computed tomography imaging showed circumferential ileal wall thickening, and endoscopy showed bleeding and friable ulcerations. Blood cultures (6/8) grew gram-positive bacilli. Vancomycin and piperacillin/tazobactam were initiated, but piperacillin/tazobactam was changed to oral levofloxacin once *B. cereus* was identified. Transthoracic echocardiography showed a non-mobile aortic valve vegetation, suggesting endocarditis. Blood cultures remained *B. cereus*-positive over 6 days of

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**Table.** Multilocus sequence typing results for *Bacillus cereus* isolates from 3 persons who inject drugs, San Diego, California, USA

Patient no.	Alleles							Sequence type
	<i>glpF</i>	<i>gmk</i>	<i>ilvD</i>	<i>pta</i>	<i>pur</i>	<i>pycA</i>	<i>tpi</i>	
1	13	8	46	28	9	12	7	100
2	105	61	95	92	92	77	68	247
3								
2013 isolate	172	71	14	29	24	14	7	787
2015 admission isolate	12	8	9	14	11	12	10	24
2015 discharge isolate	12	8	9	14	11	12	10	24

therapy, so gentamicin was added for 7 days, followed by 6 weeks' treatment with only vancomycin and levofloxacin. Crohn disease was diagnosed based on imaging findings.

In January 2015, patient 3 returned to the ED for a pulmonary syndrome, which was diagnosed as bronchitis and treated with azithromycin. Blood sample cultures (2/2) grew *B. cereus*. The patient was afebrile and had no clinical findings. His leukocyte count was within reference range, and CRP was marginally elevated (0.77 mg/dL). He admitted to resuming injection of crushed oxycodone with unused paraphernalia because of increasing right hip pain. Transthoracic echocardiography at admission showed no evidence of the previous vegetation or any new lesions, and results of a tagged leukocyte scan were negative, suggesting that neither the prior vegetation nor the prosthetic hip was the source of the new bacteremia. After 7 days of observation and multiple negative blood cultures, the patient was discharged without antimicrobial drug therapy. However, 1 of 4 cultures of blood samples obtained on the day of discharge grew *B. cereus*, so he returned to the ED, where physical examination showed no abnormalities and laboratory test results were negative. The patient refused admission and was discharged on a 6-week course of oral ciprofloxacin with outpatient follow-up. He denied use of injection drugs while an inpatient or after discharge.

To determine if isolates from the 3 patients were related, we performed MLST to characterize 7 alleles and their phylogenetic lineage based on sequence type (2,3), using the *B. cereus* MLST website (<http://pubmlst.org/bcereus/>). Isolates from each patient were genetically distinct; however, the 2 isolates from the most recent admission of patient 3 were clonal but different than his 2013 isolate (Table). These findings reinforced the presumption of a true bacteremia and that a prolonged course of antimicrobial drugs was warranted for patient 3. The persistent bacteremia from 2013 and the clonal isolates from 2015 demonstrate 2 unique episodes of infection probably associated with injection of crushed oxycodone as the common risk factor.

We identified 4 episodes of *B. cereus* bacteremia in 3 PWIDs. Although often considered a blood culture contaminant, *B. cereus* can indicate true bacteremia in the appropriate clinical context. *B. cereus* bacteremia has previously been reported in PWIDs (4), and cultures of heroin samples and injection drug use paraphernalia have been predominantly positive for *Bacillus* species (4–6). In

another study, spore-forming bacteria (*B. cereus*, *B. anthracis*, *Clostridium botulinum*) were shown to contaminate heroin (7). Endocarditis caused by *B. cereus* has also been documented in PWIDs (8).

A 2014 epidemiologic review of *B. cereus* bloodstream infections identified 51 cases meeting Infectious Disease Society of America guidelines for true bacteremia (9). The review showed increased rates of environmental isolation of *B. cereus* in summer, possibly due to a higher environmental load of *B. cereus* during this season. In line with our clinical experience, the findings from that single-center study showed no clonality, supporting the assumption that contamination may be multiclonal (9). Given that multiclonality would be identified in the clinical setting only if colonies differed in appearance, it is possible that the reported cases of bacteremia might also have been multiclonal.

Although there are no established treatment guidelines, most *B. cereus* species produce  $\beta$ -lactamases and thus show resistance to  $\beta$ -lactam antimicrobial drugs. Sensitivity to other antimicrobial drugs has been studied, but variation in in vitro methods demonstrates inconsistencies with susceptibility cutoffs (10). *B. cereus* is considered susceptible to aminoglycosides, carbapenems, and fluoroquinolones, and newer antimicrobial drugs (linezolid, daptomycin, telavancin) may have activity against *B. cereus* (10–12); however, the true effect of the in vitro MICs on clinical outcomes is unclear. To achieve clearance of bacteremia, 2 of the 3 patients in our study required the addition of gentamicin (an aminoglycoside) to their treatment regimen. At follow-up, all 3 patients were clinically well without recurrence of bacteremia.

## Conclusions

Identification of genetically distinct isolates of *B. cereus* in these temporally associated cases is consistent with findings from previous work and highlights the environment as the source of these cases of bacteremia. However, given the potential for *B. cereus* to cause serious disease, isolation of this organism should prompt a complete clinical evaluation.

## Acknowledgment

We thank Keith Jolley for development of the *B. cereus* MLST website, which is housed at the University of Oxford and was funded by the Wellcome Trust.

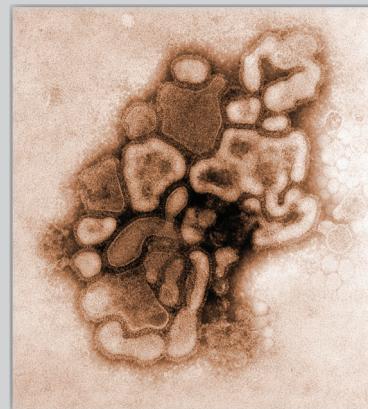
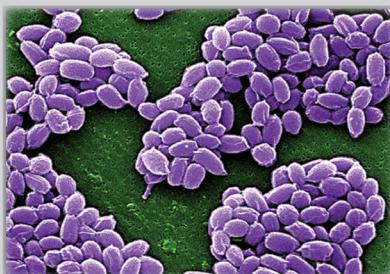
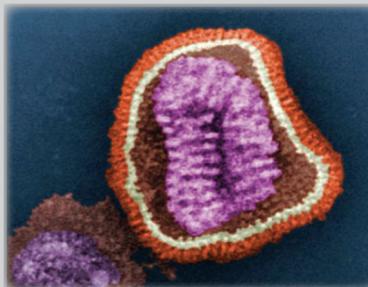
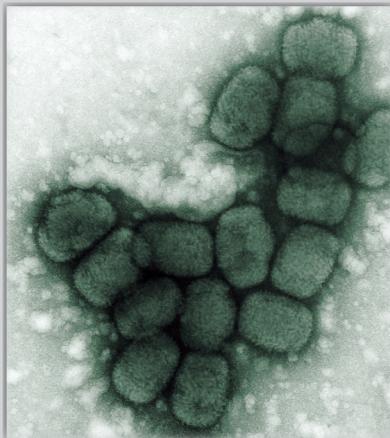
Dr. Schaefer is a resident physician in Internal Medicine at the University of California, San Diego Health. Her field of interest is hospital-acquired infections.

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# Trends in Pneumonia Mortality Rates and Hospitalizations by Organism, United States, 2002–2011<sup>1</sup>

Brandon A. Wuerth, John P. Bonnewell,  
Timothy L. Wiemken, Forest W. Arnold

Because the epidemiology of pneumonia is changing, we performed an updated, population-based analysis of hospitalization and case-fatality rates for pneumonia patients in the United States. From 2002 to 2011, hospitalization rates decreased significantly for pneumonia caused by pneumococcus and *Haemophilus influenzae* but increased significantly for *Pseudomonas* spp., *Staphylococcus aureus*, and influenza virus.

Pneumonia is the leading cause of infection-related deaths in the United States, with potential for severe complications such as respiratory failure and sepsis (1). A recent nationwide study noted that prior studies may have overestimated a temporal reduction in mortality rate (2). As pneumonia epidemiology has changed, interest in following epidemiologic trends continues, particularly for the various etiologic organisms. Emergence of influenza A(H1N1)pdm09 virus has highlighted the role of influenza viruses as etiologic agents of pneumonia (3).

The epidemiology of pneumonia is constantly changing because of advances in preventive measures, diagnostic testing, and novel therapies. Although the epidemiology, by organism, of pneumonia in hospital patients was recently clarified (4), our study also considered the effects of influenza virus and included regional information. Our objective was to provide an updated population-based analysis of hospitalized pneumonia patients to determine the major etiologic agents and associated hospitalization rates, case-fatality rates, and patient demographic differences (age, sex, and region).

## The Study

In a retrospective cohort study, we examined hospitalizations of pneumonia patients using discharge data from the publicly available Nationwide Inpatient Sample (NIS) database (5). The University of Louisville Institutional Review Board did not require a review because the project did not meet the common rule definition of human subjects' research. We completed Healthcare Cost and Utilization Project Data Use Agreement Training.

The study included patients  $\geq 18$  years of age discharged with a principal diagnosis of pneumonia according to standards of the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM). We also included those with a principal diagnosis of sepsis or respiratory failure and a secondary diagnosis of pneumonia. Only ICD-9-CM codes for which the numbers of an organism were substantial were analyzed: pneumococci, *Klebsiella* spp., *Pseudomonas* spp., *Haemophilus influenzae*, *Staphylococcus aureus*, and influenza virus. We followed US Census Bureau definitions in classifying cases by US region (6).

The NIS database contains an  $\approx 20\%$  stratified sample of US community hospitals. From that sample, the Agency for Healthcare Research and Quality calculated national estimates by weighting each discharge on the basis of hospital location, teaching status, bed number, and ownership control, relative to all US hospitals, which permitted us to estimate the number of US hospitalizations. For each patient discharged, we recorded age, sex, region, principal and secondary diagnoses (up to 15), and whether patient had died from any cause. Population estimates were obtained from the US Census Bureau (7).

Primary outcomes were temporal trends in hospitalization and case-fatality rates for pneumonia based on infecting organism as determined by ICD-9 coding. We then calculated the rate for hospitalizations caused by each organism using the number of hospitalizations from the NIS database as the numerator and US Census Bureau population estimates as the denominator. Discharge codes do not specify where the pneumonia was acquired, and although hospitalization rate was used, this does not necessarily indicate community-acquired cases. We calculated the all-cause case-fatality rate for each organism similarly, using the number of deaths.

We extracted data from the NIS using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA) and calculated adjusted odds ratios for age ranges, sex, and region using multivariate logistic regression. Study period differences were determined with the online  $z$  test calculator of the Healthcare Cost and Utilization Project ( $p < 0.05$  was significant) (8). Other descriptive statistics were completed with Excel 2013 (Microsoft Corp., Redmond, WA, USA).

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**Table 1.** Hospitalization rates (hospitalization/100,000 population) for 6 causative agents, by sex and region, United States, 2002–2011

Category	Rate (adjusted odds ratio)					
	Pneumococcus, n = 379,400	<i>Klebsiella</i> spp., n = 109,515	<i>Pseudomonas</i> spp., n = 330,302	<i>Haemophilus</i> <i>influenzae</i> , n = 80,281	<i>Staphylococcus</i> <i>aureus</i> , n = 575,573	Influenza virus, n = 270,237
Overall	12.6	3.6	11.0	2.7	19.2	9.0
Sex						
M	13.0 (1)	4.1 (1)	12.3 (1)	2.7 (1)	20.5 (1)	8.0 (1)
F	12.3 (0.68)	3.2 (0.57)	9.7 (0.57)	2.6 (0.72)	17.9 (0.64)	10.0 (0.90)
Region						
Northeast	11.9 (1)	3.1 (1)	8.8 (1)	2.1 (1)	15.9 (1)	8.8 (1)
Midwest	15.1 (1.33)	3.1 (1.05)	11.5 (1.37)	3.3 (1.61)	17.7 (1.17)	12.8 (1.51)
South	12.8 (1.16)	4.6 (1.65)	13.0 (1.62)	2.8 (1.46)	22.5 (1.57)	9.0 (1.10)
West	10.7 (1.32)	3.0 (1.48)	9.1 (1.55)	2.3 (1.64)	17.9 (1.71)	5.5 (0.90)

*S. aureus* was the most commonly identified organism that caused pneumonia, at 19.2 cases/100,000 population (Table 1). Pneumococcus and influenza virus were more frequent causes in the Midwest, *Klebsiella* and *Pseudomonas* spp. were more frequent causes in the South, and *H. influenzae* and *S. aureus* were more frequent agents in the West.

Hospitalization rates, based on etiologic agent, changed during the study period (Figure 1). Overall, the rate for pneumonia hospitalizations caused by pneumococcus decreased 23% ( $p < 0.001$ ) and for *H. influenzae*, 42% ( $p < 0.001$ ). For *Klebsiella*, the rate increased 35% ( $p < 0.001$ ); for *Pseudomonas*, 23% ( $p < 0.001$ ); for *S. aureus*, 23% ( $p < 0.001$ ); and for influenza virus, 132% ( $p < 0.001$ ).

Case-fatality rate was highest for pneumonia caused by *S. aureus*: 15.6 deaths/100 cases (Table 2). Pneumococcal pneumonia, *Klebsiella*, *Pseudomonas*, and *S. aureus* were more frequent in the Northeast, whereas pneumonia caused by *H. influenzae* and influenza virus was more frequent in the West.

Overall case-fatality rates, based on causal agent (Figure 2), decreased for pneumococcus by 18% ( $p = 0.01$ ), for *Pseudomonas* spp. by 8% ( $p = 0.29$ ), for *H. influenzae*

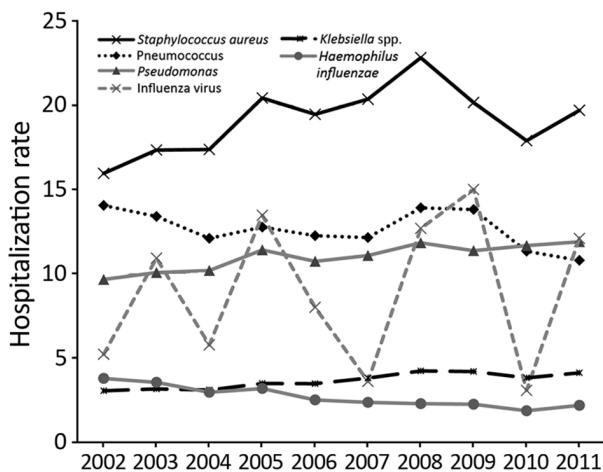
by 3% ( $p = 0.85$ ), and for *S. aureus* by 32% ( $p < 0.001$ ). Rates increased for *Klebsiella* spp. by 13% ( $p = 0.26$ ); and for influenza virus by 67% ( $p < 0.001$ ). Hospitalization and case-fatality rates by age are shown in online Technical Appendix Tables 1, 2 (<http://wwwnc.cdc.gov/EID/article/22/9/15-0680-Techapp1.pdf>).

## Conclusions

We found that, from 2002 to 2011, the hospitalization rates for pneumonia caused by *Klebsiella* spp., *Pseudomonas* spp., *S. aureus*, and influenza virus increased, and those caused by pneumococcus and *H. influenzae* decreased. The case-fatality rate of influenza virus–caused pneumonia increased, while rates of pneumococcus–caused and *S. aureus*–caused pneumonia decreased. Hospitalization and case-fatality rates were higher for men (especially elderly) than for women.

Our results coincide with those of previous studies, which found that *S. aureus*, pneumococcus, and *Pseudomonas* spp. comprised a large percentage of all pneumonia cases (9,10). Our results also coincide with those of a similar study which used the NIS database during 1993–2011: a decrease in pneumonia caused by *Streptococcus* spp., *H. influenzae*, and *Pseudomonas* spp. and an increase in pneumonia caused by *Staphylococcus* spp. (4). However, our study also examined trends in pneumonia caused by *Klebsiella* spp. and influenza virus and regional variations. Another study that used similar methods found that adjusted odds ratio of an inpatient death from pneumonia decreased from 2002 to 2005 (11). Previous studies also found that male patients  $\geq 75$  years old carried the greatest disease prevalence and considerably greater hospitalization rates (185/10,000 population in 2006) (12,13).

US regional data are limited, and comparable results, based on clinical data, were not found. However, 2 sets of data from the Centers for Disease Control and Prevention (Atlanta, GA, USA) showed the highest incidence of pneumonia in the Midwest and the lowest in the West (12,14). Drivers of geographic differences could include variations in coding practices between regions and vaccination recommendation adherence (15).



**Figure 1.** Hospitalization rates (hospitalizations/100,000 population) for patients with pneumonia for 6 causative agents, United States, 2002–2011.

**Table 2.** All-cause case-fatality rate (deaths/100 cases for 6 causative agents, by sex and region, United States, 2002–2011)

Category	Rate (adjusted odds ratio)					
	Pneumococcus, n = 25,112	<i>Klebsiella</i> spp., n = 15,687	<i>Pseudomonas</i> spp., n = 39,975	<i>Haemophilus</i> <i>influenzae</i> , n = 3,935	<i>Staphylococcus</i> <i>aureus</i> , n = 89,540	Influenza virus, n = 9,417
Overall	6.6	14.3	12.1	4.9	15.6	3.5
Sex						
M	6.8 (1)	15.4 (1)	12.6 (1)	5.1 (1)	16.6 (1)	3.8 (1)
F	6.5 (0.91)	12.9 (0.81)	11.5 (0.87)	4.7 (0.86)	14.4 (0.83)	3.3 (0.84)
Region						
Northeast	8.1 (1)	20.5 (1)	15.3 (1)	5.8 (1)	18.9 (1)	3.9 (1)
Midwest	5.6 (0.67)	12.0 (0.53)	9.8 (0.62)	3.8 (0.64)	13.1 (0.67)	3.3 (0.83)
South	6.4 (0.82)	12.4 (0.56)	11.7 (0.75)	4.8 (0.86)	14.9 (0.78)	3.3 (0.92)
West	7.2 (0.91)	16.2 (0.76)	13.2 (0.85)	5.9 (1.06)	16.9 (0.89)	3.8 (1.07)

Known changes in the epidemiology of pneumonia over the study period include the emergence of influenza A(H1N1)pdm09 virus, which caused 60.8 million cases of influenza and 12,469 deaths in 2009–2010 alone (3). We found that patients with influenza virus infections had the highest hospitalization rate during 2009. Many previous studies excluded influenza because it likely represents a separate clinical entity and the hospitalization rate can be difficult to interpret using calendar years (because early or late influenza seasons can skew yearly trends) (2,11).

Our study was limited by its reliance on ICD-9-CM coding to identify cases, potentially allowing misclassification. ICD-9-CM coding is based on discharge data, so determining whether the patient was diagnosed with pneumonia at admission or during hospitalization is not possible. Thus, we could not differentiate between community- or hospital-acquired cases. In addition, we did not examine unspecified cases of pneumonia that did not include an etiologic organism, the most commonly used pneumonia code. Decreases in hospitalization rates and case-fatality rates could be explained by improved outpatient management or increased rates of vaccination. Last, case-fatality rates were calculated by using deaths during

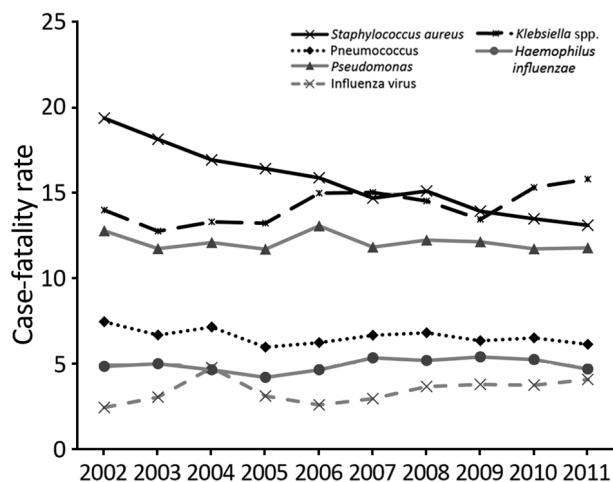
the inpatient stay and not beyond, because coding did not permit 30-day outcomes.

In conclusion, knowing which etiologic agents are increasing or have the highest case-fatality rate is critical because treatment options and prognosis vary by organism, and resources must be allocated accordingly. Knowing patient demographic characteristics for each organism is also essential, to clarify the populations at greatest risk.

Dr. Wuerth is currently a third-year internal medicine resident at the Medical University of South Carolina in Charleston, South Carolina. He plans to pursue a fellowship in gastroenterology with a research interest in inflammatory bowel diseases.

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**Figure 2.** All-cause case-fatality rate (deaths/100 cases) for patients with pneumonia for 6 causative agents, United States, 2002–2011.

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# Reduction of Healthcare-Associated Infections by Exceeding High Compliance with Hand Hygiene Practices

Emily E. Sickbert-Bennett, Lauren M. DiBiase,  
Tina M. Schade Willis, Eric S. Wolak,  
David J. Weber, William A. Rutala

Improving hand hygiene from high to very high compliance has not been documented to decrease healthcare-associated infections. We conducted longitudinal analyses during 2013–2015 in an 853-bed hospital and observed a significantly increased hand hygiene compliance rate ( $p < 0.001$ ) and a significantly decreased healthcare-associated infection rate ( $p = 0.0066$ ).

The association between hand hygiene and infection prevention has long been known (if not always fully accepted) since the time of Semmelweis (*1*). The challenge in healthcare settings is to achieve and sustain high compliance among many disciplines of personnel who interact with patients and their environment. We investigated whether an improvement in hand hygiene compliance from a baseline high level ( $>80\%$ ) to an even higher level ( $>95\%$ ) could lead to decreases in healthcare-associated infections (HAI).

## The Study

In October 2013, University of North Carolina Hospitals, an 853-bed facility, implemented a new hand hygiene program (Clean In, Clean Out; <http://news.unchealthcare.org/empnews/handhygiene>) in all inpatient areas, after a successful pilot implementation of the program in the pediatric intensive care unit (*2*). Key features were that the focus for observation was simply on cleaning hands upon entering and leaving patient rooms and that all healthcare personnel (including physicians, advanced practice providers, nurses, nursing assistants, hospital unit coordinators, housekeeping, radiology, occupational/physical/recreational therapists, nutrition and food services staff, phlebotomists, and respiratory therapists) were asked to make observations and provide immediate feedback to each other (*3*). Previously,

infection preventionists and designated nursing staff on each inpatient unit performed covert observations of hand hygiene compliance according to Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) indications for hand hygiene (*1*), and compliance reports by location were disseminated quarterly. Comprehensive surveillance for device-associated and non-device-associated HAI was assessed by 4 infection preventionists according to CDC National Healthcare Safety Network case definitions and included all hospital locations and all infections.

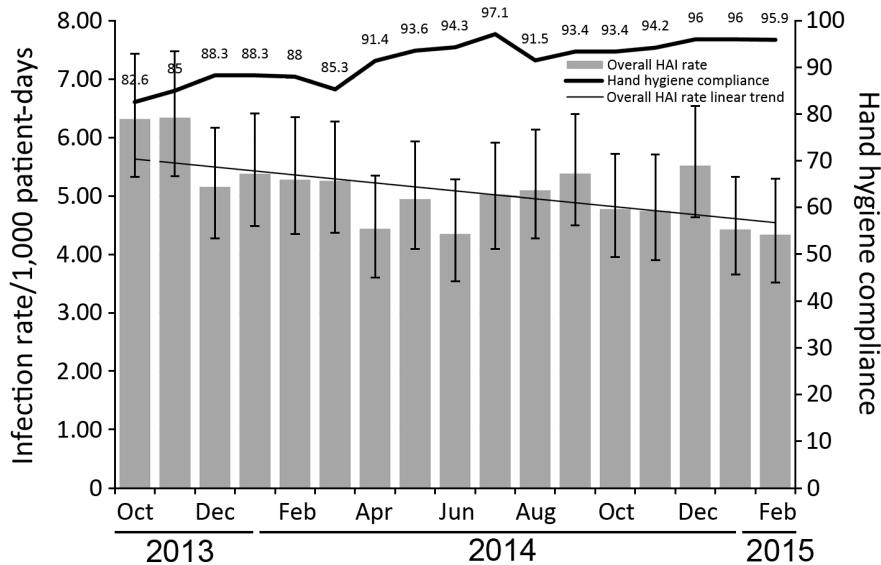
We compared hand hygiene compliance data from the last quarter of the covert observations by infection preventionists and designated nursing staff to compliance data from the first month of the new program by using a  $\chi^2$  test. Hand hygiene compliance data were collected at the unit level, and hospital-wide estimates were obtained by averaging all reporting units, weighted by patient-days for each respective unit. We also used a  $\chi^2$  to compare the average historical HAI rate from January 2013 until the implementation of the new program in October 2013 to the average HAI rate during the study period of October 2013–February 2015, after implementation of the new program.

We examined overall longitudinal hand hygiene compliance rates and HAI rates during the new program by using generalized linear models to describe overall trends. To examine the association between HAI and hand hygiene compliance, we used Poisson regression using generalized estimating equations with an exchangeable working correlation matrix using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). We used data for hand hygiene compliance and number of overall HAI, HAI with multidrug-resistant organisms (MDRO), and healthcare-associated *Clostridium difficile* infection (HA-CDI) from each nursing unit to estimate the overall association between hand hygiene and HAI rates. An offset of patient-days was used to account for varying levels of time at risk for each unit and month.

During the 17-month study period,  $>4,000$  unique observers made  $>140,000$  observations under the new hand hygiene program. We noted a significant increase in overall hand hygiene compliance rate ( $p < 0.001$ ) and a significantly decreased overall HAI rate ( $p = 0.0066$ ), supported by 197 fewer infections (Figure) and an estimated 22 fewer deaths (*4*). These reductions resulted in an overall savings of US  $\approx$ \$5 million (*5*).

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**Figure.** Overall healthcare-associated infection (HAI) rate and hand hygiene compliance by month, October 2013–February 2015. Numbers above data bar indicate monthly compliance percentages. Error bars indicate 95% CIs.

The association between hand hygiene compliance and HAI, adjusting for unit-level data, showed a 10% improvement in hand hygiene, associated with a 6% reduction in overall HAI ( $p = 0.086$ ). The association between hand hygiene compliance and HA-CDI, adjusting for unit-level data, showed a 10% improvement in hand hygiene, associated with a 14% reduction in HA-CDI ( $p = 0.070$ ). No association was noted between hand hygiene compliance and MDRO infections ( $p = 0.7492$ ).

Hospital-wide hand hygiene compliance measurements by using the previous method (covert observation by designated staff) in the final measurement quarter were not statistically different than in the first month of compliance data measured by all staff in the new program ( $p = 0.7503$ ). In addition, the average HAI rate in the 9 months before implementation of the new program was not statistically different ( $p = 0.542$ ) from the average HAI rate during the 17-month study period after implementation.

When the CDC Hand Hygiene Guideline was published in 2002, hand hygiene compliance was summarized on the basis of then-current studies to be very low (average 40%, range 5%–81%) (1). Investigators have demonstrated reductions in HAI and MDRO infections when compliance increased from low to medium levels (48% to 66%) (6). More recently, hospital epidemiologists and infection preventionists have worked to achieve and sustain higher compliance by using shared accountability, incentives, and feedback strategies (7), but until now, no analysis has demonstrated whether an improvement in hand hygiene from a baseline high level (>80%) to an even higher level (>95%) would lead to hospital-wide decreases in HAI (8). Demonstrating the importance of continuously improving hand hygiene compliance is critical for staff and hospital leaders who may underestimate the impact on HAI.

Hand hygiene compliance measurements have been studied and methods have been proposed to alleviate concerns associated with interobserver variation, sampling bias, and the Hawthorne effect (9). We overcame these concerns by simplifying the compliance measurement to only evaluate the opportunities that cover most (~87%) of the World Health Organization–defined “Five Moments” on the basis of a 24-hour validation video surveillance of activity in patient rooms; that is, 21% of episodes before patient contact, 22% of episodes after touching a patient, and 44% of episodes after touching patient surroundings (10). Furthermore, by engaging all hospital staff in measuring hand hygiene compliance, all opportunities of the hygiene program were eligible opportunities for measurement. In this way, the Hawthorne effect was a consistent presence that became the main intervention for achieving improvement. Finally, the finding that our previous hand hygiene compliance rates measured by trained, designated staff was not statistically different than the compliance rates from the beginning of the new program further supports that the new compliance metric was not affected by any new, unanticipated measurement bias.

Although we cannot eliminate the possibility that other infection prevention factors were also associated with a decreased HAI rate, no other specific hospital-wide infection prevention goals were adopted during the time period of this analysis. The associations (and absence thereof) we found with hand hygiene and specific types of infections are biologically plausible. Absence of association between MDRO HAI and hand hygiene is understandable because many MDRO infections occur in patients who may be colonized before admission, have invasive devices, and are at increased risk for becoming infected with their own flora. However, *C. difficile* infections in healthcare facilities are

predominantly spread through contact with infected patients or a contaminated environment, then carried on the hands of healthcare personnel. Therefore, a weak association between HA-CDI reduction and hand hygiene improvement is plausible. Although we adjusted the hand hygiene compliance data by patient-days, some units had patients at much lower risk for infections (e.g., psychiatric units). Despite including units of varying risk for HAI, we demonstrated that increased hand hygiene compliance improvements from already high rates can be an important strategy for achieving infection reductions, particularly for healthcare-associated *C. difficile* infections.

### Conclusions

A program designed to improve hand hygiene compliance among hospital staff successfully engaged all healthcare personnel in monitoring and improving their own hand hygiene compliance. This pursuit of excellence for hand hygiene compliance led to substantial HAI reductions hospital wide.

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We thank Judie Bringham, Rebecca Brooks, Sherie Goldbach, Lisa Teal, Katherine Schultz, Elizabeth Walters, and David Williams for support of Clean In, Clean Out throughout the facility. We thank Tracy Spears for her assistance with statistical analysis.

Dr. Sickbert-Bennett is associate director of hospital epidemiology at UNC Health Care and research assistant professor of medicine/infectious diseases and epidemiology at UNC Chapel Hill. Her research interests are in healthcare-associated infections, surveillance, and hand hygiene.

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# Nosocomial Outbreak of Parechovirus 3 Infection among Newborns, Austria, 2014

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In 2014, sepsis-like illness affected 9 full-term newborns in 1 hospital in Austria. Although results of initial microbiological testing were negative, electron microscopy identified picornavirus. Archived serum samples and feces obtained after discharge were positive by PCR for human parechovirus 3. This infection should be included in differential diagnoses of sepsis-like illness in newborns.

Parechoviruses are small, nonenveloped, single-stranded RNA viruses belonging to the family *Picornaviridae*. Although most human parechovirus (HPeV) infections cause self-limiting mild respiratory or gastrointestinal symptoms, HPeV type 3 (HPeV3) has been found in 5%–13% of newborns and young infants <3 months of age with late-onset sepsis or encephalitis (1–12). Knowledge of HPeV3 infections originates from single cases or small series of sporadic unrelated infections. One considerable outbreak, affecting ≈200 infants with obviously community-acquired diseases, was recently reported from Australia (1,2). In contrast, we describe a timely confined, and apparently nosocomial, outbreak of HPeV3 infection originating from 1 maternity ward in Austria, affecting one fifth of newborns hospitalized during that period.

## The Study

During August 5–September 7, 2014, a total of 9 newborns, 2–27 (median 5) days of age, showed signs of sepsis-like illness (fever and reduced general condition) and were admitted to the Department of Paediatrics and Adolescent Medicine, Medical University of Graz, Graz, Austria. All had been delivered within 2 weeks in the same obstetric unit at the Regional Hospital Feldbach, which is located in a small town (4,500 inhabitants) in southeastern Styria,

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a region of southern Austria of which Graz is the capital. During these 2 weeks, 44 newborns had been delivered at this obstetric unit (total 1,400/y); 9 (20.5%) born during these 2 weeks became symptomatic (Table; Figure). Diagnostic procedures were performed at the discretion of each attending physician and comprised testing for adenovirus, enterovirus, norovirus, rotavirus, herpesvirus 1, herpesvirus 2, varicella zoster virus, Epstein-Barr virus, cytomegalovirus, human herpesvirus 6, and parvovirus B19. However, no causative agent was identified.

Because the only pediatric department in southern Styria (including Feldbach) is at the Medical University of Graz, all severely ill newborns or infants are admitted there. However, no additional cases of sepsis or sepsis-like illness of unclear etiology were identified in newborns or young infants from that area during these months. The facts that the outbreak was temporally confined and all affected patients had been nursed in the same ward strongly indicated a common source within this unit. Investigations to detect a possible common source in the affected obstetric unit comprised anamnestic and clinical examination of hospital staff members and the newborns' mothers, surface swabbing (e.g. nursery rooms, baby baths, examination beds, diaper changing tables), microbiological examinations of formula, and analysis of staff roster and occupancy plans. However, a presumed common source or causative agent could not be identified.

To further seek the causative pathogen, we analyzed serum, urine, and nasal secretions from 4 patients (nos. 4, 6, 7, 8; Figure) by negative staining in a transmission electron microscope (Zeiss 906, Oberkochen, Germany) at 80 kv. Although nasal secretions revealed no particles, serum and urine of 2 of the tested patients (nos. 6, 8) contained icosahedral particles, diameter 20–30 nm, resembling picornaviruses. Because of the young ages of the patients, we assumed that the particles were HPeVs. However, by the time we received the electron microscope results indicating picornaviruses, all affected patients had been discharged. We therefore requested fecal samples from all 9 discharged patients for molecular diagnostics of HPeV. We received samples from 4 patients (nos. 5, 6, 7, 9) a median of 29 days (range 9–34) after discharge.

RNA was isolated by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For HPeV detection, we used a real-time reverse transcription PCR (RT-PCR) selective for the 5' non-translated region as described (13,14). Molecular typing of

**Table.** Clinical characteristics for 9 newborns with human parechovirus 3 infection, Austria, 2014\*

Characteristic	No. (%) patients	Median (range)†
<b>Demographic</b>		
Male sex	5 (55.6)	NA
Gestational age, wk + d	NA	40 + 1 (38 + 3 to 41 + 2)
Birth weight, g	NA	3,480 (3,240–4,160)
Vaginal delivery	6 (66.7)	
Age at onset of fever, d	NA	6 (1–27)
Age at admission to PD, d	NA	6 (2–27)
Time from discharge from maternity to admission to PD, d	NA	1 (0–24)
<b>Clinical</b>		
Fever	9 (100)	39.1°C (38.5°C–39.9°C)
Reduced general condition	4 (44.4)	NA
Tachypnea	4 (44.4)	NA
Tachycardia	3 (33.3)	NA
Drinking difficulties	5 (55.6)	NA
Circulatory centralization	3 (33.3)	NA
Increased irritability	3 (33.3)	NA
<b>Treatment</b>		
Admission to NICU or PICU	3 (33.3)	NA
Antimicrobial drug treatment, cefuroxime + amoxicillin	8 (88.9)	3 (2–5) d
Intravenous hydration	4 (44.4)	NA
Length of stay at PD, d	NA	3 (0–9)
<b>Laboratory values</b>		
Leukocytes, cells/ $\mu$ L	NA	7,690 (4,750–18,310)
Granulocytes, cells/ $\mu$ L	NA	4,595 (1,300–10,124)
Lymphocytes, cells/ $\mu$ L	NA	2,330 (590–7,100)
Lymphopenia, <2,000 cells/ $\mu$ L	4 (44.4)	NA
Monocytes, cells/ $\mu$ L	NA	1,100 (660–2,470)
Minimal thrombocytes/ $\mu$ L	NA	200,000 (101,000–270,000)
C-reactive protein, mg/L‡	NA	1.6 (<0.6–11.4)
C-reactive protein >5 mg/L	2 (22.2)	NA
Procalcitonin, ng/mL§	NA	0.18, 0.18, and 0.36
Interleukin-6, pg/mL¶	NA	21.0 and 124.8

\*NA, not applicable; NICU, neonatal intensive care unit; PD, pediatric department; PICU, pediatric intensive care unit.

†Unless otherwise indicated.

‡Upper limit of reference range = 5.0 mg/L.

§Upper limit of reference range = 0.5 ng/mL; measured for 3 patients.

¶Upper limit of reference range = 7.0 pg/mL; measured for 2 patients.

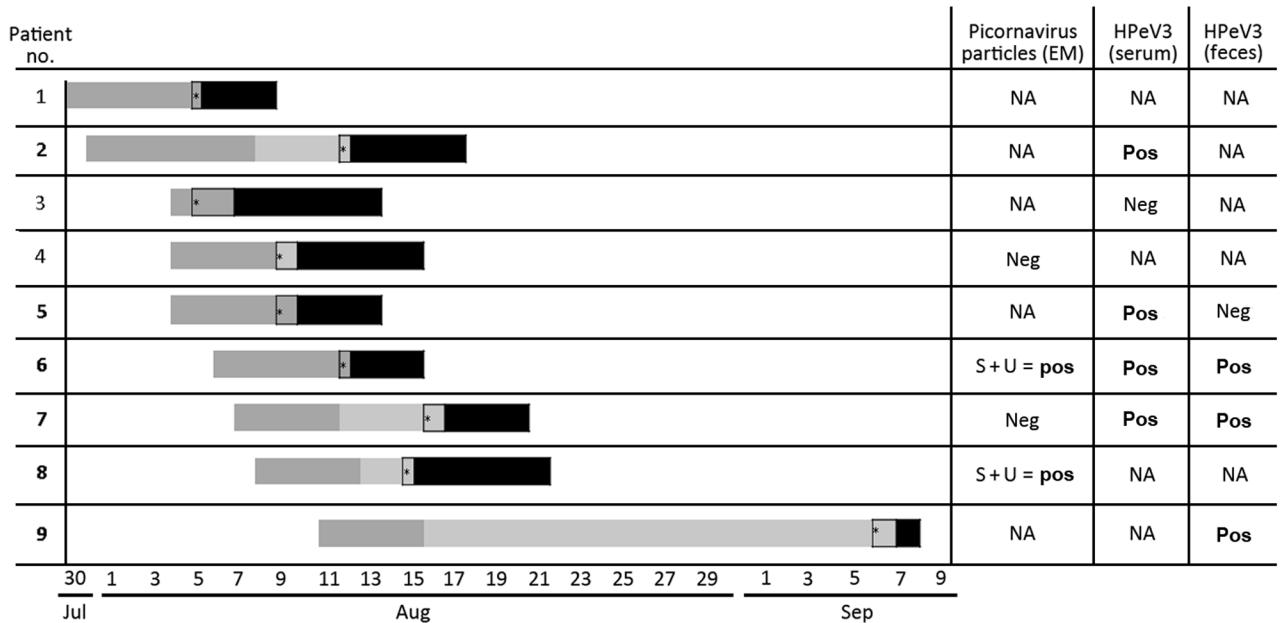
positive samples was conducted by nested real-time RT-PCR and sequencing of the partial capsid viral protein (VP) 3/VP1 region by using primers described by Harvala et al. (15). We performed reverse transcription and first-round amplification by using a One-Step RT-PCR Kit (QIAGEN). Nested amplification was performed by using a HotStarTaq Master Mix Kit (QIAGEN). The resulting fragment ( $\approx$ 300 bp) was subsequently sequenced by using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems/Life Technologies, Darmstadt, Germany) and nested PCR primers. Sequences were aligned to reference sequences in GenBank by using a BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) algorithm (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/9/15-1497-Techapp1.pdf>).

Of the 4 fecal samples tested, 3 were positive for HPeV. Molecular typing that used partial VP3/VP1 capsid protein regions revealed HPeV3 in all 3 samples. To analyze the presence of this pathogen even during acute illness, we retrieved archived serum samples from 5 patients (nos. 2, 3, 5, 6, 7; Figure). No samples had been retained for 3 other patients. For 4 of the 5 patients tested, archived serum samples were positive for HPeV-3, indicating

systemic infection with HPeV3 during the symptomatic phase of disease. Comparison of all 7 sequences (from 3 fecal samples and 4 serum samples) revealed 100% identity. Resulting sequences were submitted to GenBank (accession nos. KU556748–KU556754).

Clinical signs and symptoms and laboratory changes for these 9 patients were compatible with those published for patients with HPeV3 infection (1,2). All 9 newborns recovered without complications; no severe, long-term complications were noted 15 months later.

Despite intensive epidemiologic evaluation, we were not able to identify a human (personnel, mothers, siblings, or visitors) or nonhuman (surfaces, formula) source of infection within this ward. However, because the causative agent was identified several weeks after the end of the outbreak, testing for HPeV had not been performed on any such human or environmental specimens. Therefore, we are not able to clearly differentiate common-source infection from person-to-person transmission. Because newborns do not have direct contact with each other, an asymptomatic adult or older sibling might have been the unidentified common source. This hypothesis is in line with the fact that most



**Figure.** Chronological details for 9 newborns with human parechovirus 3 infection, Austria, 2014. Either human parechovirus 3 (HPEV3; detected by real-time reverse transcription PCR,  $n = 5$ ) or particles resembling picornavirus (detected by electron microscopy [EM],  $n = 2$ ) were detected in  $\geq 1$  of the analyzed materials for 6 (indicated in boldface) of 8 patients. For 1 patient, neither EM nor PCR had been performed. Dark gray bar, postdelivery stay in maternity ward; light gray bar, stay at home; black bar, stay at pediatric department. \*Onset of fever. NA, not applicable; neg, negative; pos, positive; S, serum; U, urine.

infections with HPEV in adults are asymptomatic. Of the 9 newborns, 4 became symptomatic while still hospitalized; thus, they were certainly infected while in the maternity ward. The other 5 became symptomatic after discharge from the obstetric unit, so they might have acquired the infection while outside the hospital. However, the interval between discharge and readmission was  $<1$  week for all but 1 patient, and no cases of community-acquired sepsis and sepsis-like illness in newborns or infants from that region without association with the affected maternity ward could be identified during that period.

Thus, the infection was most likely nosocomial for at least 8 of the 9 patients. Assuming the period of infection for these patients (i.e., during their stay in the maternity ward), we can draw conclusions with regard to the incubation period of HPEV3 infections. The observed intervals between infection and appearance of symptoms were from 1 to 12 days. Only patient 9 became symptomatic 27 days after discharge; this patient might have acquired the infection outside the hospital.

## Conclusions

For newborns, HPEV3 is a relevant pathogen; febrile illness appears as sepsis. After symptomatic infection, a newborn can shed HPEV3 in feces for at least 1 month. The contagious nature of the virus can lead to nosocomial outbreaks. Thus, timely identification of the causative agent may

prevent nosocomial transmission (by isolation and identification of the source) and unnecessary treatment with antimicrobial drugs. For newborns with sepsis-like illness, routine diagnostic considerations should include HPEV3.

Dr. Stenger is assistant professor for Pediatrics and Adolescent Medicine at the Medical University of Graz, Austria, and head of the Working Group for Infectious Diseases of the Austrian Society for Pediatrics and Adolescent Medicine. His main research interest focuses on the management of infectious diseases in children and adolescents with impaired immunity.

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# Large-Scale Survey for Tickborne Bacteria, Khammouan Province, Laos

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We screened 768 tick pools containing 6,962 ticks from Khammouan Province, Laos, by using quantitative real-time PCR and identified *Rickettsia* spp., *Ehrlichia* spp., and *Borrelia* spp. Sequencing of *Rickettsia* spp.–positive and *Borrelia* spp.–positive pools provided evidence for distinct genotypes. Our results identified bacteria with human disease potential in ticks in Laos.

*Rickettsia*, *Borrelia*, *Ehrlichia*, *Anaplasma*, and *Coxiella* spp. are tick-associated bacteria and well-described human pathogens. All of these bacteria, except *Coxiella* spp., are transmitted through tick bites and cause febrile disease with a wide spectrum of severity. Tickborne bacterial pathogens are believed to be an underrecognized cause of acute febrile illness in Southeast Asia (1).

In Laos, spotted fever group *Rickettsia* have been shown to cause undifferentiated fever in 2% of febrile hospitalized adult patients (2). However, data on bacteria in ticks in Laos are sparse. To date, 1 *Rickettsia* sp. has been identified in a *Boophilus* sp. tick from Luang Namtha Province; this species showed 99.8% similarity with the *Rickettsia* sp. FUJ98 *ompA* gene (3). No other tickborne bacteria have been reported from Laos. Therefore, we investigated *Rickettsia*, *Borrelia*, *Ehrlichia*, *Anaplasma*, and *Coxiella* spp. in ticks from Khammouan Province, Laos.

## The Study

We collected ticks in Nakai District, Khammouan Province, during the dry seasons (December–April) during 2012–2014, as previously described (4) (online Technical Appendix Figures 1, 2, <http://wwwnc.cdc.gov/EID/article/22/9/15-1969-Techapp1.pdf>). A total of 6,692 ticks were pooled ( $n = 768$  pools, 1–10 ticks/pool) according to genus, sex, developmental stage, collection period, and

site. One *Amblyomma testudinarium* nymph that contained a blood meal was processed separately.

We extracted DNA by using the NucleoSpin 8 Virus Extraction Kit (Macherey-Nagel, Düren, Germany). Pools were screened by using single quantitative real-time PCRs specific for *Rickettsia* spp. (17-kDa gene), *Borrelia* spp. (23S rRNA gene), *Anaplasma* spp. (major surface protein 2 gene), *Ehrlichia* spp. (16S rRNA gene), and *Coxiella* spp. (IS1111) (5–8) (online Technical Appendix Table 1). Five microliters of diluted (1:10) template containing 1× Platinum Supermix-UDG (Invitrogen, Carlsbad, CA, USA) and bovine serum albumin (40 mg/mL) were used for each assay. Positive and nontemplate controls were included in each run. Screening by PCR was performed once per sample. In concordance with published guidelines, results were considered positive if they had a cycle quantitation ( $C_q$ ) value  $\leq 40$  and likely positive if they had a  $C_q$  value 40–45 (9).

Sequencing was attempted for pools with  $C_q$  values  $< 40$  (online Technical Appendix Table 2) and performed by Macrogen (Seoul, South Korea). Consensus sequences were analyzed by using CLC Main Workbench 7 (<http://www.clcbio.com/products/clc-main-workbench/>) and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and submitted to GenBank. Phylogenetic trees were constructed by using the Kimura 2-parameter model and the neighbor-joining method. Bootstrap values were determined by using 1,000 replications.

A total of 768 tick pools containing 6,692 ticks were screened. Pools contained 3 genera of ticks: 59.9% (460/768) *Haemaphysalis* spp., 36.3% (279/768) *A. testudinarium*, and 3.8% (29/768) *Dermacentor auratus*. Of the pools, 3% (23/768) contained adults, 36.5% (280/768) contained larvae, and 60.5% (465/768) contained nymphs (Table 1).

*Rickettsia* spp. were identified in 5.7% (44/768) of pools, and an additional 2.3% (18/768) of pools were likely positive for *Rickettsia* spp. Sequences consistent with 5 described *Rickettsia* species or genotypes were identified: *R. tamurae*, *R. japonica*, *Rickettsia* sp. ATT, *Rickettsia* sp. Kagoshima6, and *Rickettsia* sp. TwKM01 (Table 2; Figure 1).

Three novel genotypes (Table 2) were identified that might be new species. *Candidatus Rickettsia laoensis* (pool 447) was identified in 1 *Haemaphysalis* sp. pool. Phylogenetic analysis of 2845–2920-bp concatenated sequences of *gltA*, *sca4*, and *ompB* genes suggested that this bacteria

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**Table 1.** Tick pools tested for bacteria after screening by quantitative PCR, Khammouan Province, Laos\*

Bacteria and tick species	No. positive pools/no. tested (%)				
	Total	Larvae	Nymphs	Adult males	Adult females
<i>Rickettsia</i> spp.					
All	44/768 (5.7)	6/280 (2.1)	37/465 (8.2)	0/12 (0)	1/11 (9.1)
<i>Amblyomma testudinarium</i>	27/279 (10.0)	0/61 (0)	27/217 (12.9)	0/1 (0)	0/1 (0)
<i>Haemaphysalis G1</i>	5/398 (3.8)	6/194 (3.1)	9/200 (4.5)	0/3 (0)	0/1 (0)
<i>H. hystricis</i>	1/6 (16.7)	NS	NS	0/3 (0)	1/3 (33.3)
<i>Dermacentor auratus</i>	1/29 (3.4)	0/0 (0)	1/26 (3.8)	0/2 (0)	0/1 (0)
<i>Ehrlichia</i> spp.					
All	12/768 (1.6)	4/280 (1.4)	6/465 (1.3)	1/12 (8.3)	1/11 (9.1)
<i>A. testudinarium</i>	2/279 (0.7)	0/61 (0)	2/217 (0.9)	0/1 (0)	0/1 (0)
<i>Haemaphysalis G1</i>	8/398 (2.0)	4/194 (2.1)	4/200 (2.0)	0/3 (0)	0/1 (0)
<i>H. aborensis</i>	2/6 (33.3)	NS	NS	1/3 (33.3)	1/3 (33.3)
<i>Borrelia</i> spp.					
All	12/768 (1.6)	2/280 (0.7)	8/465 (1.7)	2/12 (16.7)	NS
<i>A. testudinarium</i>	2/279 (0.7)	1/61 (1.6)	1/217 (0.5)	0/1 (0)	0/1 (0)
<i>Haemaphysalis G1</i>	6/398 (1.5)	1/194 (0.5)	5/200 (2.5)	0/3 (0)	0/1 (0)
<i>Haemaphysalis G1.2</i>	1/13 (7.7)	NS	1/13 (7.7)	NS	NS
<i>H. aborensis</i>	2/6 (33.3)	NS	NS	2/3 (66.7)	0/3 (0)
<i>D. auratus</i>	1/29 (3.4)	0/0 (0)	1/26 (3.8)	0/2 (0)	0/1 (0)
<i>Coxiella</i> spp.					
All	5/511 (1.0)†	4/187 (2.1)†	1/310 (0.3)	0/8 (0)	0/6 (0)
<i>Haemaphysalis G1</i>	5/279 (1.8)†	4/162 (2.5)†	1/117 (0.9)	NS	NS
<i>Anaplasma</i> spp.					
All	2/768 (0.3)†	0/280 (0)†	0/465 (0)†	0/12 (0)	0/11 (0)
<i>A. testudinarium</i>	1/279 (0.4)†	0/61 (0)	1/217 (0.5)†	0/1 (0)	0/1 (0)
<i>Haemaphysalis G1</i>	1/398 (0.3)†	1/194 (0.5)†	0/200 (0)	0/3 (0)	0/1 (0)

\*NS, no samples were available for screening.

†Includes samples with cycle quantitation values &lt;40 and 40–45.

belonged to the *R. massiliae* group of rickettsiae (online Technical Appendix Figure 3). *Candidatus* Rickettsia mahosotii (pools 81 and 372) was identified in *Haemaphysalis* spp. and *A. testudinarium* pools. Phylogenetic analysis of *gltA*, *sca4*, and *ompB* genes suggested that this bacteria belonged to the *R. rickettsii* group (online Technical Appendix Figure 3). *Candidatus* Rickettsia khammouanensis was identified in 1 *Haemaphysalis* sp. nymph pool (pool 120). Phylogenetic analysis of *gltA*, 17-kDa, and *ompB* genes suggested a relationship with the *R. helvetica* group (online Technical Appendix Figure 4).

In addition, 15 *A. testudinarium* pools showed dual peaks for 17-kDa gene sequences, which suggested the presence of *R. tamurae* and *Rickettsia* sp. ATT. Sequencing of *sca4*, *ompA*, and *ompB* genes from 1 of these pools (pool 239) identified unique sequences (Table 2; online Technical Appendix Figure 4).

*Borrelia* spp. were identified in 1.6% (12/768) of pools (Table 1). Two unique sequences obtained from *Haemaphysalis* spp. pools showed 99.3% (298/300) (GenBank accession no. KR733069) and 98.7% (296/300) (accession no. KR733068) identity with Shiretoko *Haemaphysalis* *Borrelia* sp. (AB897888). Phylogenetic analysis confirmed that both bacteria were closely related to Shiretoko *Haemaphysalis* *Borrelia* sp. (accession no. B897888) and belong to the relapsing fever group of *Borrelia* (Figure 2).

Twelve (1.6%) of 768 pools were positive for *Ehrlichia* spp. (Table 1); an additional 6 pools (0.8%)

were likely positive. One short sequence from a *Haemaphysalis* sp. nymph pool (pool 357) was obtained, and this sequence showed 100% identity (116/116 bases) with the genus *Ehrlichia*.

No pools were positive for *Anaplasma* spp., but 2 were likely positive (Table 1). Although not all pools were tested for *Coxiella* spp. (n = 511), 1 pool (0.2%) was positive, and 4 pools were likely positive for *C. burnetti*. No confirmatory sequences were obtained from these pools. The 1 tick that contained a blood meal (*A. testudinarium* nymph) showed negative results by screening PCRs.

## Conclusions

This study provides evidence that *Rickettsia* spp., *Borrelia* spp., and *Ehrlichia* spp. are present in ticks in Laos. Several *Rickettsia* spp. identified in this study are human pathogens. Infections with *R. tamurae* (2) and *R. japonica* are well described in Southeast Asia (10). However, the pathogenicity of *Rickettsia* sp. TwkM01 (11), *Rickettsia* sp. ATT (12), *Rickettsia* sp. kagoshima6 genotypes (13) and potential novel *Candidatus* Rickettsia laoensis, *Candidatus* Rickettsia mahosotii, and *Candidatus* Rickettsia khammouanensis is unknown. *Candidatus* Rickettsia khammouanensis is phylogenetically related to *R. helvetica*, for which there is serologic evidence for its role as a human pathogen in Laos (2). Unique *ompA*, *ompB*, and *sca4* sequences identified in this study (Table 2) might indicate the presence of

**Table 2.** Sequence data for *Rickettsia* species isolated from ticks, Khammouan Province, Laos\*

Tick pool	Tick species and stage	<i>Rickettsia</i> spp. gene, GenBank accession no., and % similarity (no. matching nucleotides/total)				
		17-kDa	<i>gltA</i>	<i>sca4</i>	<i>ompA</i>	<i>ompB</i>
110	<i>Amblyomma testudinarium</i> nymph	Unclear sequence	NS	Unclear sequence	KT753264, 100.0 (529/529) with <i>Rickettsia</i> sp. TwKM01 EF219467	NS
177, 180, 216, 220	<i>A. testudinarium</i> nymph	KR733070, 100.0 (355/355) with <i>R. tamurae</i> AB114825	KT753265, 99.8 (1,096/1,098) with <i>R. tamurae</i> AB812551	KT753266, 99.7 (607/609) with <i>R. tamurae</i> DQ113911	NS	NS
315	<i>A. testudinarium</i> nymph	KT753267, 98.8 (407/412) with <i>R. raoultii</i> JX885457	KT753268, 99.9 (1,036/1,037) with <i>Rickettsia</i> kagoshima6 JQ697956	KT753269, 96.8 (795/821) with <i>Rickettsia</i> sp. AUS 118, KF666473	Could not be amplified	KT753270, 95.0 (1,073/1,129) with <i>R. massiliae</i> CP003319
239	<i>A. testudinarium</i> nymph	KT753271, 99.7 (360/361) with <i>Rickettsia</i> sp. ATT AF483196	KT753272, 99.7 (1,048/1,051) with <i>R. tamurae</i> (AB812551)/KT753273; 99.2 (367/370) with <i>Rickettsia</i> sp. hhmj7 KC566999	KT753274, 97.1 (759/782) with <i>Rickettsia</i> sp. AUS 118 KF666473	KT753275, 87.2 (530/602) with <i>R. raoultii</i> JQ792137	KT753276, 97.5 (1,052/1,079) with <i>R. massiliae</i> CP003319
76, 337, 450, 453	<i>Haemaphysalis</i> G1 nymphs (3), <i>A. testudinarium</i> nymph (1)	KT753277, 98.4 (417/423) with <i>R. raoultii</i> JX885457	KT753278, 99.9 (1,037/1,038) with <i>Rickettsia</i> sp. kagoshima6 JQ697956	KT753279, 98.4 (794/807) with <i>R. japonica</i> AF155055	Could not be amplified	KT753280, 96.0 (410/427) with <i>R. raoultii</i> EU036984
81, 372	<i>Haemaphysalis</i> G1 nymphs, <i>A. testudinarium</i> nymph (17 kDa only)	KT753283, 99.0 (408/412) with <i>R. raoultii</i> JX885457	KT753284, 99.5 (1,090/1,096) with <i>R. sibirica</i> U59734	KT753285, 98.5 (838/851) with <i>R. japonica</i> AF155055	Could not be amplified	KT753286, 97.7 (1,118/1,144) with <i>R. massiliae</i> CP003319
120	<i>Haemaphysalis</i> G1 nymph	KT753287, 96.1 (391/407) with <i>R. helvetica</i> GU827073	KT753288, 97.1 (370/381) with <i>Candidatus</i> <i>Rickettsia</i> rara DQ365805	Could not be amplified (x2)	Could not be amplified (x2)	KT753289, 86.4 (362/419), <i>R. aeschlimannii</i> AF123705
407	<i>Haemaphysalis hysticis</i> adult	KR733074, 100.0 (413/413), <i>R. japonica</i> AP011533	KT753281, 100.0 (1,063/1,063), <i>R. japonica</i> AP011533	NS	NS	KT753282, 100.0 (1,191/1,191) with <i>R. japonica</i> AP011533
447	<i>Haemaphysalis</i> G1 nymph	KT753291, 98.6 (407/413) with <i>R. massiliae</i> CP000683	KT753290, 99.6 (961/965) with <i>R. raoultii</i> JX885455	KT753292, 97.5 (809/830) with <i>Rickettsia</i> sp. AUS118 KF66473	KT753293, 97.5 (591/606) with <i>Rickettsia</i> sp. JL-02 AY093696	KT753294, 98.4 (1,137/1,156), with <i>R. massiliae</i> CP003319

\*New sequences were compared with reference sequences. NS, not sequenced.

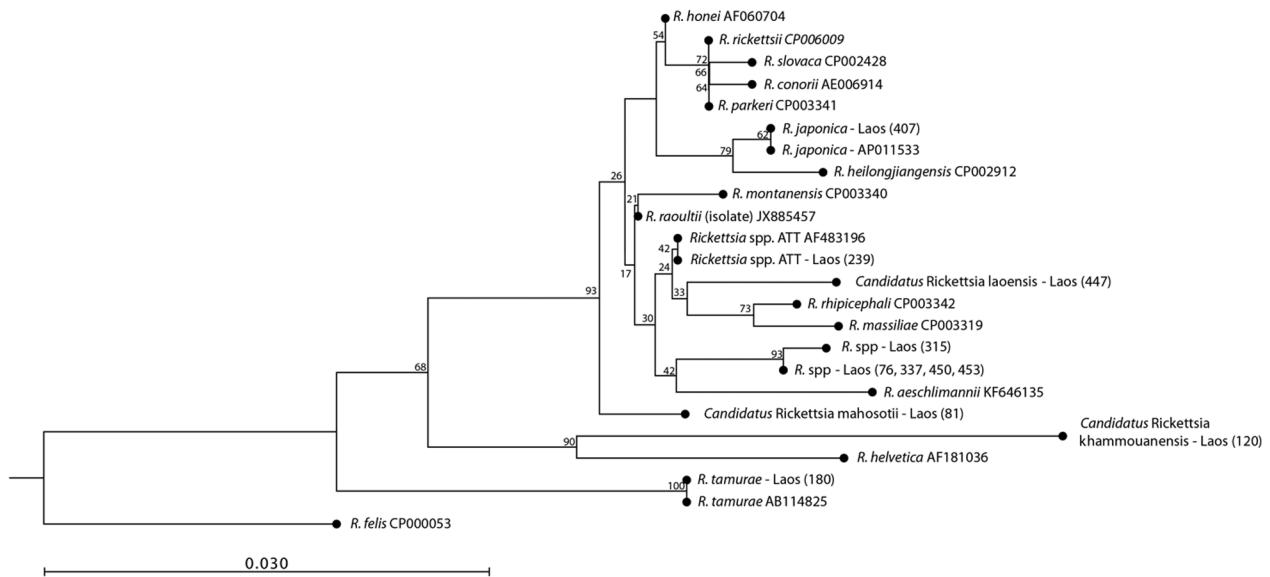
*Rickettsia* sp. ATT (12), which was previously believed to be identical to *R. tamurae* (14), and suggests that it might be a distinct species. Further studies, including whole-genome sequencing, are required to identify and confirm these novel genotypes and understand their role in human disease.

*Borrelia* spp. sequences identified in *Haemaphysalis* spp. pools were shown to have high concordance with the Shiretoko *Haemaphysalis* *Borrelia* isolated from *Haemaphysalis* spp. ticks and deer in Japan (15). The species belongs to the relapsing fever group of *Borrelia* and is related to *B. lonestari*.

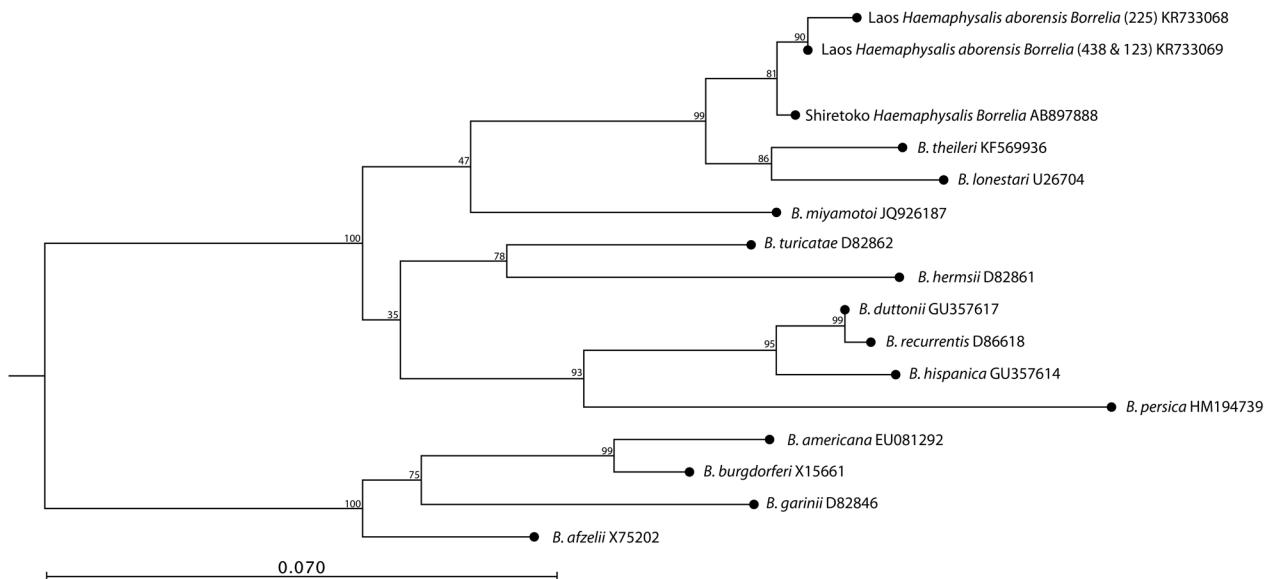
Sequence data for *Ehrlichia* spp. indicated the presence of these bacteria but were not sufficient to identify

them to the species level. The  $C_q$  values were high (40–45) for *Anaplasma* spp., but no sequence data were obtained. *Coxiella* spp. were screened by using primers for IS1111, which are not specific for *C. burnetii*, and no confirmatory sequence data were obtained. Because of limited reagents, screening of all 768 pools for *Coxiella* spp. was not completed. Further work is required to investigate the presence of these bacteria in Laos.

Our study had several limitations. First, pooling of ticks precludes an accurate assessment of prevalence of bacterial pathogens. Second, sequences obtained from some *A. testudinarium* pools had dual peaks, suggestive of multiple infections, and could therefore not be interpreted. Third, ticks were collected only from 1 area in Laos



**Figure 1.** Phylogenetic analysis of *Rickettsia* spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (350 bp) of the 17-kDa gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.



**Figure 2.** Phylogenetic analysis of *Borrelia* spp. in ticks, Khammouan Province, Laos. The tree was constructed by using partial nucleotide sequences (299–323 bp) of the *flaB* gene, the Kimura 2-parameter model, and the neighbor-joining method. Analyses were supported by bootstrap analysis with 1,000 replications. Numbers along branches are bootstrap values. GenBank accession numbers are shown for reference sequences. Sample numbers for each tick are shown in parentheses. Scale bar indicates nucleotide substitutions per site.

(Khammouan Province); thus, extrapolating findings to the entire country must be done cautiously.

Our results highlight the emergence of tickborne bacteria in Laos. These findings emphasize the need for further research of tick-associated bacteria and their role in human disease.

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# Multidrug-Resistant *Shigella* Infections in Patients with Diarrhea, Cambodia, 2014–2015

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We observed multidrug resistance in 10 (91%) of 11 *Shigella* isolates from a diarrheal surveillance study in Cambodia. One isolate was resistant to fluoroquinolones and cephalosporins and showed decreased susceptibility to azithromycin. We found mutations in *gyrA*, *parC*,  $\beta$ -lactamase, and *mphA* genes. Multidrug resistance increases concern about shigellosis treatment options.

Shigellosis is a major public health problem in developing countries. Antimicrobial therapy with fluoroquinolones is recommended to shorten the course of disease and fecal shedding. However, limitations on shigellosis treatment options have been a concern since 1993, when ciprofloxacin-resistant *Shigella* was documented (1), followed by reports of multidrug-resistant (MDR) *Shigella* and of *Shigella* that harbored extended-spectrum  $\beta$ -lactamase (ESBL) genes (2). We describe MDR *Shigella* isolated from patients with diarrhea in Cambodia during 2014–2015.

## The Study

During July 2014–April 2015, we examined stool specimens collected from patients 3 months–5 years of age and 18–60 years of age who were seen for or admitted with acute diarrhea at 3 healthcare settings in Battambang, Cambodia, as part of ongoing hospital-based surveillance of diarrhea etiology. Stool specimens were processed for identification of enteric pathogens by standard microbiology, ELISA, and PCR. *Shigella* species were identified by standard biochemical tests and the API 20E system (bioMérieux, Marcy l'Étoile, France) and serotyped by commercial antisera (Denka Seiken Co, Ltd., Tokyo, Japan). Antimicrobial drug susceptibility testing was performed with

the standard Kirby-Bauer disk diffusion method by using commercially available antimicrobial disks (Becton Dickinson, Franklin Lakes, NJ, USA). Antimicrobial drugs tested for susceptibility were ampicillin, azithromycin (AZM), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline, and trimethoprim/sulfamethoxazole. Susceptibility results were interpreted according to Clinical and Laboratory Standards Institute guidelines (3). We used zone diameter interpretive standards for *Enterobacteriaceae* for all antimicrobial drugs tested, except AZM, for which we applied the standard for *Staphylococcus* spp.

*Shigella* spp. were isolated from 11 (5%) of 212 diarrhea stool samples. Antimicrobial drug susceptibility testing showed that 10 (91%) of the 11 *Shigella* isolates were resistant to ampicillin, tetracycline, trimethoprim/sulfamethoxazole, and NAL. We selected the 10 MDR isolates for further characterization and determined MICs of AZM and CIP by Etest (bioMérieux). ESBL production was tested by using Neg Combo Panel Type 50 on the MicroScan WalkAway plus System (Siemens Healthcare Diagnostics, Newark, DE, USA). PCR and sequencing were used to characterize resistance genes (*gyrA* and *parC*) in the quinolone-resistance determining region (QRDR), the AZM resistance gene (*mphA*), and  $\beta$ -lactamase genes (4–7).

Of the 10 MDR isolates, 2 were *S. flexneri* 2a; 1 was an *S. flexneri* 2 variant; 6 were *S. flexneri* 3a; and 1 was *S. sonnei* (Table 1). CIP resistance was detected in 5 (50%) of the 10 isolates. Sequence analysis showed mutations of *gyrA* and *parC* genes with the amino acid substitutions in the QRDR (Table 2). All NAL-resistant isolates susceptible to CIP had a single mutation in *gyrA*. Isolates resistant to both NAL and CIP contained multiple mutations in *gyrA* and *parC*.

The most common mechanism of quinolone resistance in the *Shigella* spp. was mutation of *gyrA*, typically at codon 83 or 87, and of *parC* at codon 80 (7). All isolates in our study had the common mutation in *gyrA* at position 83 (Ser83→Leu); 1 isolate had another common mutation at position 87 (Asp87→Gly). A mutation in *parC* at position 80 (Ser80→Ile), detected in the *S. sonnei* isolate, was previously reported in an *S. dysenteriae* serotype 1 isolate in India (7) and in Asia travel-associated *S. sonnei* and *S. flexneri* isolates in the United States (8). A mutation at position 57 (Ser57→Arg) was detected in all 4 CIP-resistant *S. flexneri* 3a isolates, but this mutation's role in CIP resistance is unclear because position 57 is outside the QRDR region. Characterization of plasmid-mediated quinolone resistance (PMQR) genes should be further investigated because

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**Table 1.** Epidemiologic data of patients with multidrug-resistant *Shigella*, Cambodia, July 2014–April 2015

Isolate no.	Organism	Isolate collection date	Patient age	Patient sex	Antimicrobial drugs taken before enrollment
1	<i>S. flexneri</i> 2a	2015 Apr 3	3 y	M	No
2	<i>S. flexneri</i> 2a	2015 Apr 28	18 mo	M	No
3	<i>S. flexneri</i> 2v	2015 Apr 20	1 y	F	No
4	<i>S. flexneri</i> 3a	2015 Jan 22	1 y	M	Yes*
5	<i>S. flexneri</i> 3a	2015 Feb 20	6 mo	M	No
6	<i>S. flexneri</i> 3a	2014 Nov 17	4 y	M	No
7	<i>S. flexneri</i> 3a	2014 Nov 25	1 y	F	Yes†
8	<i>S. flexneri</i> 3a	2014 Dec 12	3 y	M	No
9	<i>S. flexneri</i> 3a	2015 Feb 21	13 mo	M	No
10	<i>S. sonnei</i>	2015 Mar 11	2 y 4 mo	M	No

\*Unknown type of drug taken 1 time.

†250 mg amoxicillin taken for 3 d.

coexistence of mutations in the QRDR and PMQR genes has been reported in *Shigella* isolates with decreased susceptibility to fluoroquinolones (8). PMQR may facilitate the selection of QRDR mutations, resulting in higher levels of quinolone resistance.

No clinical breakpoints for AZM have been clearly defined for *Shigella* spp., but CDC's National Antimicrobial Resistance Monitoring System for Enteric Bacteria (<http://www.cdc.gov/narms/index.html>) recommends using the term "decreased susceptibility" for reporting. We detected decreased susceptibility to AZM in *S. flexneri* 3a (isolate no. 9) with a MIC of 32 µg/mL. This isolate was found to carry the *mphA* gene encoding a macrolide 2'-phosphotransferase that inactivates macrolide antimicrobial drugs and has been reported to reduce AZM susceptibility in *Shigella* isolates (5). Emergence of decreased susceptibility

to AZM may affect treatment options for shigellosis, especially for pediatric cases because ceftriaxone is administered parenterally by injection and fluoroquinolones are not encouraged for use in children.

We detected ≥1 β-lactamase gene in all 10 *Shigella* isolates; 2 isolates that were resistant to cephalosporins revealed ESBL production (Table 2). The 8 isolates that carried β-lactamase-producing genes TEM-1 or TEM-1 and OXA-1 were cephalosporin susceptible, suggesting that TEM-1 and OXA-1 may not play a role in increased resistance to third-generation cephalosporins. Of the remaining 2 isolates, 1 *S. flexneri* (isolate no. 9), which harbored CTX-M-27 and TEM-1, showed resistance to CRO and CTX but not to CAZ, and 1 *S. sonnei* (isolate no. 10), which carried CTX-M-55, was resistant to all cephalosporins tested.

**Table 2.** Antimicrobial susceptibility results and molecular characterization of resistance genes of *Shigella* isolates collected from patients in Cambodia, July 2014–April 2015\*

Isolate no.	Organism	Antimicrobial resistance	CIP MIC, µg/mL†	Amino acid substitutions in QRDR				AZM MIC, µg/mL‡	<i>mphA</i> gene	ESBL confirmatory test	β-lactamase genes
				Ser 83	Asp 87	Ser 57	Ser 80				
1	<i>S. flexneri</i> 2a	AMP-SXT-TET-NAL	0.25	Leu	–	–	–	2.00	Neg	Neg	TEM-1, OXA-1
2	<i>S. flexneri</i> 2a	AMP-SXT-TET-NAL	0.25	Leu	–	–	–	1.50	Neg	Neg	TEM-1, OXA-1
3	<i>S. flexneri</i> 2v	AMP-SXT-TET-NAL	0.19	Leu	–	–	–	1.50	Neg	Neg	TEM-1, OXA-1
4	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL	0.25	Leu	–	–	–	1.00	Neg	Neg	TEM-1
5	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL	0.19	Leu	–	–	–	1.50	Neg	Neg	TEM-1
6	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL-CIP	4.00	Leu	–	Arg	–	0.75	Neg	Neg	TEM-1
7	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL-CIP	4.00	Leu	–	Arg	–	1.00	Neg	Neg	TEM-1
8	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL-CIP	4.00	Leu	–	Arg	–	1.00	Neg	Neg	TEM-1
9	<i>S. flexneri</i> 3a	AMP-SXT-TET-NAL-CIP-AZM-CRO-CTX	6.00	Leu	–	Arg	–	32.00	Pos	Pos	TEM-1, CTX-M-27
10	<i>S. sonnei</i>	AMP-SXT-TET-NAL-CIP-CRO-CTX-CAZ	6.00	Leu	Gly	–	Ile	4.00	Neg	Pos	CTX-M-55

\*AMP, ampicillin; Arg, arginine; Asp, aspartate; AZM, azithromycin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; ESBL, extended-spectrum β-lactamase; Gly, glycine; Ile, isoleucine; Leu, leucine; NAL, nalidixic acid; Neg, negative; Pos, positive; QRDR, quinolone-resistance determining region; Ser, serine; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; –, no amino acid substitutions found.

†CIP MIC interpretive criteria for *Enterobacteriaceae* is susceptible ≤1, resistant ≥4 µg/mL.

‡AZM MIC interpretive criteria for *Salmonella enterica* serovar Typhi is susceptible ≤16, resistant ≥32 µg/mL.

A key element that increased ceftazidimase activity was a single amino acid substitution from Asp to Gly at position 240; this substitution was identified in CTX-M-15, CTX-M-16, CTX-M-27, and CTX-M-32. CTX-M-27, first reported from France in 2003, differed from its parental enzyme, CTX-M-14, by substitution of Asp240Gly (9). Reports suggest that this Gly-240-harboring CTX-M-27 confers higher levels of resistance to CAZ in *Escherichia coli* infections, but we did not detect this characteristic in the *Shigella* isolates we examined. CTX-M-55 was first reported in ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates in Thailand in 2007; it was associated with high resistance to CRO, CTX, and CAZ (10) and was subsequently reported in other Asia countries, including Cambodia. Among fecal samples collected from children in Cambodia, 88% carried *E. coli* harboring ESBL genes containing *bla*<sub>CTX-M</sub> variants, including CTX-M-15, CTX-M-55, and CTX-M-14 (11). A case of ESBL-producing *S. sonnei* harboring CTX-M-55 was also reported in a woman traveling from Korea to China (12).

The CDC Health Alert Network has distributed a health advisory on CIP- and AZM-nonsusceptible *Shigella* infection in the United States (13). Three separate outbreaks of MDR shigellosis among men who have sex with men, international travelers, and children in daycare centers have been reported (13). We found 2 ESBL-producing, fluoroquinolone-resistant *Shigella* isolates. Moreover, *S. flexneri* 3a (isolate no. 9), which had decreased susceptibility to AZM, was also resistant to nearly all oral and parenteral drugs considered for shigellosis treatment. This isolate can ferment sorbitol, a feature found in 7% of *Shigella* spp. and possibly causing misidentification of *Shigella* spp. as other species (14). *S. sonnei* (isolate no. 10) belongs to biotype g (i.e., with biochemical reactions ONPG+ [o-nitrophenyl-β-D-galactopyranose], rhamnose-, and xylose-), which has been shown to carry integrons with multiple gene cassettes, leading to multidrug resistance (15).

## Conclusions

MDR *Shigella* is an emerging problem that raises concern about shigellosis treatment worldwide, including in Cambodia. Health authorities should implement systematic surveillance of antimicrobial drug resistance and controlled antimicrobial drug use to increase understanding of the problem and minimize unnecessary antimicrobial drug use, which contributes to increased resistance.

## Acknowledgments

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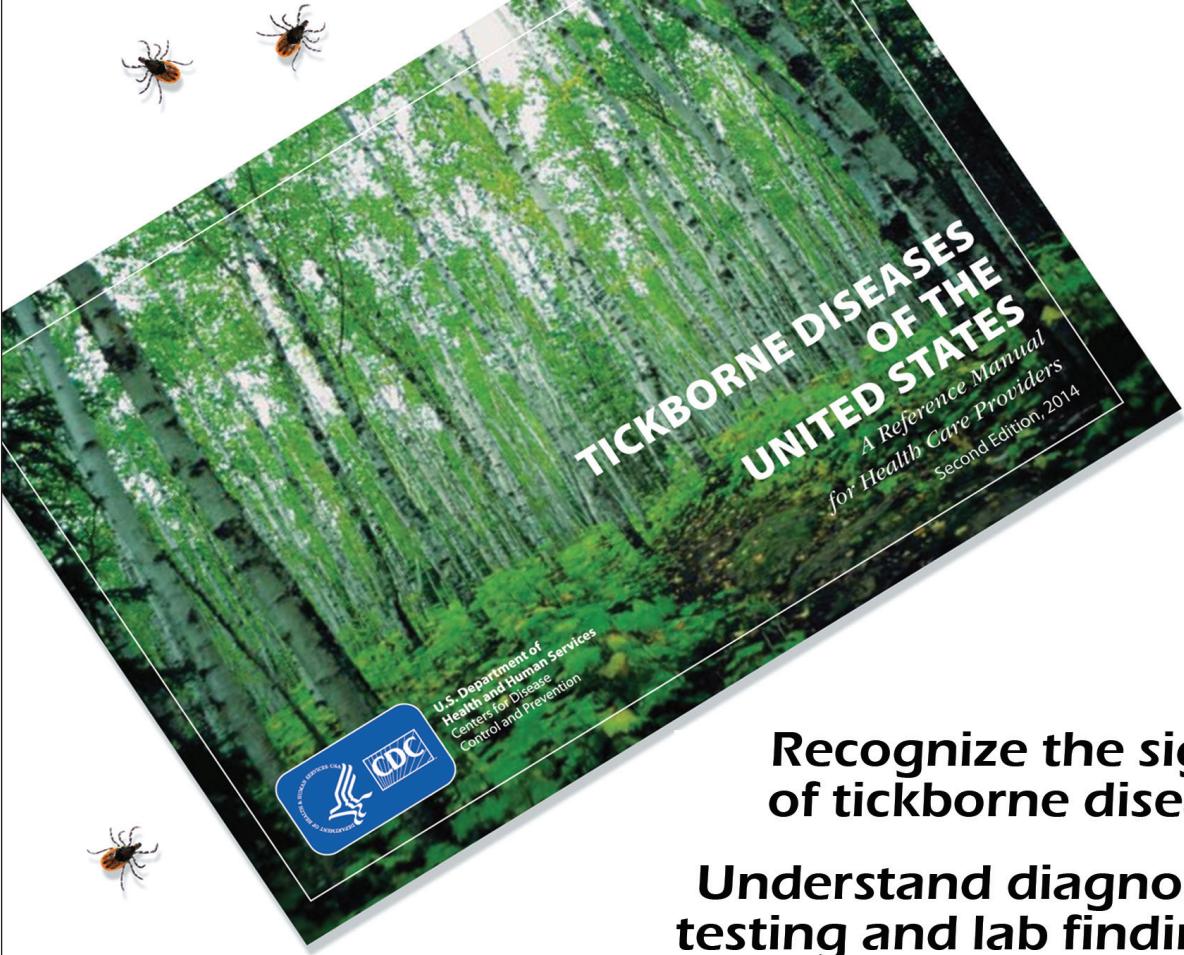
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# Contact Tracing for Imported Case of Middle East Respiratory Syndrome, China, 2015

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Jun Wang, Jiansen Li, Jie Wu, Jianfeng He,  
Jinyan Lin, Yonghui Zhang

Confirmation of an imported case of infection with Middle East respiratory syndrome coronavirus in China triggered intensive contact tracing and mandatory monitoring. Using a hotline and surveillance video footage was effective for tracing all 110 identified contacts. Contact monitoring detected no secondary transmission of infection in China.

In 2015, South Korea reported the largest outbreak of Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) infection that has occurred outside the Middle East (1). This outbreak caused 186 laboratory-confirmed cases and 36 deaths. Subsequent transmission of MERS-CoV in South Korea was associated with local hospitals and caused 3 second-generation infections (2).

Of the 186 MERS cases originating in South Korea, 1 was confirmed by China (3). A preliminary report of this patient's exposure history and onset of illness has been published (4). The patient was symptomatic in South Korea and traveled by airplane to Hong Kong, China, and then by 2 consecutive buses to Guangdong, China, on May 26, 2015. In Guangdong, his community visits included 2 hotels, 2 restaurants, and 1 enclosed meeting room. The China health authority isolated the patient on May 28 and confirmed the patient's illness as an imported case of MERS-CoV infection on May 29. To prevent local spread, we conducted a comprehensive investigation to trace all contacts of this case-patient in mainland China and also conducted mandatory monitoring.

## The Study

Because this research was a part of a public health response, our study did not require formal ethical approval from a medical ethics committee. Contact tracing was initiated immediately after we detected the case-patient. We communicated with the airline and bus operators to collect passenger information and undertook personal interviews in related communities. A hotline was set up, and the case-patient's travel information was published in the media. We investigated hotline callers and identified suspected contacts. We also reviewed video footage from closed circuit television

in hotels and restaurants visited by the case-patient, enabling us to identify contacts and measure duration and distance of exposures. Information about bus passengers was limited; consequently, with help from police departments, we analyzed video footage recorded by public surveillance cameras at bus stations and surrounding communities and traced the whereabouts of related passengers.

We identified 110 contacts in mainland China: 87 (79%) were from mainland China, 11 (10%) from South Korea, 2 (2%) from Hong Kong, 6 (5%) from Taiwan, 3 (3%) from Canada, and 1 (1%) from Japan. Of the 110 contacts, 27 were air travel contacts (passengers onboard the same flight with the case-patient); 24 were land travel contacts (stewards and passengers taking the same buses with the case-patient); and 59 were community contacts (persons who had face-to-face contact with the case-patient or who had direct contact with his belongings in hotels, restaurants, and a meeting room) (Table 1). We found 34 (58%) of the community contacts through personal interviews. The hotline resulted in 16 (59%) air travel contacts and 12 (50%) land travel contacts. Reviewing video helped trace 9 (38%) land travel contacts and 25 (42%) community contacts (Table 2). We located all community, air travel, and land travel contacts within 3 days, 6 days, and 8 days, respectively (Figure).

Among 44 contacts whom we classified as close contacts, 6 were air travel contacts who had been seated  $\leq 3$  rows from the case-patient on the flight; 24 were land travel contacts; and 14 were community contacts who had prolonged ( $>15$  minutes) face-to-face ( $<2$  m) contact with the case-patient or direct contact with his belongings. We classified the remaining 66 contacts as common contacts. Of the 44 close contacts, 40 were staying in mainland China and were quarantined in designated facilities for 14 days after their last exposure to the case-patient. Public health officials checked body temperatures twice daily and monitored symptoms. The remaining 4 close contacts, 2 from Taiwan and 2 from South Korea, had returned to their countries before they were traced. We notified local health authorities about these 4 contacts. Of 66 common contacts, 49 were quarantined in designated facilities, and 17 conducted self-monitoring at home (an alternative for common contacts) for 14 days after their last exposure to the case-patient. Public health officials visited them daily. During follow-up, fever developed in 1 contact and 2 others had sore throat.

Throat swab samples from 106 contacts and serum samples from 53 were obtained on the first and last days of follow-up. An additional set of specimens was collected from the 3 symptomatic contacts immediately after onset

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**Table 1.** Results of contact investigation of a patient with an imported case of Middle East respiratory syndrome coronavirus infection, China, 2015

Category	Contacts, no. (%), N = 110	Close contacts,* no. (%), n = 44	Common contacts, no. (%), n = 66
Sex			
M	59 (54)	34 (77)	25 (38)
F	51 (46)	10 (23)	41 (62)
Age group, y			
17–29	39 (35)	10 (23)	29 (44)
30–59	66 (60)	30 (68)	36 (55)
60–70	5 (5)	4 (9)	1 (2)
Tracing approach			
Personal interview	48 (44)	15 (34)	33 (50)
Hotline	28 (25)	18 (41)	10 (15)
Video reviewing	34 (31)	11 (25)	23 (35)
Contacts			
Air travel	27 (25)	6 (14)	21 (32)
Land travel	24 (22)	24 (54)	0
Community	59 (54)	14 (32)	45 (68)
Management†			
Quarantine in designated facility	89 (84)	40 (100)	49 (74)
Self-monitoring at home	17 (16)	0	17 (26)
Symptoms‡			
Symptomatic	3 (3)	2 (5)	1 (2)
Asymptomatic	103 (97)	38 (95)	65 (98)

\*Close contacts include 2 from Taiwan and 2 from South Korea that returned to their countries before they were identified. We notified local health authorities about these 4 contacts.

†Numbers and percentages for contacts and close contacts exclude 4 contacts who left mainland China before they were identified.

of symptoms. All specimens were tested by real-time reverse transcription PCR, as previously described (4). No specimens tested positive for MERS-CoV, and follow-up for contacts ended on June 10, 2015.

From the date of his isolation (May 28) until the date of his discharge (June 26), the case-patient received direct medical care and examination from 73 healthcare workers (HCWs). These HCWs were not considered contacts in our investigation because they all used personal protective equipment, as recommended by the World Health Organization (5). The hospital conducted follow-up with all 73 HCWs until 14 days after their last interaction with the case-patient. No HCW was symptomatic during follow-up. Throat swab and serum samples were obtained from all HCWs on day 10 after the case-patient’s admission and on day 14 after his discharge. All specimens tested negative for MERS-CoV by real-time reverse transcription PCR. Follow-up for HCWs ended on July 10, 2015.

### Conclusions

We traced 110 contacts of a patient with an imported case of MERS-CoV infection in China. Follow-up and laboratory testing indicated that no virus transmission occurred among contacts. Because of the timely notification from

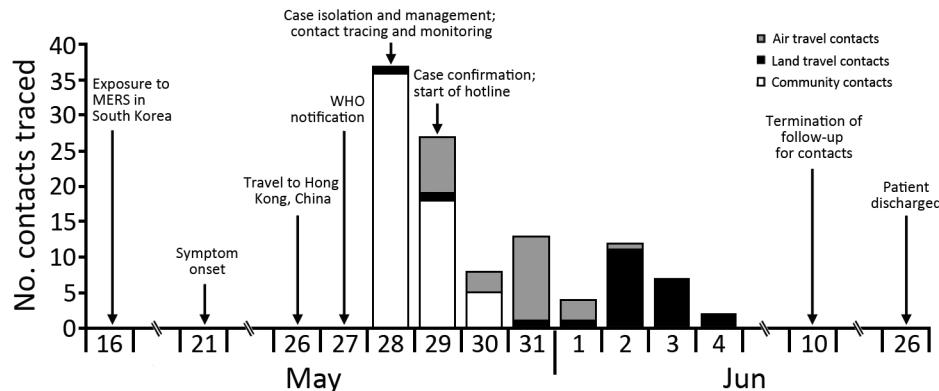
South Korea and the World Health Organization regarding the MERS-CoV outbreak (4), our hospital was able to prepare in advance for admission of the case-patient. No HCWs were infected. Our findings indicate that human-to-human transmission of MERS-CoV is still limited (6–8).

To minimize risk of local spreading, China health authorities decided to identify and trace all contacts of the initial case-patient and enforce mandatory monitoring. Evidence supports this aggressive policy. First, the MERS outbreak in South Korea, from where the case-patient traveled, was ongoing, and superspreading was observed there (9,10). Second, the patient was symptomatic and potentially infectious during his travel and stay in China. Previous clusters have been detected in hospitals and households (11–13), and MERS-CoV transmission might occur in enclosed settings. Moreover, because knowledge about MERS is still limited, cases could have been easily missed initially.

Our approach illustrates the feasibility of multiple complementary practices for contact tracing. Publicizing information and hotlines can facilitate contact tracing and risk communication and helped us identify >50% of the case-patient’s travel contacts. However, we also had to rule out large numbers of false hotline calls that resulted from inaccurate recall and excessive worry. Review of video footage

**Table 2.** Results of different contact tracing approaches for patient with an imported case of Middle East respiratory syndrome coronavirus infection, China, 2015

Tracing approaches	Air travel contacts, no. (%), n = 27	Land travel contacts, no. (%), n = 24	Community contacts, no. (%), n = 59
Personal interview	11 (41)	3 (13)	34 (58)
Hotline	16 (59)	12 (50)	0
Video reviewing	0	9 (38)	25 (42)



**Figure.** Timeline for imported case of Middle East respiratory syndrome (MERS) coronavirus infection and contact tracing investigation, China, 2015. The case-patient was identified on May 27, 2015, and quarantined beginning in the early morning of May 28, the day contact tracing began. Laboratory testing, which began on May 28, confirmed MERS on May 29, the date of the start of the hotline. WHO, World Health Organization.

is another active solution for identifying contacts, especially for anonymous contacts. In our investigation, we directly located some bus passengers who resided near the station by reviewing footage from surveillance cameras. Other bus passengers left the station by private cars, which were captured by cameras. Our inquiries into car registration information traced these contacts successfully. Reviewing video footage can also measure a contact's exposure objectively and quantitatively. Investigators should combine and compare video footage meticulously to gather pieces of information.

Our contact tracing and monitoring involved challenges. Contacts came from different countries and regions, and sites of their exposures varied. Also, no identity information for bus passengers was available, and privacy issues were concerns. We spent 8 days tracing these passengers, and some had already left China. Furthermore, lack of knowledge about MERS made some contacts less willing to comply with mandatory monitoring. Nevertheless, we traced and monitored all contacts eventually. We suggest combining multiple approaches and data sources beyond ordinary investigation to trace contacts of persons with imported cases of MERS.

### Acknowledgments

We thank all the authorities, the Huizhou Municipal Center for Disease Control and Prevention, the Hong Kong Centre for Health Protection, and field and laboratory staff for participating in contact tracing and monitoring.

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# Mutation in West Nile Virus Structural Protein prM during Human Infection

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A mutation leading to substitution of a key amino acid in the prM protein of West Nile virus (WNV) occurred during persistent infection of an immunocompetent patient. WNV RNA persisted in the patient's urine and serum in the presence of low-level neutralizing antibodies. This case demonstrates active replication of WNV during persistent infection.

West Nile virus (WNV) is a notable cause of neuroinvasive disease and febrile illness. In humans, WNV generates low viremia levels during infection (1). WNV is endemic in Israel and has been the cause of several disease outbreaks in recent years (2). Several subtypes of WNV lineage 1 have been phylogenetically identified in mosquitoes in Israel (3). However, no WNV sequence or isolation of viruses from humans in Israel had been reported since 2000 until the 2014 case we report here. In this study, we isolated and sequenced WNV lineage 1 and identified an amino acid mutation in the prM protein sequence that occurred between day 19 and day 28 of persistent viremia and viruria in a person with confirmed WNV encephalitis.

## The Study

A 56-year-old male gardener was admitted to Barzilai Medical Center in Ashkelon, Israel, on July 22, 2014, with a 7-day history of headache, abdominal pain, nausea, and fever (temperature 39°C); the date of hospital admission is designated as day 7 of his illness. His family recalled that he had received an unusually large mosquito bite 2 weeks before hospital admission. His medical history was remarkable for a thymoma B2 that was resected 3 years earlier without any evidence of myasthenia gravis. He did not receive immunosuppressive drugs or any other long-term drug therapy.

On examination, he was drowsy, disoriented, and non-cooperative. Marked neck rigidity was noted. Results of the

remainder of the physical and neurologic examination were normal. A complete blood count revealed 13,750 leukocytes/ $\mu\text{L}$ , primarily neutrophils (88%). Follow-up blood counts, 10 and 50 days after admission, showed 6,400 and 6,500 leukocytes, of which 4,800 and 4,500 were neutrophils, respectively. Blood chemistry levels were within reference ranges, except for elevated blood glucose (131 mg/dL). Results of a computed tomography scan of his brain, without and with contrast media, were normal. Cerebrospinal fluid (CSF) examination revealed clear fluid containing 412 leukocytes/ $\mu\text{L}$  (285 neutrophils and 127 mononuclear cells/ $\mu\text{L}$ ), a protein level of 234 mg/dL and glucose level of 55 mg/dL. Gram stain results were negative for bacteria. An electroencephalogram showed generalized slowing of brain electrical activity. Treatment with intravenous ceftriaxone and acyclovir (until herpes simplex virus infection was excluded by PCR) was initiated, and the patient's mental status gradually improved. On the eighth day of hospitalization, pain developed in the left shoulder along with rapidly progressive weakness and atrophy in the left upper limb muscles over several days, mainly in the deltoid, supraspinatus, biceps, and triceps muscles. Treatment with antiinflammatory drugs and physiotherapy were initiated. Electrophysiologic studies 2 weeks later showed asymmetric denervation in the muscles innervated by C4–C7 nerve roots; the denervation was more prominent on the left side, compatible with an anterior-horn cell lesion. This segmental polioliike syndrome improved slowly and was still present a year later.

CSF and serum samples obtained on day 7 tested positive for specific WNV IgM and negative for WNV IgG (WNV IgM capture DxSelect and WNV IgG DxSelect; Focus Diagnostics Inc., Cypress, California, USA). Real-time reverse transcription PCR (RT-PCR) of CSF was negative for WNV RNA, but real-time RT-PCR of serum was positive for WNV RNA, confirming the diagnosis of WNV disease. Because RNA extracted from a urine sample obtained on day 12 was positive ( $8.8 \times 10^7$  copies/mL) for WNV RNA, we monitored urine and serum samples for WNV RNA using real-time RT-PCR (4). The results demonstrated persistent viremia for 47 days and viruria for 61 days after illness onset (Table). In addition, infectious virus was isolated from 2 urine samples taken on days 12 and 15. We observed persistence of WNV RNA and virus isolation, despite the development of IgM and IgG, as well as WNV neutralizing antibodies (by ELISA and microneutralization) (Table).

Whole-genome (96%) next-generation sequencing (Ion Torrent PGM system; Life Technologies, Grand Island,

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**Table.** Serologic and molecular results in serum and urine samples from a patient with persistent WNV infection. Israel, 2014

Days after infection†	Days after illness onset‡	Serum, copies/mL	Urine, copies/mL	Serum IgM (ELISA result)§	Serum IgG (ELISA result)§	Serum neutralization titers‡	Amino acid in prM 20 (%)
14	7	6 × 10 <sup>4</sup> ¶		Pos 1.8	Neg	Neg	T(100)
19	12		8.8 × 10 <sup>7</sup> ¶	ND	ND	ND	T (100)
22	15	2.5 × 10 <sup>4</sup>	2.3 × 10 <sup>6</sup> ¶	Pos 8.07	Int 1.35	1:20	T (100)
23	16	3.6 × 10 <sup>3</sup>	2 × 10 <sup>6</sup>	Pos 8.16	Int 1.35	1:20	
24	17	8.3 × 10 <sup>3</sup>	4.1 × 10 <sup>6</sup>	Pos 8.12	Pos 1.65	ND	
25	18	1.2 × 10 <sup>4</sup>	2 × 10 <sup>6</sup>	Pos 8.29	Pos 1.98	1:20	
26	19	1.8 × 10 <sup>4</sup>	1.8 × 10 <sup>6</sup> ¶	Pos 8.04	Pos 1.95	ND	T (100)
35	28	1 × 10 <sup>4</sup>	1.3 × 10 <sup>5</sup> ¶	Pos 8.30	Pos 2.91	1:40	I (80), T (20)
50	43	6 × 10 <sup>2</sup>	8.5 × 10 <sup>3</sup>	Pos 8.20	Pos 3.27	1:40	
54	47	2 × 10 <sup>3</sup>	6 × 10 <sup>2</sup>	Pos 8.15	Pos 3.43	1:40	
61	54	Neg	1.1 × 10 <sup>4</sup>	Pos 7.94	Pos 3.49	1:40	
68	61	Neg	6.8 × 10 <sup>1</sup>	Pos 7.42	Pos 3.54	1:40	
76	69	Neg	Neg	Pos 7.35	Pos 3.61	1:40	

\*I, isoleucine; Int, intermediate; ND, not done; Neg, negative; Pos, positive; T, threonine.

†Based on patient's memory of being bitten by a mosquito.

‡Days after start of headache, abdominal pain, nausea, and fever.

§Results of ELISA IgM and IgG given in relative units.

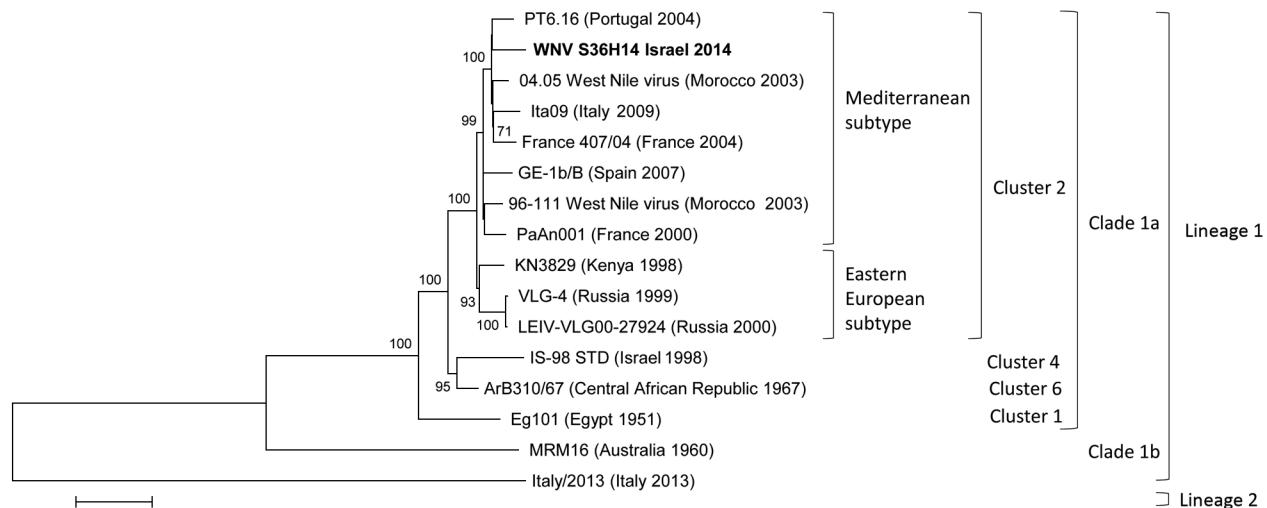
¶Sample sequenced.

NY, USA) (5) of RNA extracted from the patient's initial urine sample showed the highest identity with WNV sequences of the Mediterranean subtype within lineage 1, clade 1a, cluster 2 (Figure). Sequencing of the pre-membrane (prM), membrane, and envelope protein sequences (2,500 bp) obtained from the first serum sample showed identical sequences to those obtained from all subsequent urine samples taken until day 19. Sequencing of RNA from a urine sample obtained on day 28 revealed a single amino acid substitution, T20I (threonine to isoleucine), in WNV prM. Chromatogram results showed that this amino acid substitution was found in 80% of the sequences, displaying an almost complete change in virus sequence in <9 days.

No other nucleotide mutations in the prM or the envelope proteins were identified.

## Conclusions

The phenomenon of WNV RNA persistence has been described in urine (6,7), plasma (8,9), and whole blood (9,10). However, the reason the virus persists in some patients but not in others is unknown. In this study, we followed the kinetics of viral clearance and antibody response of a patient with WNV persistence and demonstrated that, despite the development of IgM and IgG, substantial amounts of WNV RNA persisted in serum for 47 days and in urine for 61 days after illness onset. Notably, isolation of infectious



**Figure.** Phylogenetic analysis of West Nile virus (WNV) lineage 1 isolate from a patient with persistent WNV infection, Israel, 2014, compared with reference strains. The analysis was conducted on 96% of the WNV nucleotide sequence using the neighbor-joining method implemented in MEGA 6.0 software (<http://www.megasoftware.net>). The robustness of branching pattern was tested by 1,000 bootstrap replications. The percentage of successful bootstrap replicates is indicated at nodes, showing only values of >70%. A WNV lineage 2 sequence obtained from strain ITA 13 (GenBank no. KF647252) was used as an outgroup. Bold indicates the WNV lineage 1 strain sequenced in this study. Scale bar indicates nucleotide substitutions per site.

virus from urine and appearance of an amino acid mutation in the prM of WNV on day 28 indicate not only persistence of WNV RNA but also active replication.

In assessing this case, we cannot exclude the possibility that the patient's genetic background or underlying conditions may have played a role in the control of WNV infection and persistence. Although patients with thymoma B2 may, in rare cases, exhibit hypogammaglobulinemia and cellular immune dysfunction (11), this patient was considered immunocompetent because his medical history and follow-up after this infection did not show any indication of immune deficiency. The change in neutrophil count during acute infection is intriguing because neutrophils have been shown to serve as a reservoir for WNV replication during early infection and to contribute to viral clearance at a later stage of illness (12).

The amino acid mutation identified in this study (T20I prM) is conserved in all of the WNV strains sequenced so far. A previous study found that the mutation of T20 prM to aspartic acid (instead of the isoleucine identified here) affected glycosylation, heterodimer formation, and the secretion of WNV-like particles (13). This amino acid is located in the pr section of the prM protein, which is cleaved during virion maturation into a pr protein and a small membrane-anchored M peptide. Not all prM proteins are cleaved during egress, because virions containing at least some uncleaved prM protein are found in bulk virus populations and are infectious (14,15). The presence of prM on virions has been shown to increase the sensitivity of virus particles to neutralization by some envelope-specific antibodies (15). We observed only a small increase in neutralization between a sample containing the wild type virus (1:20, day 15) and a sample that mostly contained the mutated virus (1:40, day 28) (Table). These findings suggest that development of IgG was deficient. It is currently not possible to determine whether the mutation arose due to the persistent viremia and/or the antibody deficiency, triggered it, or contributed to the mutation.

Future studies should investigate whether T20I prM modifies the ability of WNV antibodies to interact with the virus and affect viral growth, modification, and infectivity. In addition, in light of the data described here, examining the dynamics of WNV clones and mutations in other cases of persistent infection would be worthwhile.

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The Sheba Medical Center Ethical Review Board approved this study (2430-15-SMC).

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# Multidrug-Resistant *Escherichia coli* in Bovine Animals, Europe

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Of 150 *Escherichia coli* strains we cultured from specimens taken from cattle in Europe, 3 had elevated MICs against colistin. We assessed all 3 strains for the presence of the plasmid-mediated *mcr-1* gene and identified 1 isolate as *mcr-1*-positive and co-resistant to  $\beta$ -lactam, florfenicol, and fluoroquinolone antimicrobial compounds.

The dissemination of mobile genetic elements containing antimicrobial resistance genes and the emergence of carbapenem  $\beta$ -lactamases (e.g., *Klebsiella pneumoniae* carbapenemase-2 and New Delhi metallo- $\beta$ -lactamase-1) have narrowed the chemotherapeutic options available to clinicians (1,2). Treatment of infections associated with carbapenem-resistant *Enterobacteriaceae* requires the use of polymyxin B and polymyxin E (colistin). These cationic peptides are considered to be the last line of defense for infections in humans.

Colistin is a drug with a bactericidal action that targets the lipid A component of the lipopolysaccharide structure located in the outer wall of some gram-negative bacteria. Consequently, the drug exhibits a broad spectrum of activity against *Enterobacteriaceae* (3). Despite its use in animal production in certain countries, rates of resistance to colistin have so far remained low in animals and humans (3,4). Polymyxin resistance can develop after modification of the lipid A component in the lipopolysaccharide structure through mechanisms that are chromosomally mediated and result in a reduction in the affinity for these cationic peptides (5,6). In a recent report, Liu et al. (7) described the first known case of plasmid-mediated colistin resistance involving the *mcr-1* gene coding for a phosphoethanolamine transferase-like enzyme.

Considering the importance of colistin in the control of multidrug-resistant (MDR) nosocomial human infections caused by gram-negative bacteria and the use of this drug in veterinary medicine, the identification of the *mcr-1* gene in food-producing animals is of major public health importance. The objective of our study was to retrospectively investigate a large collection of *E. coli* cultured from cattle that had suspected enteric or mastitic infections.

## The Study

During 2004–2010, we cultured 150 *E. coli* strains from fecal samples collected from cattle with suspected enteric infection or milk-aliquots collected from cattle with suspected mastitis in France and Germany. We conducted antimicrobial susceptibility testing by using disk diffusion against a panel of 17 compounds consisting of penicillin G, amoxicillin, and amoxicillin/clavulanic acid; cephalothin, cefoxitin, cefotaxime, and cefepime; ertapenem, meropenem, and imipenem; marbofloxacin, ciprofloxacin, and nalidixic acid; gentamicin; tetracycline; florfenicol; and trimethoprim/sulfamethoxazole. We interpreted results according to the criteria of the Clinical and Laboratory Standards Institute where appropriate (8,9).

A subset of these *E. coli* (n = 45) were classified as MDR and expressed resistance to  $\geq 3$  drug classes. We determined plasmid profiles and PCR-based replicon types as described previously (10,11) and detected plasmids ranging in size from 2 to 200 kbp. Our PCR-based replicon type analysis identified several incompatibility (Inc) types, including IncX4 in *E. coli* strain 11-1896 and the previously reported IncHI2 type in *E. coli* strain 29957 (Table). We then determined the MICs of these 45 MDR isolates for colistin by using broth microdilution. Three of 45 demonstrated MICs  $> 2$  mg/L, which we interpreted as being colistin resistant based on breakpoint tables of the European Committee on Antimicrobial Susceptibility Testing (12). We identified these isolates as *E. coli* 22134 O9:H9 U/ST10, *E. coli* 11-1896 O9:H12 U/ST58, and *E. coli* 29957 O101:H9 A or C/ST167 (Table). All were additionally resistant to  $\geq 2$  drug classes, including aminoglycosides, aminopenicillins, cephalosporins, fluoroquinolones, phenicols, tetracyclines, and trimethoprim and sulfonamides. One of the 3 isolates (*E. coli* 29957) was resistant to all of the antimicrobial compounds tested, including  $\beta$ -lactams, florfenicol, and fluoroquinolone

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**Table.** Selected characteristics of 3 colistin-resistant *Escherichia coli* isolates cultured from cattle with suspected enteric or mastitic infections, France and Germany, 2004–2010\*

<i>E. coli</i> isolate	Year of isolation	Phylotype	ST	Plasmid size, kbp	PBRT†	Antimicrobial resistance profile	Antimicrobial resistance genotypes†	Colistin MIC, mg/L
22134	2004	U	ST10	147; 57; 36	IncFIB, IncFIC, IncFII	AML, NAL, CT	<i>bla</i> <sub>TEM-1B</sub> , <i>strAB</i> , <i>tet</i> (34), <b><i>gyrA</i></b> , <b><i>parE</i></b> , <b><i>pmrA</i></b> , <b><i>pmrB</i></b>	8
11-1896	2010	U	ST58	147; 120; 36; 28; 22; 15; 2	IncFIB, IncFII, IncI1, IncQ1, IncX4	AML, CT, CTX, KF, TE, STX	<i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>strAB</i> , <i>sul2</i> , <i>tet</i> (A), <i>tet</i> (34), <i>dfrA5</i> , <b><i>gyrB</i></b> , <b><i>pmrA</i></b> , <b><i>pmrB</i></b> , <b><i>phoB</i></b> , <b><i>eptB</i></b>	8
29957	2007	A or C	ST167	200; 147; 36	<b>IncFIA</b> , <b>IncFIB</b> , IncFIC, IncFII, <b>IncH12</b> , IncHI2A, IncQ1	AMC, AML, CN, CIP, CT, FLO, MAR, NAL, TE, STX	<i>bla</i> <sub>TEM-1A</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>aadA1</i> , <i>aadA2</i> , <i>aadB</i> , <i>aph</i> (3')-Ia, <i>aac</i> (3)-Iia, <i>strAB</i> , <i>sul1</i> , <i>sul2</i> , <i>sul3</i> , <i>tet</i> (A), <i>tet</i> (B), <i>tet</i> (34), <i>dfrA1</i> , <i>mcr-1</i> , <i>mef</i> (B), <i>catA1</i> , <i>cmlA1</i> , <i>floR</i> , <b><i>gyrA</i></b> , <b><i>parC</i></b> , <b><i>pmrB</i></b>	4

\*With the exception of *mcr-1* in *E. coli* 29957, genes shown in bold are located on the chromosome in which nonsynonymous amino acid substitutions were identified in the corresponding proteins (online Technical Appendix Tables 1, 2, <http://wwwnc.cdc.gov/EID/article/22/9/16-0140-Techapp1.pdf>) known to confer resistance to quinolones and colistin. Plasmid replicon types and *mcr-1* genes shown in bold were further confirmed by PCR. AMC, amoxicillin/clavulanate; AML, amoxicillin; CN, gentamicin; CT, colistin; CIP, ciprofloxacin; CTX, cefotaxime; FLO, florfenicol; Inc, incompatibility type, KF, cephalothin; NAL, nalidixic acid; MAR, marbofloxacin; PBRT, PCR-based replicon types; TE, tetracycline; ST, sequence type; STX, trimethoprim/sulfamethoxazole.

†Indicates plasmid replicon types and antimicrobial resistance genotypes extracted from whole genome sequencing data.

compounds (Table). In addition, PCR results indicated that this isolate was positive for the presence of the *mcr-1* gene (online Technical Appendix Figure, panel A, <http://wwwnc.cdc.gov/EID/article/22/9/16-0140-Techapp1.pdf>) (7).

We conducted whole-genome sequencing of 3 isolates with increased MICs for colistin by using the Nextera XT DNA Library Preparation Kit and the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) to produce 300-bp paired end reads (v3 chemistry). We assembled these data de novo using SPAdes version 3.6.2 (<http://bioinf.spbau.ru/spades>) and then generated queries by using the PlasmidFinder 1.3 (<https://cge.cbs.dtu.dk/services/plasmidfinder>) and ResFinder 2.1 (<http://cge.cbs.dtu.dk/services/resfinder>) databases to identify plasmid replicon types and antibiotic resistance genes using BLAST+ (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Several antibiotic resistant genotypes, including some that were acquired, were identified. Four of these genotypes occurred in *E. coli* 22134 isolates, 8 in *E. coli* 11-1896 isolates, and 21 in *E. coli* 29957 isolates (Table). These isolates harbored genes or mutations that confirmed the phenotypes detected in most of the suspected cases of infection in the cattle in our study. We also identified plasmid replicons in all 3 isolates, including 3 types in *E. coli* 22134; 5 in *E. coli* 11-1896, and 7 in *E. coli* 29957. We did not detect the *mcr-1* gene in *E. coli* 22134 or *E. coli* 11-1896; however, we identified several nonsynonymous amino acid substitutions in genes previously shown to be associated with colistin resistance, including *pmrA* and *pmrB*. We also identified *phoP* and *eptB* in *E. coli* 11-1896. Similarly, we identified the *mcr-1* gene in *E. coli* 29957 (a feature that was previously confirmed by PCR) and 1 nonsynonymous substitution in *pmrB*. The *mcr-1* gene was located in a 4,752-bp contig, which when used to query the cur-

rent databases matched an identical region containing a transposase gene, a phosphoethanolamine transferase gene (the *mcr-1* encoding gene), a hypothetical protein/phosphoesterase gene, and another transposase. The *mcr-1* gene was 100% similar at the nucleotide level to that reported in China and was found to be located distal to the same insertion sequence element IS*Apl1* that mapped to the IncHI2 type plasmid pHNSHP45 (online Technical Appendix Figure, panel B) (7).

## Conclusions

Plasmid-mediated colistin resistance identified in MDR bacteria of animal origin represents a serious risk to public health. Our data further support recent findings demonstrating that the *mcr-1* gene is not just present in Asia but can also be found in some countries in Europe (e.g., the *mcr-1* gene identified in an *E. coli* strain cultured from a food-producing animal in France in 2007) (Table). Other arrangements of the *mcr-1* gene on plasmids can occur, such as that observed in the IncX4 type (13). Liu et al. (7) reported that plasmid pHNSHP45 exhibited an in vivo transfer rate between different *E. coli* strains (measured at 10<sup>-1</sup> to 10<sup>-3</sup> per recipient) (7), a feature that could contribute to the successful dissemination of the *mcr-1* gene. Similarly, in our study, we can also confirm the transfer of the *mcr-1* gene from *E. coli* 29957 via conjugation, albeit at a reduced frequency (data not shown). Especially concerning is the extensive resistance profile of *E. coli* 29957, a feature noted in other studies, which have indicated that colistin resistance might be co-selected after the use of cephalosporins and other compounds (14,15).

The *mcr-1* gene has now been reported in food-producing animals and in humans located in different geographic regions. In several of these regions, the gene was linked to

extended-spectrum  $\beta$ -lactam and florfenicol resistance in the same bacterial isolate (15). Because *E. coli* 29957 was identified in 2007, this finding cannot be considered a recent occurrence. Given the genetic mapping reported to date, selective pressure imposed after the administration of broad-spectrum cephalosporins and other compounds might have the potential to co-select for colistin resistance and vice versa, thereby contributing to the dissemination of *mcr-1* (15). Molecular epidemiologic studies are required to discover the origin and means of transmission of this gene as a first step in attempting to limit its dissemination, particularly among pathogenic bacteria that threaten human health.

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# World Rabies Day, September 28

Rabies is a deadly disease that can kill anyone who gets it. Every year, an estimated 40,000 people in the United States receive a series of shots due to potential exposure to rabies. Each year around the world, rabies results in more than 59,000 deaths—approximately 1 death every 9 minutes.



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# Secondary Infections with Ebola Virus in Rural Communities, Liberia and Guinea, 2014–2015

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In rural communities in Liberia and Guinea, more secondary Ebola infections resulted from persons who died of Ebola virus disease at home than from persons admitted to Ebola treatment units. Intensified monitoring of contacts of persons who died of this disease in the community is an evidence-based approach to reduce virus transmission in rural communities.

Transmission of Ebola virus occurs through direct contact with blood or other body fluids of an infected person after symptoms have developed. During an outbreak of Ebola virus disease (EVD), monitoring persons (termed contacts) who have exposure to persons with EVD is the most effective way to identify and isolate new cases rapidly before transmission can occur (1). At the height of the 2014–2015 epidemic in West Africa, response teams were monitoring daily >7,000

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contacts in Liberia, 8,900 in Sierra Leone, and 2,800 in Guinea (Emergency Operations Center, Centers for Disease Control and Prevention, Atlanta, GA, USA, pers. comm.).

The World Health Organization (WHO) guidelines for monitoring contacts of persons with EVD treat all contacts equally (1). However, when resources are limited, evidence-based criteria for identifying cases of EVD most likely to result in secondary infections could help to optimize control. We analyzed data from outbreaks in rural areas of Liberia and Guinea to determine whether intensifying monitoring of contacts of persons with EVD who died at home in the community was warranted.

## The Study

Under the leadership of the Ministries of Health (MOHs) of Liberia (July–December 2014) (2) and Guinea (December 2014–June 2015), epidemiologists from multiple agencies investigated rural outbreaks of EVD. Within a community outbreak, field epidemiologists identified case-patients, monitored their contacts, and developed diagrams of infection sequences on the basis of interviews with patients, families, and community members. Transmission diagrams began with the first case identified in the community (index case) and ended when all known contacts had completed 21 days of monitoring with no new cases identified. In both countries, information from EVD case report forms included age, sex, date of symptom onset, date of isolation in an Ebola treatment unit (ETU), and date of recovery or death. Data from Guinea also included whether the person who died at home received a safe and dignified burial performed by trained teams (3). These investigations were conducted as part of the Ebola public health response in West Africa and were not considered to be human subjects research.

Data for Liberia and Guinea were combined. We used generalized estimating equations with a negative binomial distribution to compare the number of secondary infections between groups, including between persons with EVD who died at home in the community and those admitted to an ETU, between persons who were severely ill (death <3 days after admission) and those less ill (death ≥3 days of admission or recovery) at the time of admission to an ETU, and between persons who were buried safely by trained burial teams and those buried by untrained persons (in Guinea only). Additional details on statistical analyses are included in the online Technical Appendix (<http://wwwnc.cdc.gov/eid/article/22/9/16-0416-Techapp1.pdf>).

Data were available for 347 persons with EVD from 17 transmission chains; 240 (69%) persons were confirmed by

**Table.** Characteristics of persons with Ebola virus disease in rural areas of Liberia and Guinea, 2014–June 2015\*

Characteristic	Liberia, n = 165	Guinea, n = 182	Total, n = 347
No. transmission chains	9	8	17
Laboratory-confirmed EVD	114 (69)	126 (69)	240 (69)
Outcome			
Admitted to an ETU and recovered	49 (30)	29 (16)	78 (22)
Admitted to an ETU and died	51 (31)	51 (28)	102 (29)
Admitted to an ETU and unknown outcome	0 (0)	5 (3)	5 (1%)
Died at home in the community	60 (37)	97 (53)	157 (45)
Recovered in the community	4 (2)	0 (0)	4 (1)
Generated $\geq 1$ secondary EVD infections	37 (24)	62 (39)	99 (31)
Source case died in the community	31 (55)	51 (55)	82 (55)
Source case was admitted to an ETU	5 (5)	11 (16)	16 (10)
Source case survived in the community	1 (25)	0 (0)	1 (25)
No. days at risk for transmitting secondary infections in the community	5.8 (5.2–6.5)	8.1 (1.8–14.4)	6.8 (4.0–9.6)
Timing of death within an ETU			
Died <3 d after admission	12 (12)	12 (16)	24 (12)
Died $\geq 3$ d after admission or recovered	85 (88)	65 (84)	150 (86)
Burial status of those who died at home in the community			
Safely buried	NA	38 (40)	NA
Not safely buried	NA	56 (60)	NA

\*Values are no. (%) or no. (95% CI). Percentages are proportions of data not missing. ETU, Ebola treatment unit; EVD, Ebola virus disease; NA, not available.

using laboratory analysis (real-time PCR) as having EVD (Table). Most (185, 53%) persons with EVD were admitted to an ETU, of whom 102 (55%) died, 78 (42%) recovered, and 5 (3%) had a missing outcome. A total of 162 (47%) persons were not admitted to an ETU, of whom 157 (97%) died at home in the community and 4 (2%) recovered without hospitalization (3 had confirmed cases and 1 had a probable case). The overall case-fatality rate was 76% (95% CI 71%–81%).

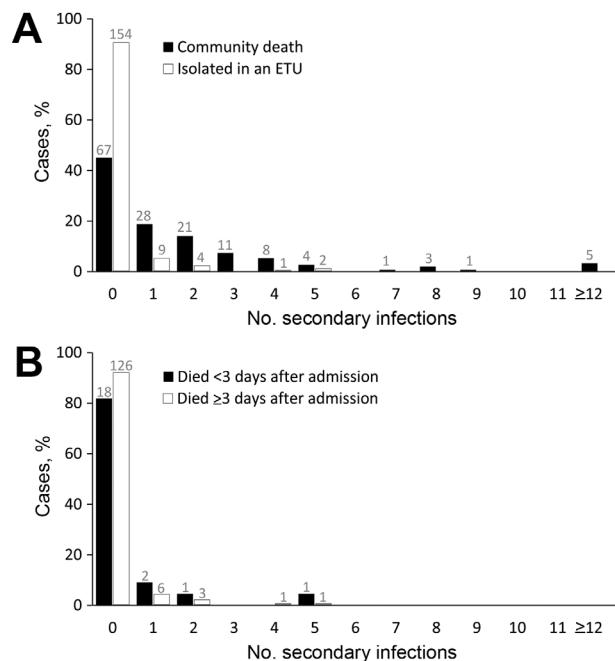
We determined the number of secondary infections for 317 (91%) persons with EVD who had outcome data; 99 (31%) resulted in  $\geq 1$  secondary infections, and there were differences by outcome status (Table). When we excluded the 4 case-patients who recovered in the community without hospitalization, the mean number of secondary infections was significantly higher for persons with EVD who died at home in the community (1.8, 95% CI 1.3–2.3) than for persons admitted to an ETU (0.2, 95% CI 0.1–0.3;  $p = 0.003$ ) (Figure 1, panel A).

We found no significant difference in the mean number of secondary infections between those who died <3 days after admission to the ETU (0.4, 95% CI 0.05–0.90) and those who died later in their hospitalization or recovered (0.1, 95% CI 0.06–0.30;  $p = 0.24$ ) (Figure 1, panel B). We also found no significant difference in the mean number of secondary infections associated with cases of EVD in persons who received a safe burial (1.2, 95% CI 0.8–1.7) versus those who did not receive a safe burial (1.8, 95% CI 1.1–2.5;  $p = 0.40$ ) (Figure 2).

## Conclusions

In rural outbreaks in Liberia and Guinea, Ebola virus transmission was driven by contact with persons who died of EVD at home, and isolation before death was associated with 88% fewer secondary infections. Possible reasons for

a larger number of secondary cases associated with deaths at home in the community include 1) a higher per-contact probability of transmission caused by higher levels of viremia or more exposure to body fluids during terminal illness and death (4); 2) a greater number of contacts between uninfected persons and persons with EVD during their terminal illness or after death; and 3) a greater number of



**Figure 1.** Percentile distribution, by number of secondary infections, of persons with Ebola virus disease (EVD) in rural outbreaks in Liberia and Guinea, 2014–2015. A) Comparison of persons with EVD who died at home in the community and those who were isolated and treated in Ebola treatment units (ETUs). B) Comparison of persons admitted to ETUs who died <3 days or  $\geq 3$  days after admission. Numbers above bars indicate actual counts.

uninfected persons having contact with persons with EVD during their terminal illness or after death.

Because we did not find an increase in secondary infections according to severity of illness at the time of ETU admission (a proxy for level of viremia), we believe that factors associated with the death are critical in transmission. Traditional burial practices in West Africa include touching and washing the body after death (5). Extensive postmortem exposures to body fluids and skin could occur during that time, and postmortem studies of nonhuman primates have shown that Ebola virus is stable in body fluids for as long as 3 weeks (6). However, it is also likely that there is an increased number of secondary infections because more persons touch the corpse while paying respect to the deceased than would touch a living patient during their illness (7).

Yamin et al. used a stochastic modeling approach to integrate epidemiologic data on Ebola from Liberia to identify potential intervention targets (8). Similar to our data, they found that secondary cases were most associated with nonsurvivors, and that isolation within 4 days of symptom onset could eliminate disease transmission. Our findings differ slightly from those of Yamin et al. because they suggest that isolation of cases of EVD at any time before death would reduce transmission.

In Guinea, we did not find burials reported as safe to have had an effect on reducing the number of secondary infections. Although some of the safe burials might have been misclassified, it is more likely that traditional mourning practices occurred before safe burial teams arrived (7). Response to future outbreaks should emphasize prevention of exposure to Ebola virus during mourning and burial, and cadavers should be classified as safely buried only if they have not been touched after death.

Classifying contacts of persons who died of EVD at home in the community as high-risk, regardless of whether they were reported to have received a safe burial, is an evidence-based approach to prioritizing those persons who should receive more rigorous monitoring. Intensive follow-

up could include assignment of highly trained staff to evaluate high-risk contacts more frequently, provision of incentives to complete the 21-day monitoring period, and housing high-risk persons in managed voluntary quarantine facilities.

### Acknowledgments

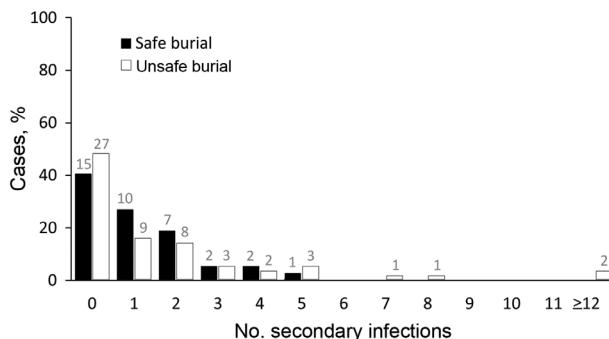
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**Figure 2.** Percentile distribution, by number of secondary infections, of persons with Ebola virus disease in rural outbreaks who died at home in the community, by safe burial status, Guinea, 2015. Numbers above bars indicate actual counts.

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# Changing Diagnostic Methods and Increased Detection of Verotoxigenic *Escherichia coli*, Ireland

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The recent paradigm shift in infectious disease diagnosis from culture-based to molecular-based approaches is exemplified in the findings of a national study assessing the detection of verotoxigenic *Escherichia coli* infections in Ireland. The methodologic changes have been accompanied by a dramatic increase in detections of non-O157 verotoxigenic *E. coli* serotypes.

Verotoxigenic *Escherichia coli* (VTEC) can cause severe disease in humans, with signs and symptoms including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (1). The primary reservoir of VTEC is ruminants, and sporadic outbreaks are commonly associated with animal contact, exposure to animal feces, food and water contamination, and person-to-person transmission (2).

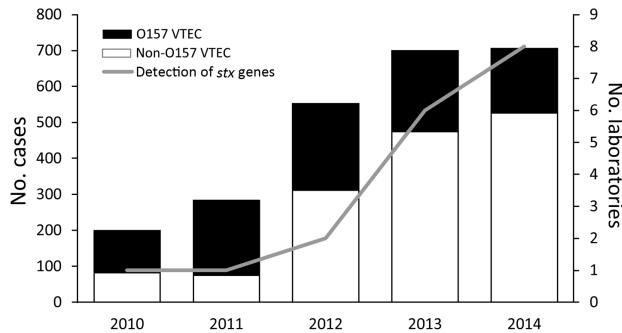
In Ireland, a country with a population of  $\approx 4.6$  million, all VTEC infections have been notifiable to the Medical Officer of Health since 2004. Initially, these infections were included under specified infections caused by enterohemorrhagic *E. coli* (EHEC), comprising any strains of serogroups O157, O26, O103, O111, and O145 (3); since an amendment of September 2011, these infections have been specified as VTEC infections (4). Data on VTEC infections in Europe have been provided since 2008 from the member states to the European Centre for Disease Prevention and Control. These data indicate that Ireland had the highest annual incidence of VTEC infection during 2009–2014, with the exception of 2011, when a large outbreak of *E. coli* O104:H4 infections occurred in Germany (5). The Health Protection and Surveillance Centre releases annual reports on the number of VTEC infection cases in Ireland, which document an increase in the number of VTEC infections in Ireland during 2010–2014 (Figure). This trend is thought to be largely attributable to an increase in non-O157 VTEC infections. A low infectious dose of  $\approx 10$  microorganisms has been described as sufficient to cause disease that can lead to hemolytic uremic syndrome (6). Real-time PCR (rPCR) has been demonstrated as a more rapid and sensitive method of VTEC detection, compared with enzyme immunoassay and culture (7).

We conducted a survey of clinical microbiology laboratories in Ireland during 2014 to assess laboratory practices for the detection and confirmation of VTEC infection. The survey included 45 questions, arranged in 4 categories: laboratory details (e.g., contact details, address, and public/private status); sample type, selection, and requirements for VTEC testing; current methods (i.e., utilization and description of enrichment, culture, biochemical characterization, antimicrobial susceptibility testing, serotyping, molecular testing, and verotoxin detection methods), past changes to methods, and intended future changes to methods for VTEC analyses; and referral and reporting procedures. Ireland is home to 25 clinical microbiology laboratories, all of which were represented in the survey findings presented here. Laboratories included the VTEC National Reference Laboratory in Cherry Orchard Hospital, Dublin, which provides the referral service for this pathogen to all clinical laboratories in Ireland, whether public or private. Laboratories were categorized into 3 groups based on their testing strategy: culture-based detection, PCR-based detection, or both. An analysis of the introduction of molecular-based detection of VTEC infection (i.e., rPCR of verotoxin genes *vt1* and *vt2*) in clinical laboratories in Ireland indicated a timeline for the implementation of these methods from 2010 to 2014. Before 2012, the reference laboratory was the sole laboratory implementing PCR-based detection of VTEC. In 2012, a regional clinical laboratory introduced an automated molecular platform for the detection of VTEC and other gastrointestinal pathogens. Four more laboratories introduced this molecular platform in 2013, with another 2 converting in 2014. Five clinical laboratories expressed an intention to introduce PCR-based detection of VTEC starting in 2015.

These data represent a shifting trend from the conventional culture-based detection of fecal coliforms (with confirmatory testing as VTEC), to a direct molecular approach in which stool specimens are tested initially for the presence of the verotoxin genes. This move toward molecular detection of VTEC infection is consistent with the increased number of non-O157 VTEC cases reported in Ireland in recent years (Figure). Similar results were reported in a study of the same nature conducted in Washington, USA (8). Along with the increased number of clinical laboratories participating in molecular detection of VTEC, a concomitant shift has occurred in the culture-based detection of VTEC. The introduction of an agar for the detection of

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**Figure.** Number of reported cases of O157 and non-O157 VTEC infection and number of laboratories performing PCR-based detection of *stx* gene, by year, Ireland, 2010–2014. *stx*, Shiga toxin; VTEC, verotoxigenic *Escherichia coli*.

Shiga-toxin–producing *E. coli* (CHROMagar STEC; Mast Diagnostika, Reinhold, Germany) to laboratories, which is selective for the top 6 non-O157 serogroups (O26, O45, O103, O111, O121, and O145), supports the recently held view that a range of VTEC serogroups are now considered a major cause of human VTEC infection (5,9).

With the exception of the reference laboratory, molecular VTEC detection in clinical laboratories in Ireland use a commercial rPCR-based platform. This method, which selects for verotoxin genes, accelerates more sensitive detection compared with traditional culture-based methods.

Our own recent observations in Ireland (10,11) may very well represent the start of a global trend toward molecular detection of verotoxin genes from clinical and other samples, to replace culture-based methods, as a first step in the detection of VTEC (12). Our findings suggest that increases in notifications of VTEC infections, and particularly those caused by non-O157 serotypes, be interpreted in light of this paradigm shift in infectious disease diagnostic methods.

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Mr. Rice is a medical scientist who is currently undertaking a PhD at Cork Institute of Technology and University College, Cork, jointly.

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## Local Persistence of Novel MRSA Lineage after Hospital Ward Outbreak, Cambridge, UK, 2011–2013

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**To the Editor:** Previously, we reported the use of whole-genome sequencing to investigate a putative methicillin-resistant *Staphylococcus aureus* (MRSA) outbreak in 2011 in the special care baby unit (SCBU) at the Cambridge University Hospitals National Health Service Foundation Trust (CUH) in the United Kingdom (1). The report identified 26 related cases of infection with or asymptomatic carriage of MRSA and showed that transmission occurred within the SCBU, between mothers on a postnatal ward, and in the community; the outbreak apparently resolved at the end of 2011. The outbreak strain, sequence type (ST) 2371, was of a novel multilocus ST related to the dominant hospital-associated lineage in the UK (ST22, EMRSA-15), but unlike most ST22 strains, this strain was Panton-Valentine leucocidin–positive (2). Since then, ST2371 has been identified as a prevalent community-associated MRSA clone in Southern India, and sporadic

isolates have also been detected by whole-genome sequencing of MRSA in Denmark (3–5).

During April 2012–April 2013, we implemented genomic surveillance of MRSA isolated at the diagnostic microbiology laboratory at the CUH (F. Coll, unpub. data). From this, we noted that 10 isolates cultured from samples submitted from general practice (n = 7) and hospital wards (n = 3) during June 2012–February 2013 were classified as ST2371. Phylogenetic comparison between these 10 isolates and the 45 isolates from the original outbreak demonstrated that these strains were highly related (staphylococcal cassette chromosome *mec* IVc, Panton-Valentine leucocidin–positive, staphylococcal protein A [*spa*] type 852) (Figure).

We undertook an epidemiologic investigation to determine whether links could be identified between these new cases and the original outbreak. The 10 isolates were cultured from 5 patients (case-patients A–E), all of whom had a direct or indirect link to the 2011 outbreak. Case-patients from the 2011 outbreak are identified by the alphanumeric code assigned during that outbreak investigation (e.g., P22) (1).

Case-patients A and B were also case-patients in the original SCBU outbreak (P22 on the postnatal ward and P14 in the SCBU, respectively). Case-patient C was born at the CUH and was not screened for MRSA, but both parents were case-patients in the SCBU outbreak (P20 and P26). Case-patient D was born at the CUH and discharged when 5 days old, which was 2 days before the birth of the presumed index case-patient of the original SCBU outbreak. The sample for the first isolate from case-patient D was collected almost 2 years later; acquisition could have occurred at the

**Figure.** Midpoint-rooted phylogenetic tree based on single-nucleotide polymorphisms (SNPs) in the core genome of methicillin-resistant *Staphylococcus aureus* isolates from 2 investigations in the United Kingdom in 2011 and 2012–2013. Isolates were mapped against the EMRSA-15 reference genome. Open circles denote 20 individual colonies from a nasal swab culture taken from a healthcare worker during an outbreak in a hospital special care baby unit (SCBU) in 2011. Gray shaded circles denote isolates from 25 patients and their family members investigated during the 2011 outbreak. Black circles denote 10 isolates from 5 persons (case-patients A–E) from whom microbiological samples were taken during the 2012–2013 study. Numbers prefixed by P indicate the original study number used for each case during the 2011 outbreak investigation. Multiple samples from the same patient are identified by an underscore followed by the sample number. Two case-patients (P22/A and P14/B) were included in both outbreaks. Scale bar indicates SNPs.



CUH or from subsequent contact with unsuspected carriers in the case-patient's family or the community. On the basis of a matching surname, case-patient E was determined to be a member of the same family as case-patients P20, P26, and likely C. Soft tissue infection was documented in all 5 case-patients, supporting the original observation that ST2371 is associated with disease. Evidence of familial transmission in the original outbreak is further supported by transmission between case-patients P20, P26, C, and E. Furthermore, 2 case-patients infected during the original outbreak, P22/A and P14/B, continued to experience disease signs and symptoms for  $\geq 15$  months after their initial diagnosis.

Our data highlight the role of hospitals as reservoirs of MRSA and subsequent failure to track the entry and spread of MRSA in the community. MRSA decolonization was advised in all cases in the original outbreak, but this process clearly proved ineffective for case-patients A and B. Potential explanations include not implementing or completing the course of decolonization; failed decolonization; or limiting decolonization to only some members of an affected family. Although the outbreak in the hospital ward was resolved, the lack of a systematic surveillance program to monitor the incidence of noninvasive MRSA infections among the case-patients' contacts and the community allowed this novel lineage to continue to cause disease in a group of linked persons. Considering recommendations to move from universal to targeted MRSA screening in hospitals in England (6), more active surveillance of any identified case-patients or carriers of MRSA in the community may be warranted.

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M.S.T., F.C., E.M.H., and S.R. undertook epidemiological and bioinformatic analysis of whole-genome sequence data. S.R. prepared the figure. S.J.P. supervised and managed the study. All authors were involved in compiling the report and approved the final version.

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## Community-Acquired *Clostridium difficile* Infection, Queensland, Australia

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**To the Editor:** In Queensland, Australia, a steady increase in community-acquired (CA) *Clostridium difficile* infections (CDI) during 2003–2012 could not be explained by patients' demographic characteristics or environmental

factors (1). Several risk factors have been implicated in the increased rates of CA-CDI, primarily exposure to antimicrobial drugs, gastric acid-suppression drugs, and corticosteroids (2). Given the recent rise in prescription of corticosteroids and proton pump inhibitors in Australia, we hypothesized that the observed increase in CA-CDI was associated with increased drug prescriptions.

To test our hypothesis, we analyzed a subset of data used in a previous study (1), which included fecal samples from patients seen by general practitioners in the community from January 2008 through December 2012. The samples were submitted to Sullivan Nicolaides Pathology (Taringa, Queensland, Australia) for *C. difficile* toxin gene detection. After samples submitted from healthcare facilities and nursing homes were excluded, the final dataset contained data from 14,330 fecal samples. We aggregated the data by patient sex, age categories, year, and statistical area level 4 (SA4). For each sex-age-year-SA4 group, we used as numerators the numbers of CA-CDI cases identified and as denominators the numbers of samples submitted for microbiological testing.

The Australian Department of Human Services provided data from the Pharmaceutical Benefits Scheme. The quantities of 11 anatomic therapeutic chemical drugs were accessed by patient sex, age group, year, and SA4. Corresponding with the CA-CDI data, medication data to be analyzed were then aggregated by sex, age group, year, and SA4.

For each medication, we built binomial logistic regression models, using CA-CDI status as the outcome, in a Bayesian framework, incorporating fixed effects for sex, age group, quantity of drug prescribed, year (2008–2012), and spatially unstructured random effects at the SA4 level. After performing an initial burn-in, we stored and summarized 1,000 values from the posterior distribution of each parameter by using descriptive statistics (posterior mean, 95% posterior credible interval [95% CrI], and p value). We examined multiple pairwise comparisons of CA-CDI and medication exposure; thus, we used the Holm adjustment for p values to avoid inflation and to control the familywise error rate.

Of the 14,330 fecal samples tested, 1,430 (10%) were positive for *C. difficile*. The proportion of positive fecal samples increased over the 5-year period, from 7.10% in 2008 to 12.72% in 2011 and 11.48% in 2012 ( $p < 0.001$ ). After adjusting the regression models for sex, age group, temporal pattern, and spatial distribution, we found that exposure to antimycobacterial drugs (odds ratio [OR] 1.09; 95% CrI 1.02–1.16) and anthelmintic drugs (OR 1.07; 95% CrI 1.01–1.13) were associated with increased odds of CA-CDI. After post hoc Holm adjustments, no statistically significant association between medication exposure and CA-CDI was observed (Table).

Our findings suggest that the increase in CA-CDI proportion was not associated with population-level medication exposure in Queensland during 2008–2012. CA-CDI epidemiology in Queensland might be driven by a group of factors other than medication exposure, such as transmission of the pathogen from food, animals, or hospitals into the community. Studies have confirmed the risk for foodborne and animalborne spread of *C. difficile* into the community (3). In Australia and New Zealand, importation of onions and garlic from the United States and Mexico might be responsible for increased CDI cases during Southern Hemisphere summers (4), and high prevalence of *C. difficile* colonization in piglets has been identified (5). However, the role of these factors in leading to CA-CDI cases remains unknown.

A recent contact tracing study in the United Kingdom demonstrated that a considerable proportion of CDIs among patients in healthcare settings originated from the community (6); this finding was supported by another study, which showed that in Queensland, more than two thirds of patients with CA-CDI required hospitalization (7). Currently, there is no evidence of a reverse-infection route (healthcare-acquired CDI being transmitted to persons in the community). However, Sethi et al. documented environmental shedding of *C. difficile* by inpatients for several weeks after resolution of symptoms (8). Therefore, the possibility that asymptomatic patients might be a source of transmission after hospital discharge needs to be examined. In recent years, epidemiologic models

**Table.** Binomial logistic regression models for medication exposure adjusted for sex, age group, temporal pattern, and spatial distribution among patients with community-acquired *Clostridium difficile* infection, Queensland, Australia, 2003–2012

Medication exposure	Odds ratio (95% credible interval)	p value	Holm-adjusted p value
Drugs for acid-related disorders	1.052 (0.943–1.163)	0.348	0.819
Drugs for constipation	1.056 (0.963–1.151)	0.235	0.781
Antidiarrheal drugs	1.106 (0.994–1.218)	0.051	0.379
Antithrombotic drugs	1.073 (0.955–1.197)	0.224	0.781
Corticosteroids for systemic use	1.043 (0.952–1.133)	0.348	0.819
Antibacterial drugs for systemic use	1.083 (0.990–1.174)	0.067	0.425
Antimycotic drugs for systemic use	1.035 (0.944–1.126)	0.454	0.819
Antimicrobial drugs for mycobacterial infections	1.089 (1.023–1.155)	0.006	0.063
Anti-inflammatory drugs	1.070 (0.970–1.170)	0.158	0.700
Antiprotozoal drugs	1.037 (0.953–1.123)	0.394	0.819
Anthelmintic drugs	1.068 (1.008–1.127)	0.021	0.189

exploring the role of CDI coming from the community into the hospital have become increasingly popular (9); however, to the best of our knowledge, only 1 modeling study described CDI dynamics within the wider community (10). Although this approach is innovative, we acknowledge some limitations. Medication exposure was used as a proxy, based on the average prescription in the community, and it cannot be applied to the individual patient. In addition, we were unable to adjust the regression model for the presence of concurrent medical conditions and other unmeasured confounders.

Exposure to medications, particularly antimicrobial drugs, probably influences CA-CDI pathogenesis (2). However, our community-based assessment indicates that a more holistic exploration is needed to identify alternative factors driving increases in CA-CDI cases in the wider population.

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## Multidrug-Resistant *Campylobacter coli* in Men Who Have Sex with Men, Quebec, Canada, 2015

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**To the Editor:** In 2015, an outbreak of multidrug-resistant *Campylobacter coli* was documented in Montreal, Quebec, Canada. We report results of an epidemiologic and molecular investigation suggesting a sexually transmitted enteric infection among men who have sex with men (MSM).

The ethics committee of Centre Hospitalier de l'Université de Montréal approved the research. During January 14–February 7, 2015, six men 35–62 years of age were documented with an enteric, erythromycin-, tetracycline- and ciprofloxacin-resistant *C. coli* pulsovar 15 infection. All 6 men had diarrhea; 5 had abdominal pain; 1 had fever  $\geq 39^{\circ}\text{C}$ ; 1 had blood in feces; and 1 had vomiting. No extraintestinal focus was documented in these patients.

Five men were evaluated in the outpatient clinic or emergency department; 1 man was hospitalized for 3 days. Five patients were treated with an antimicrobial agent.

Three were treated orally for 4–7 days: 1 with ciprofloxacin, 1 with azithromycin, and 1 with both drugs. One patient was treated for 3 days with intravenous ceftriaxone and vancomycin followed by 10 days of amoxicillin for simultaneous *Streptococcus pneumoniae* septicemia. One man was treated with 1 intramuscular ceftriaxone dose, doxycycline for 21 days, and intravenous ertapenem for 3 days for proctitis and enterocolitis. All patients recovered with treatment (in vitro susceptible or resistant agent) or without treatment.

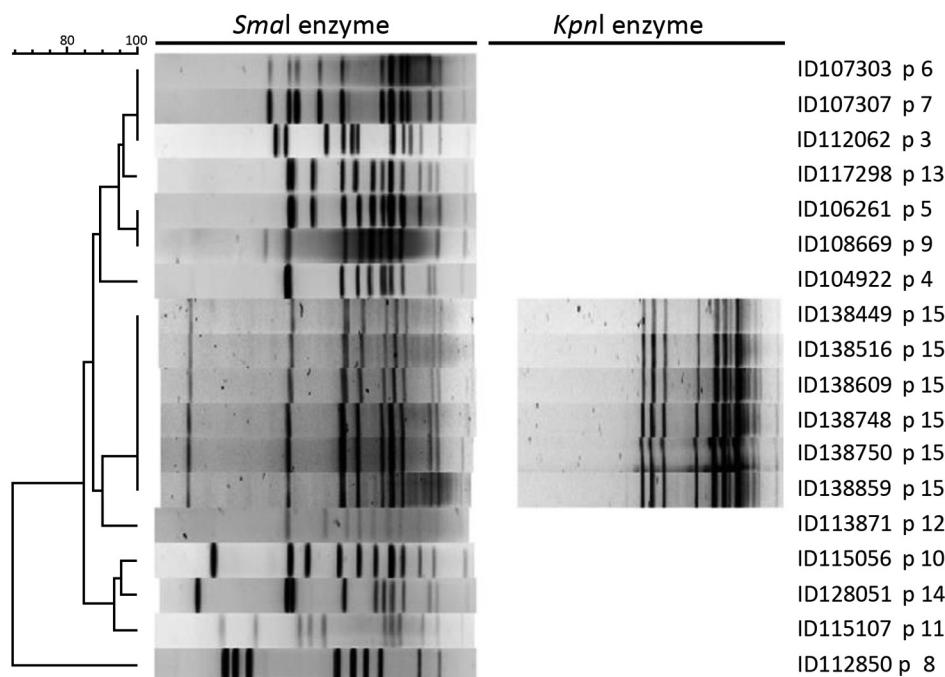
The 6 men reported to be MSM. The week before symptom onset, 4 men reported having had unprotected sex, 2 in bathhouses. Before the *C. coli* incubation period and after the outbreak started, 1 of these 2 men had traveled to the Caribbean but did not have sexual relations there. These men were not explicitly linked to each other. Five men were HIV positive; 1 was HIV negative. The 5 HIV-positive men had CD4 counts ranging from 210 to 1,150  $\times 10^6$  cells/L and HIV viral load of <40 copies/mL. Since 2010, the 6 men had 15 documented sexually transmitted infections (STIs) other than HIV, 1–3 (median 3) STIs per patient: 4 *Treponema pallidum* infections; 3 *Chlamydia trachomatis* infections (1 rectal *C. trachomatis* serovar L2b, a lymphogranuloma venereum agent); 4 *Neisseria gonorrhoeae* infections; 3 *Shigella* spp. infections; and 1 *C. jejuni* infection.

The Laboratoire de Santé Publique du Québec (LSPQ, Sainte-Anne-de-Bellevue, QC, Canada) confirmed the 6 *C. coli* infections using *cpn60* gene sequencing (1). Drug susceptibility testing was done by using disk diffusion method for nalidixic acid and Etest (AB Biodisk, Solna, Sweden)

for 12 other agents (1–3). The susceptibility and resistance breakpoints were Clinical and Laboratory Standards Institute *Campylobacter*, *Enterobacteriaceae*, and other breakpoints as reported (1–4). The 6 *C. coli* pulsovar 15 were resistant to erythromycin, azithromycin, clarithromycin, clindamycin, tetracycline, ciprofloxacin, nalidixic acid, ampicillin, and cefotaxime. All isolates were susceptible to amoxicillin/clavulanic acid, imipenem, ertapenem, and gentamicin. The 6 isolates were  $\beta$ -lactamase positive in <1 min with nitrocefin disk. Pulsed-field gel electrophoresis, done at LSPQ as described by PulseNet Canada procedures (1), showed that the 6 isolates presented the same pattern with both *Sma*I and *Kpn*I enzymes designed pulsovar 15 (Figure).

These phenotypic, epidemiologic, and molecular data confirmed a cluster of an erythromycin-, tetracycline-, and ciprofloxacin-resistant *C. coli* pulsovar 15 infections in Montreal, Quebec, Canada, during January–February 2015. Epidemiologic data suggested enteric STIs. All 6 patients reported being MSM; 4 reported having unprotected sex the week before symptom onset; 5 were HIV-positive; the 6 men had 15 other STIs; and no food was suspected to be the source of the infection.

*Campylobacter* is an important human enteropathogen bacterium, and *C. coli* is the second most frequently reported species (4–6). Few *C. coli* clusters have been reported, and the outbreaks caused by this *Campylobacter* species might be underestimated (1,7). At the LSPQ, a high heterogeneity was documented in *C. coli* isolates characterized routinely from suspected outbreaks during 2011–2015 (Figure) (1; this study). The erythromycin, tetracycline, and



**Figure.** Pulsed-field gel electrophoresis patterns of *Campylobacter coli* with *Sma*I (18 isolates) and *Kpn*I (6 isolates) enzymes tested in study of *C. coli* outbreak among 6 men in Quebec, Canada, 2011–2015. p, pulsovar. Scale bar indicates percentage similarity.

ciprofloxacin susceptibilities were epidemiologic markers in this study and in previous studies (1,8). The presence of a strong  $\beta$ -lactamase with resistance to ampicillin was also a marker in this study; epidemic *C. jejuni* and *C. coli* isolates were  $\beta$ -lactamase negative with susceptibility to ampicillin in previous outbreaks in MSM (1,8). Higher proportions of *C. coli* isolates are erythromycin- and multidrug-resistant than are *C. jejuni* isolates (4,6). When indicated, the proper antimicrobial treatment of enteric erythromycin- and ciprofloxacin-resistant *Campylobacter* spp. is not known because no clinical studies have been done for infections with such isolates, but tetracycline or amoxicillin/clavulanic acid can be used if isolates are susceptible in vitro (1,8; this study).

MSM should be counseled about preventing STIs, including enteric infections. Barriers should be used during genital, oral, and anal sex, and genital and hand washing before and after sex should be done (9,10). Our study increases evidence of clusters of *Campylobacter* STIs in MSM (1,8).

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## Biological Warfare in the 17th Century

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**To the Editor:** In an article that reviews evidence of a plot to use plague to break the siege of Candia during the Venetian–Ottoman War of the 17th century, Dr. Thalassinou and her colleagues (1) identify an incident previously unknown to historians of biological warfare. However, the authors' effort to broaden the context for biological weaponry is undermined by a reference to an often repeated allegation for which no credible evidence exists: namely, that during a siege occurring in the Swedish–Russian War of 1710, the Russians catapulted bodies of plague victims into the Swedish-held city of Reval.

Danish historian Karl-Erik Frandsen conducted a careful study of the plague outbreak affecting the Baltic area during 1709–1713 and found no evidence to support this allegation (2). Plague was first detected in Reval on August 10, 1710, while the army from Russia was still approaching the city. Reval was not besieged, and the Russians merely camped outside the city while attempting to isolate it. The army dumped corpses into a stream that flowed into Reval, but evidence does not show that the dead were plague victims, nor does evidence exist that clarifies whether the intent was contamination of the water supply or disposal of bodies. Original accounts provide no evidence to suggest that Russians hurled bodies into the city, much less plague-infected bodies. Frandsen estimates that about three quarters of the 20,000 persons in Reval died during the outbreak (2).

Intentional introduction of disease has been rare (3). Consequently, the incident identified by Thalassinou and her colleagues arouses readers' interest and inspires speculation.

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## ***Bifidobacterium longum* Subspecies *infantis* Bacteremia in 3 Extremely Preterm Infants Receiving Probiotics**

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**To the Editor:** Metaanalysis of randomized trials that tested different probiotics showed a reduction of ≈50% in necrotizing enterocolitis and all-cause deaths in preterm infants (1). Use of probiotics is increasing worldwide (2,3), and cases of probiotic sepsis were not reported among >5,000 infants in an updated review (1).

In Norway, a consensus-based protocol recommending prophylactic probiotic supplementation for preterm infants at highest risk for necrotizing enterocolitis (gestational age <28 weeks, birthweight <1,000 g) was introduced in 2014. After considering the safety profile, we investigated use in preterm infants of a widely used combination of oral

probiotics (Infloran; Laboratorio Farmacéutico Specialità Igienico Terapeutiche, Mede, Italy) that contained 10<sup>9</sup> *Lactobacillus acidophilus* (ATCC 4356) and 10<sup>9</sup> *Bifidobacterium longum* subspecies *infantis* (ATCC 15697).

*B. longum* is a microaerotolerant, anaerobic bacterium susceptible to many antimicrobial drugs (Table). This bacterium is a rare cause of neonatal infections; until 2015, only 2 *Bifidobacterium* bacteremia cases in premature newborns had been reported (4,5).

A total of 290 extremely preterm infants received oral probiotics during April 2014–August 2015 in Norway. Three patients were given a diagnosis of *B. longum* bacteremia: 2 patients in a neonatal unit in which 17 patients were given oral probiotics and 1 patient in a neonatal unit in which 31 patients were given oral probiotics (Table).

All 3 infants had respiratory distress syndrome and received mechanical ventilation after birth. Enteral feeding with human milk was begun on day 1. Oral probiotics (½ capsule, 1×/d) were given during the first week of life and increased to 1 capsule/day after 4–7 days.

We identified *B. longum* in blood cultures by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Billerica, MA, USA). Whole-genome sequencing (MiSeq, Illumina, San Diego, CA, USA) and comparative analysis of nucleotide-level variation by using variant call format in SAMtools (<http://samtools.sourceforge.net>) showed that all 3 blood culture isolates and a *B. longum* strain cultured from an oral probiotic capsule were identical.

Patient 1 had sepsis and severe hypotension 8 days after birth. A blood culture was prepared, and the patient was given antimicrobial drugs and vasoactive support. Abdominal distention, gastric residuals, and feed intolerance developed the next day, but the patient was cardiorespiratory stable. On day 12, abdominal radiographs showed pneumoperitoneum. Surgery showed multiple ileal perforations and bowel necrosis. Histologic analysis showed classical features of necrotizing enterocolitis. The patient received an ileostoma and improved after treatment with antimicrobial drugs. Blood culture was positive for gram-positive rods, which were identified as *B. longum*. Subsequent clinical course was uneventful.

Patient 2 had apnea, bradycardia, and temperature instability 12 days after birth. A blood culture was prepared, and the patient was given antimicrobial drugs. Blood culture was positive for gram-positive rods, which were identified as *B. longum*. Use of oral probiotics was discontinued. The patient recovered rapidly, and subsequent clinical course was uneventful.

Patient 3 had sepsis and necrotizing enterocolitis 9 days after birth. Ultrasound showed free abdominal fluid. A blood culture was prepared, and the patient was given antimicrobial drugs. Surgery showed 2 separate bowel perforations, and

**Table.** Characteristics of 3 extremely preterm infants with *Bifidobacterium longum* subspecies *infantis* bacteremia, 2015\*

Characteristic	Patient 1	Patient 2	Patient 3
NICU	A	B	A
Sex	M	M	F
Date of onset	Apr	Jul	Sep
Gestational age, wk	24	23	24
Birth weight, g	730	500	697
Mode of delivery	Vaginal	Vaginal	Caesarean section
Appgar score at 1, 5, and 10 min after birth	4, 5, 5	Unknown, 0, 4	2, 2, 3
Reason for prematurity	Preterm rupture of membranes, maternal infection	Sudden preterm rupture of membranes, delivery not attended by healthcare personnel	Placental abruption
Age at onset of sepsis, d	8	12	46
Maximum CRP level, mg/L, $\leq$ 48 h of symptom onset	147	25	242
Age at discharge, wk	40	41	43
Weight at discharge, kg	3.3	3.4	3.3
Bacterial culture medium and conditions	BacT/ALERT,† aerobic, 36°C	BACTEC Plus,† aerobic, 35°C	BacT/ALERT,† aerobic, 36°C
Bacterial growth in blood culture, d	2	3	2

\*Patients were given ½ to 1 capsule/day of oral probiotics (Infloran; Laboratorio Farmaceutico **Specialità Igienico Terapeutiche**, Mede, Italy) that contained  $10^9$  *Lactobacillus acidophilus* (ATCC 4356) and  $10^9$  *B. longum* subspecies *infantis* (ATCC 15697). MICs (mg/L) for antimicrobial drugs tested were 0.016 for meropenem, 0.032 for ampicillin, 0.064 for penicillin, 0.064 for piperacillin/tazobactam, 0.250 for cefotaxime, 0.250 for clindamycin, 0.250 for vancomycin, and 4.000 for ciprofloxacin. All bacterial strains were inherently resistant to aminoglycosides. ATCC, American Type Culture Collection (Manassas, VA, USA); CRP, C-reactive protein; NICU, neonatal intensive care unit.

†bioMérieux (Marcy l'Étoile, France).

the patient received an ileostoma and colostoma. Histologic analysis did not show necrosis or inflammation. *Enterococcus faecalis* grew in the blood culture obtained on day 9. The patient had a complicated clinical course and received prolonged mechanical ventilation. However, the patient gradually tolerated full feeds. Use of oral probiotics was continued.

On day 46, the condition of patient 3 suddenly deteriorated; hypotension and metabolic acidosis developed, and the patient was again given antimicrobial drugs. A blood culture was positive for *B. longum*. Supplementation with oral probiotics was discontinued. The patient recovered from the infection, but secondary ileus developed. The patient had a complicated clinical course until discharge.

Recently, 5 other *B. longum* bacteremia cases among 5 preterm infants at 26–31 weeks gestation were reported (6,7). All 5 infants had received oral probiotics; 3 had severe gastrointestinal complications, similar to patient 1 in our report, and 2 patients were moderately compromised, similar to patient 2 (6,7).

We do not know whether *Bifidobacterium* organisms in blood culture for patient 1 were a consequence of intestinal necrosis and bacterial translocation or the cause of necrotizing enterocolitis. Patient 3 probably had a leaky gut that predisposed this patient to bacterial translocation. All 3 patients were extremely premature (23–24 weeks gestation) and had impaired immune systems, which predisposed them to infections with bacteria with low virulence. A recently published case of *Bifidobacterium* bacteremia in a 2-year old boy with leukemia highlights impaired immunity as a risk factor (8).

Only aerobic blood cultures are prepared for neonates. We detected *Bifidobacterium* bacteremia by using

2 automated blood culture systems and aerobic bottles. However, the sensitivity of these systems for detecting *Bifidobacterium* bacteremia is unknown. Thus, the incidence of *Bifidobacterium* bacteremia is theoretically underestimated. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry improves species detection and its use might be 1 reason for the apparently recent increase in probiotic-associated bacteremia.

We report that systemic infection with probiotic bacteremia might have a severe clinical course in extremely preterm infants. Clinical suspicion and appropriate blood culture conditions are essential for proper diagnosis and management.

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linezolid, and tigecycline. Although the clinical significance of these findings is unknown, the decline in drug effectiveness against *S. aureus* infections represents a looming threat to patient health and highlights the possibility of a return to illness and death rates similar to those before antimicrobial drugs were available.

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## Colistin-Resistant *Enterobacteriaceae* Carrying the *mcr-1* Gene among Patients in Hong Kong

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**To the Editor:** Colistin belongs to the last line of bactericidal antimicrobial drugs active against multidrug-resistant gram-negative bacteria such as carbapenemase-producing *Enterobacteriaceae* and carbapenem-resistant *Acinetobacter baumannii*. Consequently, the discovery of the plasmid-mediated colistin-resistant gene *mcr-1* in *Escherichia coli* (1) raises concern in the medical community because colistin might be useless in treating infections caused by *mcr-1*-carrying *Enterobacteriaceae*.

During December 8, 2015–January 8, 2016, we conducted prospective laboratory surveillance of *mcr-1*-carrying *Enterobacteriaceae* and *Acinetobacter* species in a university-affiliated tertiary hospital serving a population of ≈0.53 million in Hong Kong, China. Clinical specimens were processed by using standard operating procedures for different specimen types (2). All *Enterobacteriaceae* and *Acinetobacter* spp. isolates were plated onto MH1 agar, which is Mueller-Hinton agar (BD Diagnostics, Sparks, MD, USA), supplemented with 1 µg/mL colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) for overnight incubation at 37°C in air. Intrinsically colistin-resistant organisms, including *Proteus* spp., *Providencia* spp., *Serratia* spp., and *Morganella morganii*, were excluded. *E. coli* ATCC 25922 was used as a negative control. We screened bacteria that grew on MHC1 for *mcr-1* by real-time PCR that used specific primers MCR1\_22697\_F1 (5'-CACT-TATGGCACGGTCTATGA-3') and MCR1\_22810\_R1 (5'-CCCAAACCAATGATACGCAT-3') and the hydrolysis probe MCR1\_22763\_Pb1 (FAM-TGGTCTCGG/ZEN/CTTGGTTCGGTCTGTAGGGC-3IABkFQ) (Integrated DNA Technologies, Coralville, IA, USA). The complete *mcr-1* gene found in PCR-positive isolates was amplified and sequenced by specific primers. The colistin MIC of positive isolates was measured by using Etest strips (BioMérieux, Marcy l'Etoile, France). Susceptibility to other antimicrobial drugs was determined by using the Kirby-Bauer disk diffusion method, according to Clinical and Laboratory Standards Institute guidelines (3). We retrieved clinical details of patients whose sample had *mcr-1*-carrying *Enterobacteriaceae* from the hospital clinical management system.

A total of 1,324 *Enterobacteriaceae* and 103 *Acinetobacter* spp. isolates were screened on MHC1 agar and isolated from blood, urine, stool, or respiratory samples; wound swab specimens; and other sterile and nonsterile body fluids, tissues, or swab specimens. Of the total 1,427 isolates, 62 (4.3%) grew on MHC1: 26 *E. coli*, 24 *Klebsiella* spp., 7 *Enterobacter* spp., 4 *Salmonella* spp., and 1 *Citrobacter* sp. Among these 62 isolates, 1 *Enterobacter cloacae* and 4 *E. coli* isolates were *mcr-1* positive. All gene sequences were 100% identical to that of *mcr-1* in *E. coli* strain SHP45 (GenBank accession no. KP347127), which was isolated from a pig farm specimen in China (1,3). Of the 5 *mcr-1*-positive isolates, 2 were from blood cultures from patients with biliary tract infection, 1 from a mid-stream urine specimen from a patient with symptomatic urinary tract infection, and 2 from stool specimens from asymptomatic patients. The range of colistin MICs of the 5 *mcr-1*-positive isolates was 3–64 µg/mL; all were susceptible to carbapenem. One *E. coli* isolate (from patient 4) exhibited extended-spectrum β-lactamase activity (Table). Patient 3 resided in mainland China before this admission; patient 2 received a liver transplant in China in 2004. None of the 5 patients had a history of colistin treatment.

Finding the *mcr-1* gene in 0.4% of *Enterobacteriaceae* clinical isolates in Hong Kong is expected because of the high proportion of livestock and meat imported from China, where prevalence of colistin-resistant isolates is up to 25.4% and 28.0% in pigs and retail chicken meat,

respectively (5,6). Our findings highlight several issues. We noted a wide range (3–64 µg/mL) of colistin MICs in the *mcr-1*-carrying *Enterobacteriaceae*; the *E. cloacae* isolate exhibited the highest MIC. This wide variation in MICs has been noted previously (1,7). Whether the variation results from the differential expression of the *mcr-1* gene or from potentially unidentified colistin resistance mechanisms co-existing in some isolates is unknown (8).

Our discovery of the *mcr-1* gene in an *E. cloacae* isolate adds diversity to the *Enterobacteriaceae* species known to be *mcr-1* carriers (e.g., *E. coli*, *Klebsiella pneumoniae*, and *Salmonella* sp.) (1,9). An in vitro study showed transfer of *mcr-1*-carrying pHNSHP45 (i.e., polymyxin-resistant plasmid) to *Pseudomonas aeruginosa* (1). Consequently, surveillance for the *mcr-1* gene should include all clinically relevant *Enterobacteriaceae* species, and screening for other gram-negative organisms (e.g., *P. aeruginosa*) infecting humans should be considered.

We show a potential workflow for screening *mcr-1* isolates by sequential use of MHC1 agar and real-time PCR. Clinical and Laboratory Standards Institute guidelines have no recommended colistin breakpoints for *Enterobacteriaceae* (3); however, the European Committee on Antimicrobial Susceptibility Testing recommends a breakpoint of ≥4 µg/mL to define colistin resistance in *Enterobacteriaceae* (10). Given that some *mcr-1*-positive isolates may have a colistin MIC of 2 µg/mL (1,7), which is lower than the

**Table.** Clinical details of 5 patients infected with *mcr-1*-carrying *Enterobacteriaceae*, Hong Kong\*

Patient ID† (age, y)	Underlying conditions	Time from admission to collection of specimen (specimen type)	Antimicrobial drug use <1 mo before isolation	Outcome	<i>mcr-1</i> -positive species (colistin MIC, µg/mL)
1 (55)	Acute myeloid leukemia 4 mo after bone marrow transplant	4 mo (stool sample‡)	Piperacillin/tazobactam, meropenem	Asymptomatic colonization	<i>Enterobacter cloacae</i> complex (64)
2 (68)	Primary sclerosing cholangitis with liver transplant in China in 2004; currently on sirolimus and prednisolone; right hepatectomy in 2008 for right diffuse ischemic bile injury; history of recurrent cholangitis	On admission with sepsis workup for biliary sepsis resulting from biliary anastomotic stricture (blood culture)	None	Recovered	<i>Escherichia coli</i> (3)
3 (2)	Autologous bone marrow transplant for stage IV neuroblastoma	14 d (stool sample‡)	Piperacillin/tazobactam	Asymptomatic colonization	<i>E. coli</i> (3)
4 (57)	Hepatitis B virus-related hepatocellular carcinoma; recurrent pyogenic cholangitis; recurrent biliary sepsis with portal vein thrombosis; cerebellar stroke in 2013	On admission with sepsis workup for biliary sepsis resulting from biliary stricture and recent transarterial chemoembolization (blood culture)	None	Recovered	ESBL-producing <i>E. coli</i> (4)
5 (80)	Duke's B carcinoma of rectum [lower anterior resection in 1996]; carcinoma of thyroid [post-thyroidectomy]; hypertension; diabetes mellitus	On admission with sepsis workup for symptomatic urinary tract infection (mid-stream urine sample)	None	Recovered	<i>E. coli</i> (4)

\*ID, identifier; ESBL, extended-spectrum β-lactamase.

†Patient 4 was male; others were female.

‡Routine surveillance of stool samples for multidrug-resistant organisms according to infection control protocol (4).

recommended breakpoint, we designed MHC1 with a colistin concentration of only 1 µg/mL to minimize false-negative results. However, some colistin-susceptible organisms might grow on MHC1 (<5% in our study), resulting in the low PCR-positive rate for *mcr-1* among isolates.

Exact epidemiology of the *mcr-1* gene is unknown, indicating a need to conduct accurate surveillance of the gene's prevalence in humans. Additional mechanisms unique to the *mcr-1* gene may contribute to colistin resistance, suggested by the wide variation in colistin MICs among *mcr-1*-carrying *Enterobacteriaceae*.

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## *Cryptococcus gattii* Meningitis Complicated by *Listeria monocytogenes* Infection

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**To the Editor:** Among immunocompetent persons with cryptococcal disease, infection with a second organism is rare; all reported cases have involved concomitant mycobacterial infections (1). Immunosuppression is not a necessary precondition for infection with *Cryptococcus gattii* (2), and among immunocompetent persons, *C. gattii* infection confers high mortality rates: up to 24% according to a large case series (3). In addition, cryptococcomas are frequently observed in patients with *C. gattii*, as opposed to *C. neoformans*, infection, commonly necessitating longer courses of treatment. We report a fatal case of *C. gattii* and *Listeria monocytogenes* co-infection in an immunocompetent woman with cryptococcomas.

The patient was a previously healthy 23-year-old Hispanic woman who was hospitalized in 2009 after weeks of headache and recent-onset diplopia. Lumbar puncture revealed elevated opening pressure of 52 cm H<sub>2</sub>O; elevated leukocytes (1,030 cells/µL: 31% neutrophils, 55% lymphocytes, 14% monocytes); elevated protein concentration (117 g/L); and decreased glucose concentration (30 mg/dL). Cerebrospinal fluid (CSF) cryptococcal antigen (CrAg) titer was 1:64, and culture grew *C. gattii*. HIV antibody test result was negative. Magnetic resonance imaging of the brain demonstrated scattered enhancing round lesions within the cerebrum and cerebellum, consistent with cryptococcomas. The patient was prescribed intravenous amphotericin B (1 mg/kg/d) and intravenous flucytosine (2 g/6 h) (Table); after 5 days of therapy, culture of a repeat lumbar puncture sample was negative. The patient was then given intravenous liposomal amphotericin at 7 mg/kg, and after a 14-day induction period she was discharged with instructions to take fluconazole orally (400 mg 2×/d) and to continue amphotericin B infusions (3×/wk) (Table).

**Table.** Clinical events, management, and parameters for patient with *Cryptococcus gattii* meningitis complicated by *Listeria monocytogenes* infection\*

Clinical event (day)	Therapy (days)	Opening pressure, cm H <sub>2</sub> O (day)	Leukocyte count, cells/ $\mu$ L (day)	Protein, g/L (day)	Glucose, g/L (day)	CrAg titer (day)	Culture result (day)
Days 1–15: induction therapy	AMB 1 mg/kg (1–4); 5FC 2 g q6h (1–14); L-AMB 7/mg/kg (5–14)	52 (1), 12 (12)	1,030 (1), 123 (12)	117 (1), 104 (12)	30 (1), 29 (12)	1:64 (1)	<i>Cryptococcus gattii</i> (1), negative (12)
Days 16–30: discharge, outpatient infusion, readmission	L-AMB 7/mg/kg M,W,F (15–22); FLZ 400 mg q12h (15–22); L-AMB 5 mg/kg (23–30); FLZ 600 mg q12h: (23–30); 5FC 3 g q6h (23–30)	46 (23)	111 (23)	81 (23)	34 (23)	1:8 (23)	Negative (23)
Days 31–45: inpatient therapy	L-AMB 5 mg/kg (31–45); FLZ 600 mg q12h (31–45); 5FC 3 g q6h (31–45)	44 (38)	17 (38)	66 (38)	64 (38)	NA	Negative (38)
Days 46–60: inpatient therapy	L-AMB 5 mg/kg (46–60); FLZ 600 mg q12h (46–60); DEX 2 mg q6h (46–60)	35 (48)	18 (48)	25 (48)	85 (48)	NA	Negative (48)
Days 61–75: discharge and outpatient infusion	L-AMB 5 mg/kg (61–65); FLZ 600 mg q12h (61–75); DEX 2 mg q8h (61–75); L-AMB 7/mg/kg M,W,F (66–75)	13 (63)	8 (63)	28 (63)	91 (63)	1:4 (63)	Negative (63)
Days 76–83: readmission/coma (80); death (83)	L-AMB 7/mg/kg M,W,F (76–79); DEX 2 mg q12h (76–79); FLZ 600 mg q12h (76–83); CRO 2 gm q12h (80–83); AMP 2 gm q4h (80–83); TMP/SMX 320–1,600 mg (2 double-strength tablets) q8h (80–83)	>55 (80)	1,010 (80)	258 (80)	17 (80)	1:4 (80)	<i>Listeria monocytogenes</i> (80)

\*5FC, flucytosine; AMB, amphotericin B; AMP, ampicillin; CrAg, cryptococcal antigen; CRO, ceftriaxone; DEX, dexamethasone; F, Friday; FLZ, fluconazole; L-AMB, liposomal amphotericin; M, Monday; NA, not available; q, every; TMP/SMX, trimethoprim/sulfamethoxazole; W, Wednesday.

One week after hospital discharge, the patient experienced recurrent headache and low-grade fever and was readmitted. Repeat lumbar puncture indicated an opening pressure of 46 cm H<sub>2</sub>O but improvement of all other clinical parameters (Table). CSF CrAg titer was 1:8 and culture was negative. Repeat brain magnetic resonance images revealed no hydrocephalus, minimal edema, and decreased size and number of cryptococcomas. She was again given amphotericin B (5 mg/kg/d) and intravenous flucytosine (3 g/6 h) and fluconazole (600 mg/12 h). Placement of a ventricular-peritoneal shunt was deferred, and the patient required frequent lumbar punctures to relieve elevated intracranial pressure. After 3 weeks of therapy, she began taking oral dexamethasone (2 mg 4 $\times$ /d) to reduce intracranial pressure and control symptoms consistent with immune reconstitution inflammatory syndrome. After 30 days of antifungal therapy during this second hospitalization, she experienced symptomatic improvement and was discharged with amphotericin B (5 mg/kg to be infused 3 $\times$ /wk), fluconazole (600 mg 2 $\times$ /d), and dexamethasone (tapering dosage).

Two weeks later (11 weeks after initial admission), she returned to the hospital with worsening headache and fever. Lumbar puncture demonstrated a leukocyte count of 1,010 cells/ $\mu$ L (74% neutrophils, 12% lymphocytes, 14% monocytes), glucose 17 mg/dL, protein 258 g/L, and an opening pressure of >55 cm H<sub>2</sub>O. CSF culture grew *L. monocytogenes*. The patient was prescribed ceftriaxone, ampicil-

lin, and trimethoprim/sulfamethoxazole. Shortly after the lumbar puncture, she experienced status epilepticus and became comatose. Despite emergent craniotomy for relief of intracranial pressure, she remained comatose for several days; subsequently, supportive care was withdrawn and the patient died shortly thereafter.

This case highlights the difficulties of managing severe cryptococcal disease. This patient experienced headache over 3 months and symptom relapse during 10 weeks of anticryptococcal therapy. As was done in this case, practice guidelines recommend a longer duration of polyene antimycotic induction for patients with cryptococcomas than for those without (4), and longer courses of therapy are commonly described for infections caused by *C. gattii* than for those caused by *C. neoformans* (5). Corticosteroids are commonly used to treat immune reconstitution inflammatory syndrome associated with cryptococcal meningitis (6), although recently, they have been associated with adverse outcomes (7). As indicated by this case, corticosteroids remain a risk factor for secondary infection with several pathogens, including *Listeria*. No epidemiologic exposure to *Listeria* was identified for this patient.

*C. gattii* infection has been reported in 8 states, including California (3); we have found the pathogen in the soil south of Los Angeles, California, particularly in association with Canary Island pines and sweet gum trees (8). Some patients with *C. gattii* infection have autoantibodies to

granulocyte–macrophage (GM) colony-stimulating factor (9). Although these autoantibodies have not been reported in patients with *Listeria* infections, susceptibility to infection caused by this bacterium is increased in GM–colony-stimulating factor  $-/-$  mice (10). Autoantibodies against GM–colony-stimulating factor or perhaps other cytokines might have impaired the patient’s host defense against these organisms; unfortunately, our report is limited by lack of serum for further testing.

This case demonstrates the difficulties of managing patients with *C. gattii* infection and an unusual co-infection with *L. monocytogenes*. Initiation of corticosteroids for the management of severe cryptococcal disease should be undertaken with caution. The differential diagnosis for worsening cryptococcal disease should include acute or subacute bacterial meningitis, particularly when the patient is receiving corticosteroids for the management of immune reconstitution inflammatory syndrome or associated complications.

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## Melioidosis in Travelers Returning from Vietnam to France

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**To the Editor:** Melioidosis, a potentially fatal infectious disease, occurs predominantly across much of Asia and in northern Australia because of the soil and water bacterium *Burkholderia pseudomallei* (1). We report 2 related cases of suppurative cervical lymphadenitis, an unusual adult presentation of melioidosis, in 2 men who returned to France from Vietnam on the same trip (2).

Patient 1, a 28-year-old previously healthy man, was admitted to our hospital in Lyon, France, in October 2013 for the evaluation of a palpable neck mass, which had been growing steadily for the previous 2 months. Examination of the head and neck revealed a fluctuant, tender mass located in the inferior angle of the right side of the mandible, mimicking lymph node tuberculosis. Ultrasonographic investigation confirmed a level II enlarged cervical lymph node

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that was 2.8 cm in diameter. The routine bacterial culture from an ultrasound-guided fine needle aspiration showed a microorganism that was identified as *B. pseudomallei* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis with an extended database. An investigation by local medical staff revealed that the symptoms of our patient began in August 2013, when he returned to France after attending a wedding ceremony in Vietnam, a country to which *B. pseudomallei* is known to be endemic (3). No environmental risk factors, such as the percutaneous inoculation of contaminated material, ingestion, or inhalation, which are the main routes of transmission of melioidosis, were reported (1). The interview of patient 1 identified a co-traveler with similar symptoms (patient 2), who was subsequently admitted to the same hospital.

Patient 2, a 31-year-old previously healthy man, reported a 2-month history of a painful, inflamed, gradually enlarging, right-sided neck mass, accompanied by weight loss, night sweats, and intermittent fevers. Examination showed an enlarged cervical lymph node that was confirmed as level III, 3 cm in diameter. After noncontributory culture results from an ultrasound-guided fine needle aspiration, we performed an open biopsy under general anesthesia. We excised an adherent, enlarged, pus-filled lymph node and necrotic tissue for microbiologic testing. A real-time PCR assay specifically targeting type-3 secretion system genes (*orf11* and *BpSCU2*) quickly revealed the presence of *B. pseudomallei* DNA, and the diagnosis of melioidosis was confirmed by culture, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and antibiogram (4).

For both patients, a cervical, chest, abdominal, and pelvic computed tomographic scan showed no other foci of infection, and a 14-day regimen of intravenous ceftazidime therapy was administered, followed by oral treatment with cotrimoxazole for 6 months (5). However, the neck mass of patient 1 was still swollen after 1 month of treatment, and oral amoxicillin/clavulanic acid was added to cotrimoxazole for 2 months based on an antibiogram from a new bacterial isolation. For patient 2, cotrimoxazole was switched to amoxicillin/clavulanic acid after a presumed adverse drug reaction. At last report, both patients had been disease free for 20 months.

As soon as the second case of melioidosis was confirmed, local and national public health authorities in France were notified, and a larger contact investigation was initiated because 16 other travelers attended the same wedding ceremony before returning to their home countries. None of these travelers had any symptoms of melioidosis. No serologic testing was performed.

Although sporadic cases of travel-associated melioidosis are regularly reported, such case clusters occurring in returning travelers is rare but underscores the role of the

contact investigation in this context (6–8). Phylogenetic analyses, performed by a 7-locus multilocus sequence typing analysis, revealed that the 2 isolates shared the same sequence type (381) (identification nos. 4488 and 4489, <http://pubmlst.org/bpseudomallei>), which was previously identified in Thailand and Cambodia (9), suggesting a clonal infection from a single-point source. The epidemiologic assessment will be completed by whole-genome sequencing.

Most cases of oropharyngeal melioidosis have been reported in children and were believed to be associated with an oral contamination (1,3). The ingestion of unchlorinated or inefficiently chlorinated water from local residences and hotels has been involved in melioidosis outbreaks and could have been the route of infection for these patients. However, the source of infection might also be linked to a scooter ride taken by both men together around a lake in the vicinity of Hanoi (10).

These patients had no known individual risk factor for melioidosis, such as diabetes, hazardous alcohol use, chronic lung or renal disease, thalassemia, glucocorticoid and other immunosuppressive therapy, or cancer, whereas up to 70% of patients with travel-associated melioidosis had  $\geq 1$  predisposing factor (1,8). However, the percentage of patients with an underlying risk factor dropped to 37.5% when the data excluded patients who were born in melioidosis-endemic countries or others who had a long-term stay in a melioidosis-endemic country (6). This finding makes us cautious not to repeat making the common assumption about the link between underlying conditions and the risk for melioidosis, especially in regard to conventional tourists traveling in melioidosis-endemic areas.

#### Acknowledgments

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## ***mcr-1*–Positive Colistin-Resistant *Escherichia coli* in Traveler Returning to Canada from China**

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**To the Editor:** A 61-year-old man underwent transurethral prostate resection in Vancouver, British Columbia,

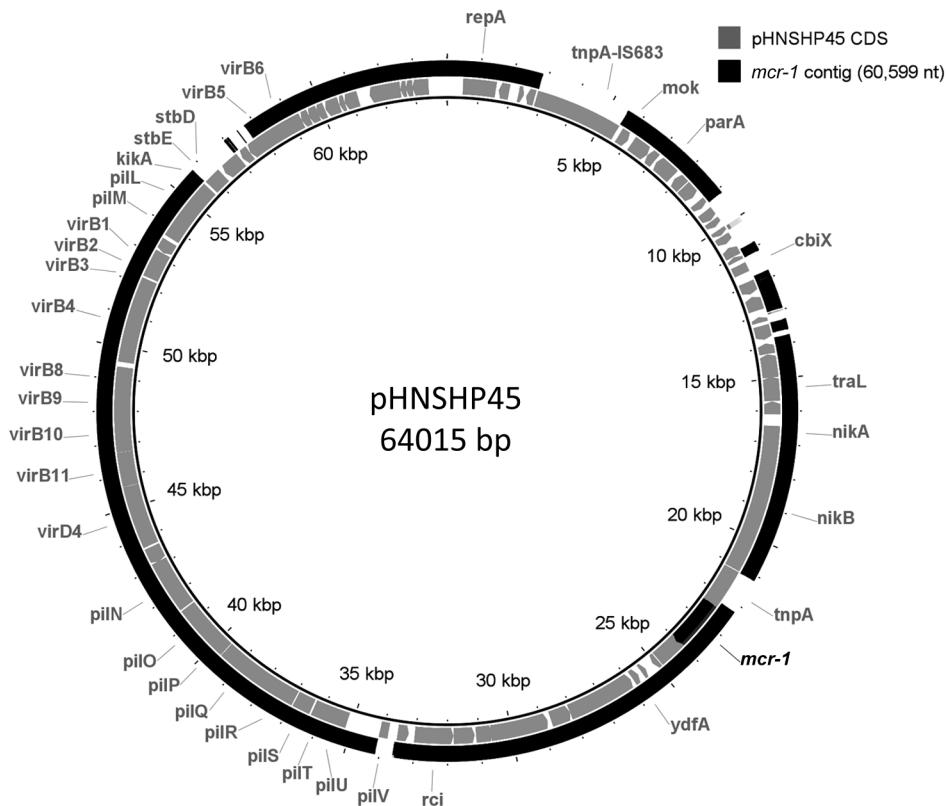
in January 2016. On postoperative day 1, he was febrile (39.1°C) and had leukocytosis (12.7 × 10<sup>9</sup> cells/L). Blood and urine cultures were ordered on postoperative day 2, and ceftriaxone was started. On postoperative day 3, urine culture grew *Escherichia coli* (>100 million CFU/L). Susceptibility testing (VITEK2, bioMérieux, Quebec, Canada) indicated a possible extended-spectrum β-lactamase producer and showed resistance to ampicillin, cefazolin, ceftriaxone, gentamicin, ciprofloxacin, and trimethoprim/sulfamethoxazole; intermediate resistance to tobramycin; and susceptibility to amoxicillin/clavulanate, piperacillin/tazobactam, ertapenem, meropenem, and nitrofurantoin. Treatment was switched to amoxicillin/clavulanate. The urinary catheter was removed 48 hours later. The patient was discharged on postoperative day 5 and completed 14 days of oral amoxicillin/clavulanate. Blood cultures were negative after 7 days' incubation.

The *E. coli* cultured from the patient underwent further testing and grew in equal amounts on Columbia Colistin-Nalidixic acid Agar (CNA) with 5% sheep blood and Columbia agar with 5% sheep blood (OXOID, Ontario, Canada). This result was brought to the attention of the hospital's medical microbiologist. A colistin Etest (bioMérieux, Quebec, Canada) showed a MIC of 3 μg/mL; EUCAST defines colistin resistance as >2 μg/mL for *Enterobacteriaceae* (1).

A real-time PCR to detect the mobile colistin resistance (*mcr-1*) gene was developed at the Provincial Public Health Laboratory by using primers MCR-1F (5'-CATC-GCTCAAAGTATCCAGTGG-3'), MCR-1R (5'-CCATG-TAGATAGACACCGTTCTCAC-3'), and probe MCR-1P (5'-Cy5-TGCAGACGCACAGCAATGCCTATGAT-TAO-3') with TaqMan Fast Advanced Master Mix (Life Technologies, Burlington, Ontario, Canada), on an ABI 7500 FAST thermocycler (Applied Biosystems, Foster City, CA) by using manufacturer's recommended conditions. The *mcr-1* gene was confirmed by Sanger sequencing by using previously described oligonucleotides (2). The isolate was also PCR-positive for a *bla*<sub>CTX-M</sub> gene. The strain was sequenced by using MiSeq (Illumina, Victoria, British Columbia, Canada), and predicted to be sequence type 3944 based on multilocus sequence typing databases (<http://github.com/tseemann/mlst>; <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>) and serotype O159:H4 (3). Sequence type 3944 does not belong to any clonal groups; 1 isolate from Asia is in the MLST database. Abricate (<http://github.com/tseemann/abricane>) and PlasmidFinder (4) were used to query the SPAdes-assembled genome (5). Results showed that this isolate carries 3 plasmids that have IncR, IncFIA/HI, and IncI2 replicons. The *mcr-1* gene was found on a 60,599-nt contig with the IncI2 replicon; this contig is ≈87% identical to pHNSHP45 (Figure) (2). The *bla*<sub>CTX-M-27</sub> gene was found on the same contig as the IncFIA/HI replicon, and no resistance genes were found with the IncR replicon.

At the time of *mcr-1* detection, the patient and all other patients who shared a hospital room with this patient,

<sup>1</sup>These authors contributed equally to this article.



**Figure.** The *mcr-1*-containing contig from *Escherichia coli* isolated from a traveler returning to Canada from China compared to pHNSHP45 plasmid. BRIG (6) was used to generate a visual representation of the 60,599-nt contig assembled from the sequencing of the *mcr-1*-positive isolate to pHNSHP45 (2). Coding regions are represented in the inner ring; the *mcr-1*-containing contig is represented in the outer ring. The *mcr-1* gene is indicated in a black segment in the inner ring.

all for <24 h, had been discharged. According to a public health representative, and on the basis of the limited exposure duration of roommates, discharged roommates were not screened. The patient improved clinically, so no changes to therapy were indicated.

The patient had traveled to China in November 2015 for 2 weeks, where he required catheterization in a hospital emergency department in Zhejiang Province for acute urinary retention. He experienced acute urinary retention and fever 6 days after catheter removal, requiring another catheter insertion and 3 days of intravenous antimicrobial drugs in Guangdong Province. He denied contact with farm animals, live poultry markets, or undercooked meat. On return to Canada, obstructive urinary tract symptoms persisted, requiring 5 emergency department visits before prostate resection.

Colistin is a last-resort antibacterial drug because of its toxic effects and is increasingly used for treating carbapenem-resistant *Enterobacteriaceae* (CRE). Plasmid-mediated resistance genes have been described in agricultural animals and meat, as well as in humans. Initial reports described the *mcr-1* gene in China and Southeast Asia (2,7). Retrospective reviews have detected *mcr-1* in *Enterobacteriaceae* from Europe, South America, Africa, and Japan (7).

Unlike laboratory detection of CRE, where screening media and automated susceptibility panels were available,

no commercial screening media exist for *mcr-1*. MIC testing is only recommended for *Enterobacteriaceae* resistant to all other antimicrobial classes, and molecular testing may not be accessible. Furthermore, some *Enterobacteriaceae* are intrinsically resistant to colistin. Only Etest (<http://etest.net/>) could be performed in our laboratory, which is a limitation that may underestimate the actual MIC (8). However, the reference broth microdilution method is unavailable to most clinical laboratories. This isolate was identified by a technologist who recognized heavy growth of *E. coli* on a CNA plate, an unusual occurrence because CNA plates are used for the isolation of gram-positive bacteria while inhibiting gram-negative bacteria. Despite serendipitously identifying *mcr-1* on the CNA, this method is an inadequate for detection of *mcr-1*.

Retrospective screening has identified *mcr-1* isolates in Canada (9). However, we describe a prospectively identified patient in Canada with *E. coli* harboring the *mcr-1* gene. The patient's travel history suggested that acquisition occurred in China, although only 1% of inpatients with infection in Guangdong/Zhejiang Provinces harbor *mcr-1* (2).

Limited laboratory screening procedures have implications for laboratories and public health. Routine colistin testing for *Enterobacteriaceae* would be costly and low-yield; however, without such testing, the real prevalence of *mcr-1* will be underestimated. A coordinated approach to

the prevention of *mcr-1* dissemination is needed, particularly to prevent the proliferation of an organism harboring a plasmid with *mcr-1* and a carbapenemase (10).

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## Carbapenem-Resistant *Enterobacter* spp. in Retail Seafood Imported from Southeast Asia to Canada

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**To the Editor:** Carbapenems, antimicrobial drugs of last resort, are recommended only for severe community- and healthcare-associated multidrug-resistant bacterial infections. In Canada, carbapenem-resistant infection rates in hospitals remained low ( $\leq 0.25$  cases/1,000 patient admissions) over 5 years' (2009–2014) surveillance (1). Carbapenemase-producing bacteria have rarely been detected in the food chain in industrialized countries. However, carbapenemase genes were detected in bacteria isolated from produce in Switzerland (2) and seafood in Canada (3); implicated food items originated from Southeast Asia. We conducted targeted sampling to assess, using selective media, the occurrence of carbapenem-resistant *Enterobacteriaceae* in imported seafood products sold in Canada.

For testing, we selected 1,328 retail seafood samples: 928 were imported fresh and frozen raw shrimp collected during 2011–2015 by CIPARS (the Canadian Integrated Program for Antimicrobial Resistance Surveillance), and 400 comprised an assortment of imported niche-market fresh and frozen raw seafood collected specifically for this study during January–April 2015. Product information and origin country were recorded for each sample. We used chromID CARBA agar (bioMérieux, St. Laurent, QC, Canada) to select putative colonies. To determine carbapenemase production on nonsusceptible (zone of inhibition  $< 25$  mm) isolates, we used disk diffusion susceptibility to ertapenem and meropenem (10  $\mu$ g each) and the Carba NP test as previously described (4). Isolates were identified to species using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics Ltd, Milton, ON, Canada) and tested for susceptibility using the Sensititre Complete Automated System with the Sensititre NARMS Gram Negative Plate (CMV3AGNF)

(Trek Diagnostic Systems, Oakwood Village, OH, USA). We used single and multiplex PCR to screen isolates for the major carbapenemase-conferring ( $bla_{NDMP}$ ,  $bla_{KPC}$ ,  $bla_{IMP}$ ,  $bla_{VIM}$ ,  $bla_{GES}$ ,  $bla_{OXA-48}$ -like,  $bla_{NMC}$ ) and  $\beta$ -lactamase-conferring ( $bla_{SHV}$ ,  $bla_{TEM}$ ,  $bla_{CTX-M}$ ,  $bla_{OXA-1}$ ,  $bla_{CMY-2}$ ) genes (5). We performed pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing (Illumina Inc., San Diego, CA, USA) on isolates requiring further comparative testing (6). In silico multilocus sequence typing and replicon typing were conducted using the assembled sequence data (SPAdes 3.5.0 [St. Petersburg genome assembler], <http://spades.bioinf.spbau.ru/release3.5.0/manual.html>) and services of the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>). The transferability of resistance genes was determined by transformation experiments using electrocompetent *Escherichia coli* DH10B cells.

Using selective media methodology, we detected carbapenem-resistant *Enterobacteriaceae* in 8 (0.6% [95% CI 0.26–1.18]) of the 1,328 seafood samples; all 8 were from Southeast Asia (Table). Of the 928 shrimp samples collected as part of CIPARS sampling, 2 (0.2% [95% CI 0.03–0.78]) imported from Vietnam contained *Enterobacter cloacae* harboring  $bla_{IMI-1}$ , and 1 (0.1% [95% CI 0.003–0.599]) from Bangladesh contained *E. aerogenes* harboring  $bla_{IMI-2}$ . Of 101 mollusk samples, 3 (3.0% [95% CI 0.62–8.44]) clam samples imported from Vietnam contained *E. cloacae* harboring  $bla_{IMI-1}$ , and 2 (2.0% [95% CI 0.24–6.97]) clam samples from Vietnam contained *E. cloacae* harboring  $bla_{NDM-1}$ ,  $bla_{TEM}$ , and  $bla_{OXA-1}$ . All isolates with carbapenemase genes were phenotypically resistant to ampicillin, cefoxitin, and amoxicillin/clavulanic acid; some were multiclass-resistant (Table).

Isolates harboring  $bla_{IMI-1}$  genes contained no plasmid DNA. However, using electroporation into *E. coli*, we showed that the  $bla_{IMI-2}$  gene was plasmid-mediated; the plasmid contained the IncFII(Yp) replicon. The  $bla_{NDM-1}$  genes were nontransformable into *E. coli*, although the 2 isolates contained IncHI2, IncFIB, and IncFII replicons. The location

of the  $bla_{NDM-1}$  gene may therefore be chromosomal or plasmidic. Six different sequence types (STs) of *E. cloacae* were shown by multilocus sequence typing. PFGE results showed that the 2 *E. cloacae* ST479 isolates were indistinguishable, whereas the other isolates were distinct. The *E. cloacae* ST479 isolates harbored  $bla_{NDM-1}$ ,  $bla_{OXA-1}$ , and  $bla_{TEM}$ ; were phenotypically resistant to 12 tested antimicrobials drugs; and were from clam samples collected at different retail outlets on different dates. Comparison of ST373 fingerprints with the National Microbiology Laboratory PFGE database containing >170 *E. cloacae* of human origin showed that a human-sourced *E. cloacae* ST373 isolate harboring  $bla_{IMI-1}$  shared >75% similarity with a clam-sourced *E. cloacae* isolate. In addition to the carbapenem-resistant *Enterobacteriaceae* findings described here, our findings also show that 1 sample, from a black tiger shrimp (*Penaeus monodon*) originating from India, contained a non-O1, non-O139 *Vibrio cholerae* with a novel class A carbapenemase gene named  $bla_{VCC-1}$  (GenBank accession no. KT818596); this isolate has been described elsewhere (6).

Seafood, such as shrimp and clams, are raised in aquatic environments with a known potential for water-source contamination (7,8). We found multiple retail seafood samples containing *Enterobacter* spp. harboring  $bla_{NDM-1}$  and  $bla_{IMI-type}$  genes. This finding suggests that, for humans, the source of carbapenemase-producing *Enterobacter* spp. may not be limited to exposure during travel; contaminated food products may also be a source of exposure (9). The identification, in imported clams, of *E. cloacae* with the same ST and similar DNA fingerprint pattern as an isolate from a human raises concerns of a possible association; however, more work is required before a linkage and direction of transfer can be inferred. Our findings highlight the need for antimicrobial resistance surveillance systems to consider the use of selective media methodology to increase sensitivity for the detection of rare or emerging resistance genes.

**Table.** Carbapenem-resistant *Enterobacter* species detected in retail seafood products imported from Southeast Asia to Canada\*

Sample type, resistant species	No. (%) samples [95% CI]	Origin of seafood	Gene	Antibiogram profile	ST†
Shrimp, n = 928					
<i>E. cloacae</i>	2 (0.2) [0.03–0.78]	Vietnam	$bla_{IMI-1}$	AMC-AMP-(AZM)-FOX‡	ST411; ST412
<i>E. aerogenes</i>	1 (0.1) [0.003–0.599]	Bangladesh	$bla_{IMI-2}$	AMC-AMP-FOX	NA
Bivalve mollusks, n = 101					
<i>E. cloacae</i>	2 (2.0) [0.24–6.97]	Vietnam, clam	$bla_{NDM-1}$ , $bla_{TEM}$ , $bla_{OXA-1}$	AMC-AMP-FOX-TIO-CRO- CHL-CIP-GEN-STR-FIS- TET-TMP/SXT	ST479
<i>E. cloacae</i>	3 (3.0) [0.62–8.44]	Vietnam, clam	$bla_{IMI-1}$	AMC-AMP-(AZM)-FOX§	ST477; ST478; ST373
Cephalopods, n = 240	0 [0.00–1.53]	NA	NA	NA	NA
Miscellaneous, n = 59	0 [0.00–6.06]	NA	NA	NA	NA

\*AMC, amoxicillin/clavulanic acid; AMP, ampicillin; AZM, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; FIS, sulfisoxazole; CRO, ceftriaxone; FOX, cefoxitin; GEN, gentamicin; NA, not applicable (no scheme found); ST, sequence type; STR, streptomycin; TET, tetracycline; TIO, ceftiofur; TMP/SXT, trimethoprim/sulfamethoxazole.

†Determined by multilocus sequence typing.

‡ST412 resistant to AZM.

§ST477 and ST373 resistant to AZM.

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## Fluoroquinolone-Resistant *Mycoplasma genitalium*, Southwestern France

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**To the Editor:** *Mycoplasma genitalium* is a sexually transmitted bacterium involved in nongonococcal urethritis in men and associated with cervicitis and pelvic inflammatory disease in women. Azithromycin regimens have been commonly used as a first-line treatment of these conditions, but a recent increase in *M. genitalium* with azithromycin resistance has been described worldwide; in 2012, resistance in the organism was detected in France at a prevalence of 14% (1). In case of azithromycin failure, moxifloxacin is a second-line treatment; however, moxifloxacin treatment failures have also been reported and are associated with mutations in ParC or GyrA (2).

Prevalence of *M. genitalium* infection was ≈4% in 2013–2014 at Bordeaux University Hospital (Bordeaux, France). To evaluate the prevalence of fluoroquinolone resistance in *M. genitalium* in southwestern France, we examined the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes in *M. genitalium*-positive specimens obtained during 2013–2014. We retrospectively collected (from the Department of Bacteriology, Bordeaux University Hospital) 369 *M. genitalium*-positive urogenital specimens and DNA extracts from 344 patients. The *gyrA* and *parC* QRDRs were amplified and sequenced as described (3,4). We also assayed macrolide resistance-associated mutations using real-time PCR and melting curve analysis (1). To determine resistant genotypes A2058G or A2059G, we sequenced PCR products. Nucleotide positions in the 23S rRNA and amino acid positions in GyrA and ParC were identified according to *Escherichia coli* numbering.

From the 344 *M. genitalium*-positive patients, 200 specimens underwent complete analysis for the *gyrA* and *parC* genes, specimens from 221 patients were investigated for macrolide resistance, and specimens from 168 patients were examined for 23S rRNA, *gyrA*, and *parC* genes. Unsuccessful amplifications could be attributed to low bacterial loads of *M. genitalium* or to the degradation of frozen DNA during storage. Strains from 12/200 patients (6%; 95% CI 3.47%–10.19%) had QRDR mutations, with rates of 6.4% (6/93) for 2013 and 5.6% (6/107) for 2014. This prevalence is in accordance with the 4.5% moxifloxacin resistance described in the United Kingdom in 2011 (3) but lower than prevalences found in small numbers of strains in Japan and Australia during 2006–2014, which ranged from 10% to 47% (4–8).

Strains from 11 patients (patient nos. 6, 8, 12, 20, 23, 28–31, 46, 47) harbored alterations in the ParC QRDR (Table) at positions 80 (Ser→Asn or Ile) or 84 (Asp-84→Tyr or Asn). These mutations have been previously described for *M. genitalium* (4,6–8). In addition, 1 new amino acid alteration, Asn-96→Ser (strain from patient 20), was found in ParC. We detected a GyrA modification with the Ala-93→Thr transition in a strain from 1 patient (patient 3). These 2 amino acid changes were not

**Table.** Fluoroquinolone resistance–associated amino acid changes in GyrA and ParC and macrolide resistance–associated mutations in the 23S rRNA gene in *Mycoplasma genitalium*, France, 2013–2014\*

Patient no.	Date of collection	Patient		Specimen type	Mutation in the 23S rRNA gene	Amino acid changes in	
		sex				GyrA	ParC
1	2013 Jan 9	F		Vaginal swab	A2058G/A2059G	NA	NA
2	2013 Feb 1	F		Vaginal swab	A2058G	–	–
3	2013 Feb 1	M		Urethral swab	A2058G/A2059G	Ala-93→Thr	–
4	2013 Feb 18	M		Urethral swab	A2058G/A2059G	–	–
5	2013 Feb 20	M		Urine	A2058G/A2059G	–	–
6	2013 Mar 11	M		Urine	A2058G/A2059G	–	Asp-84→Tyr
7	2013 Mar 28	F		Vaginal swab	A2058G/A2059G	NA	–
8	2013 Apr 3	M		Urine	Wild-type	–	Asp-84→Tyr
9	2013 Apr 8	F		Vaginal swab	A2058G/A2059G	–	–
10	2013 Apr 11	F		Vaginal swab	A2058G/A2059G	NA	NA
11	2013 Apr 16	F		Vaginal swab	A2058G	–	NA
12	2013 Apr 19	F		Vaginal swab	Wild-type	–	Ser-80→Asn
13	2013 May 21	M		Urethral swab	A2059G	–	–
14	2013 Jul 4	M		Urine	A2059G	–	–
15	2013 Jul 4	M		Urethral swab	A2059G	–	–
	2013 Jul 19	M		Urine	A2059G	–	–
16	2013 Jul 19	M		Urine	A2058G	–	–
17	2013 Aug 9	F		Vaginal swab	A2059G	NA	–
18	2013 Sep 30	F		Vaginal swab	A2058G/A2059G	–	–
19	2013 Sep 30	F		Vaginal swab	A2058G/A2059G	–	–
20	2013 Oct 29	F		Vaginal swab	Wild-type	–	Asn-96→Ser
21	2013 Nov 22	F		Vaginal swab	A2058G/A2059G	NA	NA
22	2013 Nov 29	F		Vaginal swab	A2062T	–	–
23	2013 Dec 1	F		Vaginal swab	Wild-type	–	Asp-84→Tyr
24	2014 Jan 21	F		Vaginal swab	A2059G	–	–
25	2014 Jan 29	F		Vaginal swab	A2059G	–	–
26	2014 Jan 30	F		Vaginal swab	A2058G/A2059G	NA	–
27	2014 Feb 13	F		Vaginal swab	A2059G	–	–
28	2014 Feb 18	M		Urine	Wild-type	–	Ser-80→Ile
29	2014 Feb 24	F		Vaginal swab	Wild-type	–	Asp-84→Asn
30	2014 Mar 5	F		Vaginal swab	Wild-type	–	Asp-84→Asn
31	2014 Mar 14	M		Urine	NA	–	Ser-80→Asn
32	2014 Apr 3	F		Endocervical swab	A2058G	–	–
33	2014 Apr 7	M		Urethral swab	A2059G	–	–
34	2014 Jun 24	F		Vaginal swab	A2059C	–	–
35	2014 Jul 9	F		Endocervical swab	A2059G	NA	–
36	2014 Jul 25	F		Urine	A2058G/A2059G	NA	NA
37	2014 Jul 25	F		Vaginal swab	A2058G	–	–
38	2014 Aug 19	F		Endocervical swab	A2062T	–	NA
39	2014 Aug 28	F		Vaginal swab	A2058G/A2059G	–	–
40	2014 Sep 24	F		Vaginal swab	A2059G	–	–
41	2014 Oct 7	F		Vaginal swab	A2059G	–	–
42	2014 Oct 15	F		Vaginal swab	A2058G/A2059G	–	–
43	2014 Oct 31	M		Urine	A2058G	–	–
44	2014 Nov 5	F		Vaginal swab	A2058G/A2059G	NA	–
45	2014 Nov 28	F		Vaginal swab	A2058G	–	–
46	2014 Dec 3	F		Vaginal swab	Wild-type	–	Asp-84→Asn
47	2014 Dec 3	M		Urethral swab	Wild-type	–	Asp-84→Asn
48	2014 Dec 4	M		Urine	A2059G	–	–

\*A2058/A2059G indicates a macrolide–resistant (A2058G or A2059G) genotype. Positions in the 23S rRNA and in GyrA and ParC are identified according to *Escherichia coli* numbering. NA, not available; –, no amino acid change.

previously reported; however, mutations at the next positions (97 in ParC and 95 in GyrA) have been described for *M. genitalium* (4,7), and these positions are within the QRDRs, suggesting their involvement in fluoroquinolone resistance. As previously described, *M. genitalium* ParC alterations predominate over GyrA alterations.

None of the 12 patients with strain *parC* or *gyrA* mutations had a history of fluoroquinolone treatment. Six patients received no treatment; 4 patients received azithromycin

(1 g); 2 patients received extended azithromycin (1.5 g), 1 patient after azithromycin (1 g) failure, and 1 after receiving doxycycline for 7 days. Therapeutic outcomes were not available except for 1 patient, who experienced clinical failure after 2 azithromycin treatments.

Regarding macrolide resistance, 38 of 221 patients (17.20%; 95% CI 12.79%–22.72%) had *M. genitalium* with macrolide resistance–associated 23S rRNA mutations; prevalence was 17% (19/112) for 2013 and 17.4% (19/109)

for 2014. This prevalence is increasing compared to that described in France in 2012 (14%). We found 35 A→G substitutions at position 2058 or 2059, two A2062T mutations and one A2059C mutation (Table) (1,9). Notably, in patients 15 and 33, who were infected with strains with macrolide resistance-associated mutations, *M. genitalium* infection was unsuccessfully treated with azithromycin, with treatment failures after azithromycin (1 g) and extended azithromycin (1.5 g for 5 d), but moxifloxacin treatment was effective. Patient 15 had been treated 1 year earlier with azithromycin (1 g) for nongonococcal urethritis.

Among the 168 patients whose isolates were examined for the 23S rRNA, *gyrA*, and *parC* genes, strains from 2 patients (patients 3 and 6) had both macrolide- and fluoroquinolone-associated mutations (1.2%; 95% CI 0.33%–4.24%). Both patients received azithromycin (1 g), and patient 6 received additional azithromycin (1.5 g) after failure of azithromycin (1 g). Patient 6 experienced azithromycin failure again after the extended regimen. *M. genitalium* multidrug resistance is described in France at a prevalence of 1.2%, lower than prevalence described in Australia (7.5%) (7) and Japan (30.8%) (10).

In conclusion, *M. genitalium* fluoroquinolone resistance is emerging in France, with a prevalence of 6% in 2013–2014. Further, macrolide resistance also increased during this period, to a rate of 17.2%. Patients infected with *M. genitalium* strains containing both macrolide and fluoroquinolone resistance mutations associated with therapeutic failure raise concerns about untreatable *M. genitalium* infections.

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## Possible Transmission of *mcr-1*-Harboring *Escherichia coli* between Companion Animals and Human

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**To the Editor:** Plasmid-mediated, colistin-resistance mechanism gene *mcr-1* was first identified in *Escherichia coli* isolates from food, food animals, and human patients in November 2015 (1). Reports on detection of *mcr-1* in *Enterobacteriaceae* from humans and food animals

soon followed from  $\approx 12$  countries (2–5). Here we report detection of *mcr-1* in colistin-resistant *E. coli* isolated from companion animals and the possible transmission of *mcr-1*-harboring *E. coli* between companion animals and a person.

Three *mcr-1*-harboring *E. coli* clinical isolates were identified from specimens of 3 patients admitted to a urology ward of a hospital in Guangzhou, China. *E. coli* isolate EC07 was identified in the urine of a 50-year-old male patient with glomerulonephritis in October 2015. Isolate EC08 was cultured from the urine of a 48-year-old male patient with prostatitis in December 2015. Isolate EC09 was identified in the blood of an 80-year-old male patient with bladder cancer 3 weeks after EC08 was identified.

Review of medical records identified the patient carrying *E. coli* isolate EC07 as a worker at a pet shop. In light of this finding, we collected a total of 53 fecal samples from 39 dogs and 14 cats in the pet shop where the man worked. We isolated and identified colonies consistent with *E. coli* from fecal samples on MacConkey agar plates (Thermo Fisher, Beijing, China) and API 20E system (bioMérieux,

Durham, NC, USA). We prepared crude DNA samples of isolates for PCR testing by boiling cells in water. Among them, 6 were positive for *mcr-1* by PCR and sequencing (4 from dogs and 2 from cats). All 6 isolates were resistant to colistin, polymyxin B, cephalosporin, gentamicin, and ciprofloxacin by using the agar dilution method, in accordance with the European Committee on Antimicrobial Susceptibility Testing (<http://www.eucast.org>) for colistin and polymyxin B and Clinical and Laboratory Standards Institute guidelines (<http://www.clsi.org>) for the other antimicrobial drugs. We identified various resistance genes accounting for the multidrug resistance in these 9 *mcr-1*-positive isolates (6,7) (Table). We noted that *E. coli* isolate EC09 was also resistant to carbapenems and positive for *bla*<sub>IMP-4</sub>. We observed co-production of *mcr-1* and IMP-type metallo- $\beta$ -lactamase in *E. coli*.

We subjected all isolates to multilocus sequence typing, in accordance with the protocol described at <http://mlst.warwick.ac.uk/mlst/dbs/Ecoli>, and pulsed-field gel electrophoresis as described previously (8–10). We identified 5 *mcr-1*-positive isolates from 4 dogs (PET02–04 and PET06) and isolate EC07 as sequence

**Table.** Characteristics of 9 *mcr-1*-positive *Escherichia coli* isolates from companion animals and human patients, Guangzhou, China\*

Characteristic	Isolate								
	PET01	PET02	PET03	PET04	PET05	PET06	EC07	EC08	EC09
Isolation date	2016 Jan 1	2016 Jan 1	2016 Jan 2	2016 Jan 2	2016 Jan 2	2016 Jan 4	2015 Oct 10	2015 Nov 2	2015 Nov 21
Specimen source	Cat	Dog	Dog	Dog	Cat	Dog	Human	Human	Human
Specimen type	Feces	Feces	Feces	Feces	Feces	Feces	Urine	Urine	Blood
Phylogenetic group	B2	D	D	D	B2	D	D	B1	B1
ST†	ST93	ST354	ST354	ST354	New	ST354	ST354	ST156	ST156
PFGE type	IV	I	I	I	V	I	I	II	III
Resistance genes	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>qepA</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>rmtB</i> , <i>qnrS</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>fosA3</i> , <i>aac(6)-lb-cr</i>	<i>mcr-1</i> , <i>bla</i> <sub>TEM-1</sub> , <i>bla</i> <sub>CTX-M-55</sub> , <i>fosA3</i> , <i>rmtB</i> , <i>qepA1</i>	<i>mcr-1</i> , <i>bla</i> <sub>IMP-4</sub> , <i>bla</i> <sub>TEM-1</sub> , <i>fosA3</i> , <i>rmtB</i> , <i>qepA1</i>
MIC, $\mu$ g/mL									
Colistin	16	8	8	16	16	8	8	8	64
Polymyxin B	16	16	16	32	16	16	8	8	64
Ampicillin	>256	>256	>256	>256	>256	>256	>256	>256	>256
AMX/CLV	16	32	32	32	256	16	32	16	16
Cefotaxime	64	>256	256	>256	256	>256	256	256	>256
Ceftazidime	16	256	128	256	64	256	128	32	>256
Cefepime	8	256	128	256	16	256	64	64	>256
Gentamicin	128	>256	>256	>256	256	>256	>256	>256	>256
Amikacin	4	>256	>256	>256	>256	>256	>256	>256	>256
Ertapenem	<0.25	1	0.5	0.25	<0.25	1	0.5	<0.25	>16
Imipenem	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	>16
Meropenem	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	>16
Fosfomycin	32	>512	>512	>512	>512	>512	>512	>512	>512
Tigecycline	<2	<2	<2	<2	<2	<2	<2	<2	4
Nitrofurantoin	<16	32	32	32	128	32	64	64	32
Ciprofloxacin	256	128	128	128	64	128	128	256	256

\*AMX/CLV, amoxicillin clavulanic acid; PFGE, pulsed-field gel electrophoresis; ST, sequence type.

†By multilocus sequence typing.

type (ST) 354. Isolates PET01 and PET05, identified from cats, belonged to ST93 and a new ST strain, respectively. Isolates EC08 and EC09, from the patients who shared the same hospital room with the pet shop worker, were ST156 (Table). Results of pulsed-field gel electrophoresis were consistent with multilocus sequence typing results and showed that isolates consisted of 5 types (types I to V; online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/9/16-0464-Techapp1.pdf>). Isolate EC07 was clonally related to 4 *E. coli* strains from dogs, according criteria described by Tenover et al. (10), suggesting possible transmission of *mcr-1*-harboring *E. coli* between dogs and the patient. Colistin resistance was successfully transferred to *E. coli* C600 through conjugation in all isolates, suggesting that *mcr-1* was located on transferable plasmids.

These findings suggest that *mcr-1*-producing *E. coli* can colonize companion animals and be transferred between companion animals and humans. The findings also suggest that, in addition to food animals and humans, companion animals can serve as a reservoir of colistin-resistant *E. coli*, adding another layer of complexity to the rapidly evolving epidemiology of plasmid-mediated colistin resistance in the community.

### Acknowledgments

We sincerely thank the patients and the owners of companion animals for giving written consent for publication.

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## ***Acetobacter indonesiensis* Bacteremia in Child with Metachromatic Leukodystrophy**

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**To the Editor:** *Acetobacter indonesiensis*, first described in 2000 (1), belongs to the group of acetic acid bacteria (AAB), which includes the genera *Acetobacter*, *Gluconobacter*, *Asaia*, *Granulibacter*, and others in the family *Acetobacteriaceae*. AAB are of great industrial interest for use in vinegar fermentation processes because they oxidize alcohols or sugars incompletely, which leads

to acetic acid accumulation (2). AAB are widespread in nature and can be isolated from various sources, including vinegar, alcoholic beverages, tropical fruits, and flowers (1,2). AAB have rarely been associated with human disease. We describe a case of *A. indonesiensis* bacteremia in a child in Germany.

A 9-year-old girl with late-infantile metachromatic leukodystrophy was admitted to Marienhospital Herne, Herne, Germany, on February 9, 2015, for elective fundoplication. Because of her advanced neurologic disability, she required extensive nursing care and had several invasive medical devices, including a port catheter (detailed patient data in online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/9/16-0566-Techapp1.pdf>). Soon after her admission, fever developed, and C-reactive protein levels increased (online Technical Appendix Table 1). Physical examination and further diagnostic investigations (e.g., chest radiograph) revealed no focus of infection. Because she had experienced recurrent pneumonia and candidemia (the latter led to port catheter exchange 1 month previously), antimicrobial drug treatment with piperacillin/tazobactam and caspofungin was administered.

One blood culture, drawn from the port catheter on day 3 of the hospital stay, yielded slowly growing multidrug-resistant bacteria (agar diffusion indicated zones of inhibition only for imipenem, meropenem, fosfomicin, and tigecycline). By partial sequencing of the 16S rRNA gene, we identified the isolate as *A. indonesiensis*. Details of microbiological analyses, colony morphologic features, 16S rDNA-based phylogenetic analysis, and antimicrobial drug susceptibility testing results are given in the online Technical Appendix.

Because the patient clinically responded to piperacillin/tazobactam and caspofungin treatment, therapy was continued for 15 days, although piperacillin/tazobactam showed no *in vitro* activity against the *A. indonesiensis* isolate. Despite the patient's improved condition, 1 control blood culture drawn from the port on hospital day 10, while she was receiving antimicrobial treatment, yielded *A. indonesiensis*, although another blood culture drawn peripherally on hospital day 14 yielded no growth. Port catheter exchange was advised but was not performed, according to the parents' wishes.

The first report of human infection with AAB can be traced to 2004, when peritonitis, associated with *Asaia bogorensis*, was reported in a peritoneal dialysis patient (3). Further reports include a description of *Granulibacter bethesdensis* as a cause of lymphadenitis in patients with chronic granulomatous disease (4), isolation of *Gluconobacter* spp. from a culture of blood from an intravenous drug user and of *Gluconobacter* spp. and *Asaia* spp. from sputum samples of cystic fibrosis

patients (5), a case of *A. bogorensis* bacteremia in an intravenous drug user (6), and central venous catheter-associated cases of *Asaia lannaensis* bacteremia in a child with cancer who had received a bone marrow transplant (7) and in children who had idiopathic dilated cardiomyopathy (8).

Regarding *Acetobacter* spp., only 2 reports on human infection have been published: *A. cibinongensis* bacteremia in a patient receiving chronic hemodialysis with signs of an infected arteriovenous fistula and suspected intravenous drug abuse (9) and *A. indonesiensis* pneumonia in a cystic fibrosis patient who was receiving immunosuppressive treatment because of a recent lung transplant (10). Similar to the case we report, species identification in those cases was achieved only with the help of sequencing methods in both cases. In the *A. indonesiensis* pneumonia case, results of antimicrobial drug susceptibility testing found that the bacteria showed multidrug resistance, as in the case we report, but susceptibility to aminoglycosides.

Of note, the aforementioned AAB infections all occurred in chronically ill patients or intravenous drug users. Similarly, children with metachromatic leukodystrophy are prone to healthcare- and device-associated infections involving opportunistic pathogens, and frequent use of broad-spectrum antibiotics may predispose the children for infection with multidrug-resistant bacteria. In the case we report, frequent accessing of the port, including for parenteral nutrition, may have further promoted microbial colonization.

Because a focus of infection was not apparent and because *A. indonesiensis* grew in 2 blood cultures independently drawn from the port but not in the blood culture obtained from peripheral venipuncture, we assume the patient's port catheter harbored the infectious agent. The fact that several previously reported AAB infections were catheter-associated may further support our suspicion. However, we could not confirm this assumption because the port was not removed and cultured.

The patient clinically responded to piperacillin/tazobactam and caspofungin treatment, despite a lack of *in vitro* activity against the *A. indonesiensis* isolate. Although this response might be explained by the presence of a second pathogen (which was not cultured but covered by the given antimicrobial agents), the control blood culture drawn from the port still yielded *A. indonesiensis* and at least argues for persistent colonization of the port. Because of pathogen persistence in blood culture and limited therapeutic options owing to the multidrug-resistance of the isolate, we believe the port should have been removed in this case.

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Informed consent was obtained from the patient's parents for publication of this case report.

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## Autochthonous Chikungunya Fever in Traveler Returning to Japan from Cuba

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**To the Editor:** Chikungunya fever is a febrile illness caused by mosquito-transmitted chikungunya virus (CHIKV; genus *Alphavirus*, family *Togaviridae*). Clinical signs and symptoms typically begin with high-grade fever after an incubation period of 2–4 days (1). Other common symptoms include polyarthralgia, which is usually symmetric and involves multiple and distal joints, and skin involvement manifesting as a macular or maculopapular rash (2). Peripheral lymphadenopathy (most often cervical) and conjunctivitis might also occur (3).

Since late 2013, several outbreaks of illness caused by CHIKV have occurred in the Americas, including South America, the Caribbean, and the United States, which are outside this virus's former distribution area (3). Although autochthonous transmission of chikungunya fever has been reported in most Caribbean islands, only imported cases have been previously reported in Cuba (4). As increased numbers of US tourists visit Cuba after improved diplomatic relations in July 2015, reports of chikungunya fever cases in Cuba are of interest for travelers and health-care providers. We describe a case of autochthonous chikungunya fever in a man who had traveled from Japan to Cuba.

In late February 2016, a previously healthy 27-year-old man visited a travel clinic in the National Center for Global Health and Medicine (Tokyo, Japan) with fever and rash. In mid-February, he had traveled to Havana and Santiago de Cuba in Cuba by way of Toronto, Ontario, Canada, for 11 days of sightseeing. He used no insect repellent during the trip and was unaware of any mosquito bites. When he sought care, he reported a high-grade fever (39°C) for 24 hours and several symptoms since the day of his return: retro-orbital pain, malaise, congested conjunctivas, and a

rash on his anterior chest. Over the previous few days, his knee and ankle joints also had mild arthralgia.

On physical examination, the patient's body temperature was 38.7°C, and he had congested bulbar conjunctivas, cervical lymphadenopathy, and maculopapular rashes on his face, trunk, and extremities (online Technical Appendix Figure, panels A, B, <http://wwwnc.cdc.gov/EID/article/22/9/16-0603-Techapp1.pdf>). Laboratory tests revealed lymphopenia (701 cells/ $\mu$ L) and mild elevation of C-reactive protein (0.87 mg/dL). Real-time reverse transcription PCR detected CHIKV RNA in his serum sample. Phylogenetic analysis was performed on the basis of nucleotide sequences of the E1 gene from the sample by using the maximum likelihood method with 1,000 bootstrap replicates and MEGA 6.0 software (5). This sequence (GenBank accession no. LC146714) was 99.9% (1,319 of 1,320 sequences) was identical to that of a CHIKV strain isolated from the Dominican Republic in 2014 (GenBank accession no. KR559498) (Figure; online Technical Appendix Table). The positive-to-negative ratio of CHIKV-specific IgM was negative in a serum sample collected on day 4 after fever onset but was positive in a sample taken 7 days later (positive-to-negative ratios 5.6 and 21.9, respectively; ratios were considered positive if >11). Because the patient's serum samples contained no dengue or Zika virus, infections from these viruses were excluded, and chikungunya fever was diagnosed.

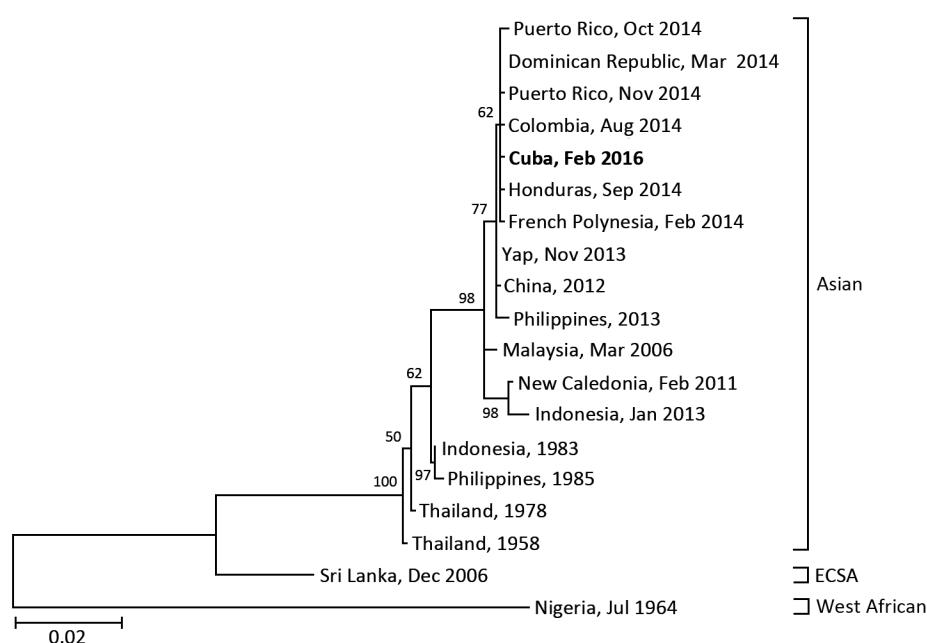
One day after the patient's first visit to the clinic, rashes on his extremities became worse and slightly itchy. Pain also developed in his wrists and metacarpophalangeal joints of his hand, followed by cervical pain and slight rigidity on the hand's distal and proximal interphalangeal

joints. The patient was initially treated with acetaminophen (600 mg 3 $\times$ /d 2 d); after diagnosis of chikungunya fever, he was treated with loxoprofen and rebamipide (60 mg and 100 mg, respectively, 3 $\times$ /d 7 d). The congested bulbar conjunctivas and rash on his trunk improved; soon thereafter, all symptoms resolved.

CHIKV was first isolated in 1953 in Tanzania during an epidemic outbreak in East Africa (6). Mosquitoes, predominantly *Aedes aegypti* and *Ae. albopictus*, transmit the virus (2). *Aedes* spp. are also the common vector of dengue and Zika viruses, and localized dengue outbreaks occurred in Santiago de Cuba in 1997 and in Havana in 2000–2001 because of the persistence of *Aedes* mosquito infestation in Cuba (7,8). Furthermore, autochthonous Zika virus infection in Cuba was first reported in March 2016 (9).

Differentiation between chikungunya fever, dengue fever, and Zika virus infection is difficult because of similar signs and symptoms and common endemic areas. We suspected chikungunya fever in this patient because of high-grade fever and maculopapular rash, although he also had prominent conjunctivitis, which is uncommon in CHIKV-infected patients but frequent in persons infected with Zika virus (3,10). Phylogenetic analysis of the virus isolated from this patient revealed a high sequence homology with recent strains discovered in Caribbean and Central American countries in 2014. Homology between the isolate from this patient and a 2014 Asian lineage isolate from the Dominican Republic was 99.92% at the nucleotide level.

This case highlights the potential threat of a chikungunya fever outbreak in Cuba. Physicians should consider chikungunya fever in the differential diagnosis for febrile



**Figure.** Phylogenetic analysis of the chikungunya virus sequence obtained from a patient returning to Japan (in bold) from Cuba in February 2016, compared with reference sequences. Virus lineages are shown at right. Scale bar represents substitutions per nucleotide position. ECSA, Eastern/Central/South African lineage.

travelers returning from Cuba with a rash, similarly to patients returning from other countries in which dengue fever, chikungunya fever, and Zika virus infection are endemic. Preventive measures, including advice to travelers on proper use of insect repellents, are critical for preventing CHIKV infection.

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## Inactivation and Environmental Stability of Zika Virus

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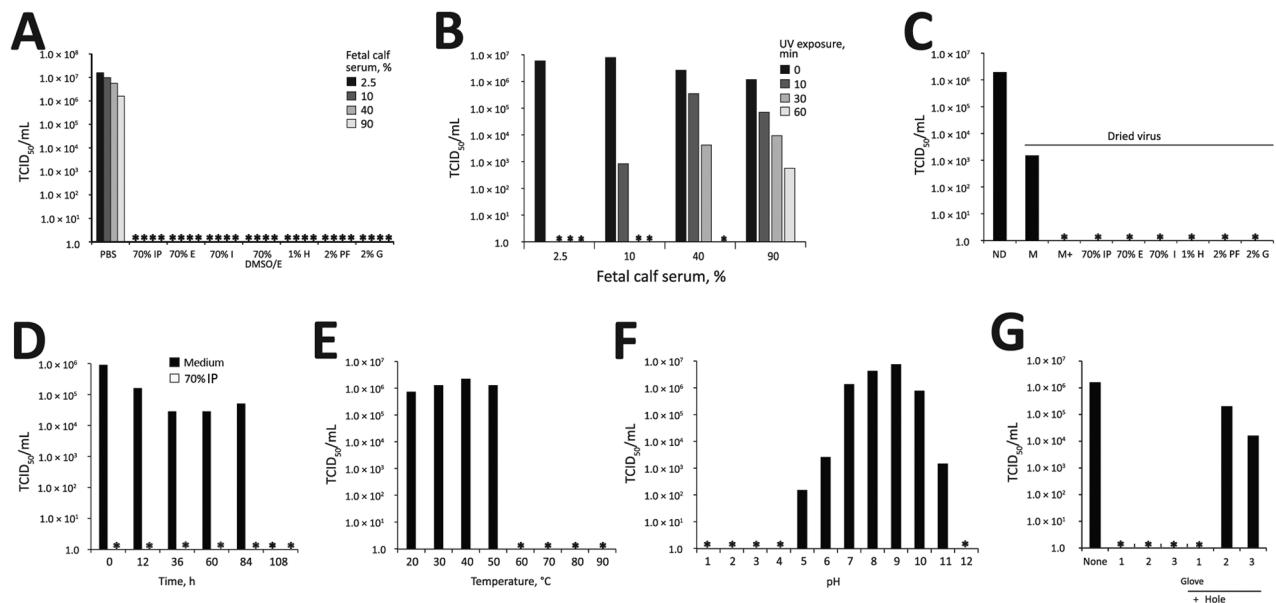
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**To the Editor:** Zika virus is an emerging virus that has spread to most countries in Latin America and the Caribbean (1,2). It is transmitted by mosquitoes and through sexual intercourse (3). Most persons infected with Zika virus are asymptomatic or experience mild symptoms (4). However, in a pregnant woman, infection may cause severe pregnancy and birth complications, most notably microcephaly in children infected in utero (5–7). Zika virus infection might also be associated with an increased incidence of Guillain-Barré syndrome (8). Thus, the virus represents a threat to healthcare workers who manage infected patients or diagnostic samples and researchers who work with infectious virus in laboratories.

Working with Zika virus, a Biosafety Level 2 (BSL-2) pathogen in the European Union, except for the United Kingdom (where it is BSL-3), requires specific safety precautions (9). No inactivation data specific for Zika virus are available (9); consequently, disinfection guidelines are based on protocols to inactivate other flaviviruses. To gain experimental evidence regarding inactivation and disinfection for Zika virus, we determined its susceptibility to various disinfectants and inactivation methods.

To test susceptibilities, we determined the 50% tissue cell infectious dose per milliliter (TCID<sub>50</sub>/mL) (10) of the Zika virus MR766 strain (1) before and after the virus was exposed to disinfectants or other inactivation procedures (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/9/16-0664-Techapp1.pdf>). We then determined the effect of alcohol-based disinfectants on viral infectivity. Using Zika virus stock containing 2.5% fetal calf serum (FCS) mixed 3:10 (vol/vol) with indicated alcohols, we incubated the mixture for 1 minute and then used it for infection (Figure, panel A). All alcohols entirely inactivated



**Figure.** Inactivation and environmental stability of Zika virus. Asterisks (\*) indicate lack of infection. A) Virus stocks containing 2.5%, 10%, 40%, or 90% fetal calf serum were incubated with alcohol-based disinfectants for 1 min. All disinfectants inactivated the virus. B) Virus stocks containing indicated concentrations of fetal calf serum were exposed to the ultraviolet (UV) light of a laminar flow hood. Higher concentrations of serum required more time to inactivate the virus. C) Virus stock was dried for 18 h and was then reconstituted in medium or the indicated disinfectants for 5 min or exposed to 10 min UV light before reconstitution. All disinfectants inactivated the virus. D) Virus was dried and incubated for indicated periods of time. Thereafter, dried virus was reconstituted in medium or 70% (vol/vol) isopropanol. Isopropanol inactivated the virus, but dried virus in medium remained infectious even after 84 h of incubation. E) Zika virus was incubated for 5 min at indicated temperatures. Temperatures  $\geq 60^{\circ}\text{C}$  inactivated the virus. F) Stocks were adjusted to indicated pH values and incubated for 10 min. pH levels  $\leq 4$  or  $> 11$  deactivated the virus. G) Finger tips of laboratory gloves were cut off, with or without introducing a hole by pinching with a needle, and put into medium. Glove tips were filled with virus stock and incubated for 90 min at room temperature. All gloves without needle holes were protective against transmission; 2 of 3 gloves with needle holes allowed virus transmission. For detailed experimental description, see online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/22/9/16-0664-Techapp1.pdf>). DMSO, dimethyl sulfoxide; E, ethanol; G, glutaraldehyde; I, incindin, IP, isopropanol; M, medium, M+, medium plus 10 min UV; ND, not dried; PBS, phosphate-buffered saline; PF, paraformaldehyde; TCID<sub>50</sub>, 50% tissue culture infective dose; UV, ultraviolet.

the virus. Complete loss of infectivity was also observed after virus exposure to 1% hypochlorite (often used to inactivate virus in liquid wastes in BSL-2/3 laboratories), 2% paraformaldehyde (used to inactivate virus for subsequent flow cytometry), and 2% glutaraldehyde (often applied to fix virus for subsequent electron microscopy analysis) (Figure, panel A). Thus, routinely used disinfectants and inactivation procedures are sufficient to inactivate Zika virus in laboratory virus stocks. Next, we repeated these experiments in the presence of a high protein load using Zika virus preparations supplemented with FCS in increasing concentrations (10%, 40%, 90%), to mimic virus found in clinically relevant material. Again, all treatments entirely disrupted Zika virus infectivity (Figure, panel A).

Ultraviolet (UV) radiation inactivates viruses by chemically modifying the genome. We exposed 200  $\mu\text{L}$  of Zika virus preparations containing increasing concentrations of serum to UV light of a laminar flow for up to 60 minutes. Exposure for 10 minutes entirely inactivated Zika virus in the presence of 2.5% FCS serum; increasing concentrations

of serum reduced the antiviral effects of UV light (Figure, panel B). When Zika virus containing 90% serum was exposed for 60 min to UV light, infectivity was reduced by 99.95%; however, some residual infectivity was detected (Figure, panel B).

Next, we evaluated environmental stability by drying 100  $\mu\text{L}$  of Zika virus stock for 18 hours. Thereafter, dried virus was reconstituted in the same volume of medium or disinfectants. Endpoint titrations showed that the reconstituted virus remained infectious, although TCID<sub>50</sub> was reduced by  $\approx 3$  orders of magnitude (Figure, panel C). All disinfectants and UV radiation entirely inactivated dried Zika virus (Figure, panel C). Additional experiments demonstrated that dried Zika virus remained infectious for  $> 3$  days (Figure, panel D) suggesting, for example, that dried droplets can be infectious, confirming that proper surface disinfection is essential.

We also assessed the environmental stability of Zika virus to heat and change in pH. The virus was stable at temperatures up to  $50^{\circ}\text{C}$  but lost all infectivity at temperatures

of  $\geq 60^{\circ}\text{C}$  (Figure, panel E). Thus, virus-contaminated materials such as surgical instruments can be decontaminated by heat. We also found that Zika virus infectivity was highest after adjusting the stock to a pH of  $\approx 9$  (Figure, panel F). In contrast, adjusting Zika virus to pH 12 or to  $\leq \text{pH } 4$  abrogated infectivity (Figure, panel F).

Finally, we analyzed whether gloves routinely used in BSL-2 laboratories protect against Zika virus. For this, we cut off fingertips of nitrile and latex gloves, filled tips with a Zika virus suspension, and placed them into cell culture plates containing medium. Virus-containing fingertips were inserted in such a way that diffusion would only occur if the virus passed through the nitrile/latex barrier. As a control, we made a hole of  $< 1$  mm in the fingertips. All 3 tested gloves prevented virus diffusion (Figure, panel G). However, if glove integrity was disrupted by a pin, the virus passed through in 2 of 3 cases (Figure, panel G).

We demonstrated that Zika virus is destroyed by classical disinfectants and inactivation methods and that nitrile and latex gloves are protective. We also showed that UV light of a laminar flow hood inactivates Zika virus, but particularly if the virus is in a protein-rich environment, the exposure time range should be in hours rather than in minutes. Although we expected that Zika virus would be inactivated by alcohol and disinfectants, we conducted a thorough experimental verification to exclude uncertainties surrounding work with this emerging pathogen.

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## ESBL-Producing Strain of Hypervirulent *Klebsiella pneumoniae* K2, France

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**To the Editor:** *Klebsiella pneumoniae* is mainly responsible for hospital-acquired urinary tract infections, bacteremia, pneumonia and intra-abdominal infections. However, since the mid-1980s, *K. pneumoniae* has also been described as the cause of highly invasive community-acquired infections (1,2). The *K. pneumoniae* isolates associated with such infections are often hypermucoviscous and frequently belong to the capsular serotypes K1 or K2. Two of the most extensively studied genes associated with invasive infections are a mucoviscosity-associated gene A (*magA*) in serotype K1 and a regulator of mucoid phenotype A (*rmpA*). These strains of hypervirulent *K. pneumoniae* (hvKP) are now circulating worldwide (1,2).

At the same time, a substantial increase of high-level antimicrobial resistance acquired by non-hvKP strains has also been observed. Clonal complexes of hvKP and multi-drug-resistant (MDR) strains had been considered independent (3) until 2014, when extended-spectrum  $\beta$ -lactamase (ESBL)— or carbapenemase-producing hvKP were first identified in China (4). Here we report an ESBL-producing strain of hvKP isolated from a patient in France.

The patient was a 56-year-old woman, born in Algeria, who alternately resided in France and Algeria for several years without travel to any other country. She underwent liver transplant in 2007 for primary biliary cirrhosis. In 2012, she had a routine posttransplant liver biopsy indicating

granulomatosis, resulting in several examinations to determine its etiology. The patient was afebrile with no inflammatory syndrome. A thoracic computed tomography scan indicated no abnormalities. We performed a bronchoalveolar lavage, and bacteriologic examination of the specimen indicated a strain of *K. pneumoniae* with a hypermucoviscosity phenotype. We identified the ESBL phenotype with a positive double-disk synergy test between clavulanic acid and third-generation cephalosporins and aztreonam. We conducted antimicrobial susceptibility tests according to guidelines issued in 2013 by the Comité de l'Antibiogramme de la Société Française de Microbiologie (<http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM2013vjuin.pdf>). These tests revealed high-level resistance to third-generation cephalosporins and to all aminoglycosides except amikacin. The strain was susceptible to cefoxitin, piperacillin/tazobactam, carbapenems, all fluoroquinolones, fosfomicin, and trimethoprim/sulfamethoxazole. The patient did not receive any antimicrobial drug treatment and after 3 years of follow-up reported no signs of pneumopathy.

Multiplex PCR and sequencing of the identified strain indicated the presence of *bla*<sub>CTX-M-3</sub> (5). We extracted plasmid carrying the ESBL gene with the DNA Plasmid Miniprep Kit (QIAGEN, Valencia, CA, USA) and transferred the gene by electroporation into *Escherichia coli* DH10B cells. Relaxase typing, which detects major replicon groups, revealed only IncL/M plasmid (6). We performed another multiplex PCR to determine capsular serotypes K1 or K2 and the presence of major virulence factors. The capsular serotype was K2 (7). We identified *rmpA*, the plasmid-mediated gene regulating extracellular polysaccharide synthesis, and *iutA*, *entB*, *mrkD*, and *ybtS* genes by this PCR test. The isolate belonged to sequence type (ST) 86 as determined by multilocus sequence typing (<http://www.pasteur.fr/mlst>).

The earliest-described strains of hvKP, which were isolated from liver abscesses, were predominantly serotype K1 and ST23 (2). The most frequently isolated non-K1 hvKP strains are currently serotype K2, known to cause hepatic abscesses and severe cases of pneumonia and other hvKP-associated infections such as necrotizing fasciitis (2). The K2 strains isolated to date appear to originate from a much broader range of ST groups that include ST86 and many others (e.g., ST65, ST66, ST373, ST374, ST375, ST380, and ST434) (8,9).

This patient, despite being immunocompromised, was only colonized with what is normally considered a highly pathogenic strain. In general, the acquisition of antimicrobial resistance genes reduces fitness, which could have been the case in this patient. However, other strains carrying MDR and high-virulence genes, namely *E. coli* ST131, have no loss in fitness (10). These clones might harbor other biologic factors providing a competitive advantage.

MDR strains of *K. pneumoniae* have emerged in recent years and have been identified as a major threat to public

health by the US Centers for Disease Control and Prevention. hvKP, with its high pathogenic potential, has also been on the rise during the same period. In the past, MDR and hvKP strains evolved separately in distinct clonal groups (2), but the recent emergence of hvKP harboring the gene for MDR, such as the one identified in our study, raises newfound concerns. Our patient was colonized with an ST86 CTX-M-3-producing strain of K2 hvKP, raising the question of whether MDR hvKP strains could circulate in Europe.

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## Chromosomal Locations of *mcr-1* and *bla*<sub>CTX-M-15</sub> in Fluoroquinolone-Resistant *Escherichia coli* ST410

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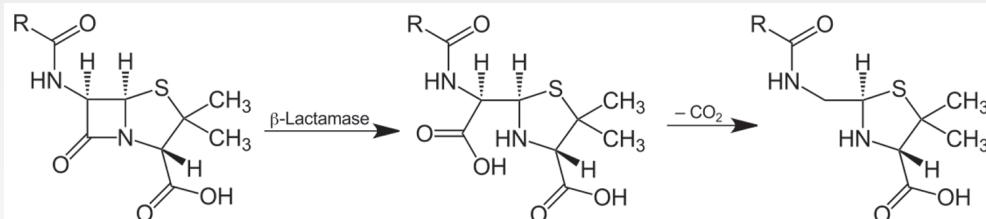
**To the Editor:** Recently, Yi-Yun Liu et al. reported on the discovery of *mcr-1*, a plasmidborne resistance gene mediating resistance to colistin, in isolates obtained from humans and animals (1). Since the original publication, *mcr-1* with or without the insertion element IS*AplI* has been detected on plasmids of different incompatibility groups, including IncI2, IncHI2, and IncX4, and in many different countries (1–3). Because colistin is a last-resort parenteral antimicrobial drug, the transfer of *mcr-1* by

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## etymologia

### β-Lactamase [ba'tə lak'tə-mās]

Enzymes that catalyze the cleavage of β-lactam rings in penicillins, cephalosporins, monobactams, and carbapenems were first described by Abraham and Chain in 1940. These enzymes confer resistance to β-lactam antibiotics on bacteria that produce them. β-lactamases are ancient, theorized to have evolved 1–2 billion years ago, but the emergence and spread of penicillin-resistant staphylococci in hospitals in the 1950s showed how penicillin use could select producers from a population of nonproducers. “Lactam” is a portmanteau of “lactone” (from the Latin *lactis*, “milk,” since lactic acid was isolated from soured milk) and “amide.” The “β” refers to the nitrogen’s position on the second carbon in the ring. The suffix “-ase,” indicating an enzyme, is derived from “diastase” (from the Greek *diastasis*, “separation”), the first enzyme discovered in 1833 by Payen and Persoz.



Action of β-lactamase and decarboxylation of the β-lactam ring. Equation by Jü, own work, public domain, <https://commons.wikimedia.org/w/index.php?curid=11204303>

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conjugation or through mobilizable plasmids raises concern about the emergence of pan-resistant *Enterobacteriaceae*.

We previously described extended-spectrum  $\beta$ -lactamase (ESBL)-producing and carbapenemase-producing isolates obtained from livestock and a human in Germany that harbored the *mcr-1* gene (2). Because the transfer of *mcr-1* through the food chain is highly likely, we looked for its presence in 62 whole-genome sequenced ESBL-producing *Escherichia coli* isolates obtained during 2012–2013 from food products sampled in Germany (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/8/16-0692-Techapp1.pdf>). We detected 4 isolates harboring the *mcr-1* gene (*E. coli* RL138, RL145, RL158, and RL465) that displayed a colistin MIC of 4 mg/L (online Technical Appendix Table 1). The raw sequencing reads and the assembled contigs of the *mcr-1*-positive isolates were deposited in the European Nucleotide Archive under project accession no. PRJEB13470. We conducted conjugation experiments to analyze the transferability of *mcr-1* (online Technical Appendix). For all isolates except RL465, *mcr-1* was transferable to *E. coli* J53  $Az^r$ . For isolates RL138, RL145, and RL158, the *mcr-1* gene was present on IncX4 and IncHI2 plasmids (Figure, panel A, <http://wwwnc.cdc.gov/EID/article/22/8/16-0692-F1.htm>; online Technical Appendix Table 2). The sequence type (ST) 410 *E. coli* isolate RL465 was detected in a turkey hen meat sample from 2013 and harbored *bla*<sub>CTX-M-15</sub> and *mcr-1*, a gene combination hitherto identified only in travelers from the Netherlands and children from China (4). Both the *bla*<sub>CTX-M-15</sub> and *mcr-1* genes were not transferable, indicating that neither gene was plasmid-encoded. Examination of the genetic environment of *mcr-1* in the assembled gapped genome showed a chromosomal location for the *mcr-1* transposition unit that included an IS*Apl1* element (Figure 1, panel A; online Technical Appendix Figure 1, panel A) flanked by the inverted repeats (IR-R1, IR-R2, and IR-L1). We verified the chromosomal location for the *mcr-1* gene by sequencing the genome to completion, using long-read single-molecule real-time sequencing (Pacific Biosciences, Menlo Park, CA, USA; online Technical Appendix Figure 2); the resulting contigs of *E. coli* RL465 were deposited in the European Nucleotide Archive under accession no. PRJEB14095. One copy of the IS*Apl1*-*mcr-1* transposition unit was located in the region between a predicted 4Fe-4S ferredoxin-type protein (*vdhY*) and *ldtE* (L,D-transpeptidase) (bp 2652307–2665241), and flanked on either side by a 2-bp direct repeat (CA). We observed a similar situation for the IS*Ecp1*-*bla*<sub>CTX-M-15</sub>-*orf477* transposition unit (online Technical Appendix Figure 1, panel B). However, this insertion mapped to a different chromosomal location in a region encoding a defective lambdoid prophage inserted between the molybdate ABC transporter operon (*modABC*) and the biotin biosynthesis operon

(*bioABCDF*) (bp 1662140–1716472). It was flanked by direct repeats (TGGTT).

We reexamined our collection of 424 genome sequenced ESBL- and carbapenemase-encoding *E. coli* isolates, obtained during 2010–2014 (2), for isolates that harbored *bla*<sub>CTX-M-15</sub> at a chromosomal location identical to that found in *E. coli* RL465. We detected 3 such isolates from 2010–2011 from companion animals and livestock (R107, sock swab dairy cattle farm, 2011; R208, sock swab pig fattening farm, 2011; V177, sick dog, 2010), and 11 consecutive isolates from a hemato-oncologic patient (5), obtained within an 11-month period during 2011–2012 (E006910, E007337, E007651, E007825, E000565, E002592, E002816, E003488, E005417, E006587, E006874) (Figure, panel B). All of these isolates were ST410 and negative for the *mcr-1* gene. Phylogenetic analysis of the core genome of these isolates with *E. coli* RL465 using the program Harvest Suite (6) indicated they were highly related and separated from *E. coli* V177 (the oldest isolate) by 66 (E006910, E007651) to 110 (E007337) single-nucleotide polymorphisms (core genome size 94%, representing 4.58 Mbp). Thus, our results suggest that transposition of the IS*Apl1*-*mcr-1* unit to the chromosome in *E. coli* RL465 is a later event and probably occurred after transfer of the *bla*<sub>CTX-M-15</sub> allele to the distinct chromosomal location into this *E. coli* ST410 subclone.

These findings highlight 2 independent points. First, our results extend data on the mobility of IS*Apl1*-*mcr-1* to a chromosomal location and reveal a new dimension in the transmissible nature of *mcr-1* in colistin-resistant *Enterobacteriaceae* isolates and their ecology. Second, clonal isolates of ST410 have been isolated from diverse environments, livestock, companion animals, and humans and, as we demonstrate here, in turkey hen meat (7,8). Thus, the simultaneous spread of the *mcr-1* and *bla*<sub>CTX-M-15</sub> genes mediated by a single bacterial clone is real and suggests that *mcr-1* is already present in the diverse reservoirs inhabited by these isolates.

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L.F., C.I., and T.C. conceived the study; S.E.W., K.G., H.G., S.D., B.B., C.S., and J.O. performed experiments; A.I., J.F., H.S., B.G., and A.K. contributed isolates and reagents; L.F., B.B., C.I., and T.C. analyzed the data; and T.C. and L.F. wrote the manuscript, which all authors approved.

B.G. is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA. The other authors have nothing to proclaim.

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## Specificity of Dengue NS1 Antigen in Differential Diagnosis of Dengue and Zika Virus Infection

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**To the Editor:** Circulation of new arboviruses of the genus *Flavivirus* poses a major problem for differential diagnosis. Zika virus, a mosquito-borne virus of the family *Flaviviridae*, is closely related to other arboviruses circulating in the Americas, including dengue, yellow fever, Saint Louis encephalitis, and West Nile viruses (1,2). Serologic cross-reactivity between these arboviruses is common; thus, to ensure optimal patient care and accurate epidemiologic surveillance, an effective differential diagnosis is required in regions with active transmission of dengue virus and circulation of Zika virus (2–4).

Cross-reactivity between flaviviruses has been reported in antibody assays and in tests for Dengue nonstructural 1 glycoprotein (NS1) antigen. Gyurech et al. (5) reported false-positive test results for dengue NS1 antigen in a patient with acute Zika virus infection. Of the 3 NS1 tests used in that study, only the SD Bioline Dengue Duo (Standard Diagnostics, Inc., Gyeonggi-do, South Korea) showed positive results for 3 of 4 sequential serum samples from the patient.

Cross-reactivity in NS1 dengue tests (ELISA and immunochromatographic) using serum samples from patients with acute Zika virus infection would have medically significant consequences. We therefore conducted a retrospective analysis of the differential diagnosis for dengue and Zika virus infections since the beginning of the Zika virus outbreak in French Guiana, a department of France on the northeast coast of South America.

French Guiana is subject to endemoepidemic circulation of dengue and experienced a large outbreak of chikungunya in 2014. We conducted our study from December 17, 2015 (the time of biologic confirmation of the first case of Zika virus disease in French Guiana), through March 2, 2016. During that time, the incidence of dengue virus infection in French Guiana was low, and only 1 sporadic case was confirmed. We studied clinical samples collected during this period from all patients with suspected arbovirus infection.

**Table.** RT-PCR results for 65 persons with clinical samples tested during the acute phase of Zika virus infection, French Guiana, December 17, 2015–March 2, 2016\*

Days from symptom onset to sample collection	No. cases	RT-PCR results, by clinical sample					
		Serum only		Urine only		Serum and urine	
		No. samples	C <sub>t</sub> , mean ± SD	No. samples	C <sub>t</sub> , mean ± SD	No. samples	C <sub>t</sub> , mean ± SD
0	8	5	31.8 ± 2.8	1	34.6†	2	Serum: 28.6 ± 10.45; urine: 28.9 ± 11.3
1	12	4	29.9 ± 4.6	2	36.5 ± 0.3	6	Serum: 36.0 ± 1.7; urine: 33.6 ± 4.4
2	20	13	32.8 ± 3.0	1	33.7†	6	Serum: 34.2 ± 3.6; urine: 33.0 ± 1.4
3	15	8	33.6 ± 3.6	2	34.2 ± 1.5	5	Serum: 33.6 ± 2.8; urine: 34.1 ± 1.6
4	7	5	31.7 ± 2.4	1	28.9†	1	Serum: 32†; urine: 32†
5	3	0	NA	1	31.7†	2	Serum: 35.1 ± 0.8; urine: 32.9 ± 3.9
Total	65	35	32.5 ± 3.1	8	33.8 ± 2.6	22	Serum: 33.6 ± 3.8; urine: 32.9 ± 3.8

\*C<sub>t</sub>, cycle threshold; NA, not applicable; RT-PCR, reverse transcription PCR.  
†SD not determined.

Samples were analyzed for the differential diagnosis of dengue, chikungunya, and Zika virus disease. Zika virus diagnosis was conducted by the National Reference Centre for Arboviruses (NRC) at the Institut Pasteur of French Guiana, in Cayenne, according to the real-time reverse transcription PCR (rRT-PCR) protocol described by Lanciotti et al. (3). Dengue diagnosis was routinely performed by all medical diagnostic laboratories using various rRT-PCR techniques or dengue NS1 test kits. We used the same SD Bioline Dengue Duo test used by Gyurech et al. (5); this test was performed at the laboratory of the Centre Hospitalier de l'Ouest-Guyanaise in Saint-Laurent du Maroni, French Guiana. We also used the Platelia Dengue NS1 Ag kit (Bio-Rad, Marnes-la-Coquette, France); the assay was performed at NRC.

Since Zika virus first appeared in French Guiana, the NRC has investigated 270 samples collected 0–5 days after fever onset for molecular diagnosis of Zika virus and dengue NS1. Of the 270 suspected patients, 65 were confirmed positive for acute Zika virus infection by rRT-PCR of serum, urine, or both. The mean cycle thresholds (± SDs) were 33.0 (± 3.4) for serum samples and 33.2 (± 3.5) for urine samples (Table). Of the 65 acute-phase Zika virus–positive serum samples, 36 were also tested with the Platelia Dengue NS1 test, 21 were tested with the SD Bioline Dengue Duo test, and 8 were tested with both tests; none of the results were positive. Of the 205 Zika virus–negative samples, 204 were also negative for dengue NS1; only 1 patient had a positive dengue NS1 test result, and the infection was confirmed by molecular investigations to be a case of acute dengue-1 disease.

This retrospective analysis of dengue and Zika virus diagnoses indicates that no false-positive dengue NS1 test results occurred among samples with acute-phase Zika virus infection. Indeed, samples from all 65 patients with rRT-PCR–confirmed acute Zika virus infection were negative by both dengue NS1 tests. Zika virus is closely related to dengue virus, and during the acute phase of disease, Zika

virus might release NS1 into patients' serum; however, this putatively released nonstructural protein does not appear to cross-react with the dengue NS1 tests used in our study. No Zika NS1 antigen assay currently exists, and acute-phase release of Zika NS1 has not been verified. If a Zika virus NS1 test is developed, it should be evaluated for cross-reactivity with serum from patients with acute dengue infection. The false-positive result reported by Gyurech et al. (5) for dengue NS1 antigen in a patient with acute Zika virus infection requires further investigation. Little is known about false-positive NS1 tests. Zika virus might show cross-reactivity with other flaviviruses and possibly cytomegalovirus, and hematologic disorders might cause NS1 positivity (6,7).

The co-circulation of Zika virus and dengue virus in the Americas is causing a health emergency. Our findings show that dengue NS1 antigen assays are still entirely appropriate for dengue surveillance, even during the epidemic circulation of Zika virus.

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## ***Vibrio cholerae* O1 Imported from Iraq to Kuwait, 2015**

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**To the Editor:** The etiologic agent of the sixth pandemic of cholera was classical biotype of *Vibrio cholerae* O1. The ongoing seventh pandemic is caused by El Tor biotype. The biotypes are differentiated by phenotypic and genotypic characteristics. However, this differentiation blurred when *V. cholerae* O1 strains were detected in Matlab, Bangladesh, in 2006, in which characteristics were mixed. Genetically, the differences occurred in *tcpA*, which encodes the major adherence antigen *rstR* that regulates site-specific recombination of CTX $\phi$  phage and *ctxB* that encodes the B subunit of cholera toxin. These genes had the characteristics of classical biotype in Matlab variants of El Tor strains. Later, various types of El Tor variants were reported in Southeast Asia, Africa, and Haiti. Differentiating features also occur in repeat toxin A gene (*rtxA*), chromosomal location of CTX $\phi$ , the number of heptad repeats in *ToxR* binding region, and the occurrence of vibrio seventh pandemic islands I and II (1,2).

Kuwait is free of endemic cholera, but imported cases occur there (3). Cholera is endemic to neighboring Iraq. An outbreak caused by *V. cholerae* O1 Inaba serotype started in Iraq in September 2015 (4). However, a full characterization of the strain is lacking. A thorough characterization of the strain assumes urgency in light of the spread of variants. We characterized isolates from 2 recent cholera cases imported to Kuwait from Iraq.

The first case was in a 19-year-old Kuwaiti man who visited Najaf and Karbala in Iraq in September 2015; the second case was in a 52-year-old Kuwaiti woman who visited the same 2 locations in October 2015. Both had watery diarrhea 3–4 times daily and vomiting; they returned to Kuwait and were admitted to Al Amiri Hospital (Sharq, Kuwait). They gave histories of drinking local water in Iraq, had moderate dehydration, and were treated with intravenous rehydration solution and a single doxycycline dose (500 mg). Diarrhea resolved after 2–3 days.

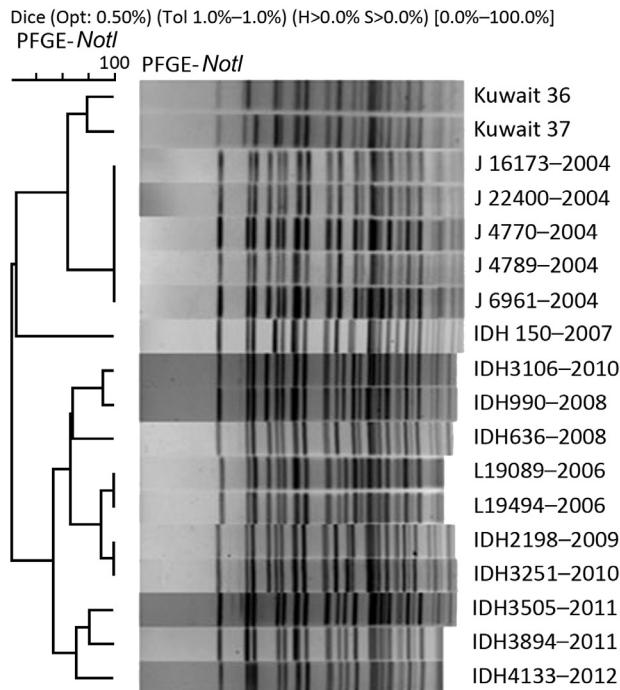
Fecal specimens collected at admission from both patients grew yellow colonies on thiosulfate bile salt sucrose agar (Eiken, Tokyo, Japan); these colonies were confirmed as *V. cholerae* O1 Inaba serotype by biochemical reactions and agglutination with specific antiserum (Denka Seiken, Tokyo, Japan). The woman's isolate was designated as Kuwait 36 and the man's as Kuwait 37. The isolates were positive for chicken cell agglutination and Voges-Proskauer tests and were polymyxin B resistant, characteristics of El Tor biotype. The isolates were resistant to nalidixic acid but susceptible to ciprofloxacin, norfloxacin, ofloxacin, tetracycline, meropenem, ampicillin, ceftriaxone, trimethoprim/sulfamethoxazole, chloramphenicol, erythromycin, azithromycin, streptomycin, neomycin, and gentamicin by disk diffusion test. Tetracycline susceptibility confirmed favorable response to doxycycline.

We studied the genotype of *ctxB* using a double-mismatch amplification mutation PCR (i.e., mismatches in both primers). PCR with classical *ctxB*-specific primers *ctxBF4/ctxBRvCla* yielded an amplicon of 191 bp, but not with Haitian *ctxB* specific primers *ctxBF3/ctxBRvCla*, indicating that the isolates had a *ctxB* of classical biotype (genotype 1) (5,6). Mismatch amplification mutation assay PCR (MAMA-PCR, i.e., mismatch in only 1 primer) with Haitian-specific *tcpA* primers *tcpAF2/tcpARev* produced an amplicon of 167 bp but not with El Tor *tcpA*-specific primers *tcpAF1/tcpAEIRev*, suggesting these isolates had the Haitian variant *tcpA* (2). MAMA-PCR for *rtxA* with El Tor-specific primer pair *rtxAF/rtxAR1* yielded a 187-bp amplicon but no amplicon for Haitian variant primer pair *rtxAF/rtxAR2*, suggesting the occurrence of *rtxA* of El Tor variety (2). The isolates possessed El Tor type *rstR* because they produced a 500-bp amplicon with primer pair *rstR2/rstA3R* (7). The isolates were positive for *rstC*, a repeat sequence activator found in El Tor biotype, because they yielded an amplicon of 238 bp with primer pair *rstC1/rstC2* (8). *rstB* is required for CTX $\phi$  phage integration. The Haitian strain has a GTA deletion at positions 77–79. MAMA-PCR with primer pair *rstB F1/rstB R1* produced a 160-bp amplicon, suggesting the absence of deletion in El Tor type *rstB* (2). The isolates had CTX $\phi$  integrated in the large chromosome with RS element downstream because they produced a 766-bp amplicon with CII F/CII R primers

(9). PCR sequencing with primers Zot F/ctxA R indicated the presence of 4 heptad (TTTTGAT) repeats in the *ToxR* binding region of *ctxAB* promoter, similar to El Tor biotype (2). Both isolates possessed vibrio seventh pandemic islands I and II, typical of El Tor biotype as assessed by PCRs with a variety of primers (10). Clonal relationship studied by pulsed-field gel electrophoresis suggested that isolates from the Kuwaiti patients were similar to each other and closer to Indian isolates of 2004 (Figure). Cholera is endemic to India; many El Tor variants circulate there (2).

We showed that the strain causing cholera in Iraq did not have the typical El Tor characteristics but instead had mixed characteristics of El Tor, classical, and Haitian strains. Altered strains of *V. cholerae* O1 might have implications for disease severity and vaccine efficacy (1). El Tor variants seem to be sweeping the world. We wonder whether they could replace the archetypal El Tor strain and become the causative agent of the eighth pandemic of cholera.

This study was supported in part by the Indian Council of Medical Research, Government of India. P.S. received a Junior Research Fellowship from the Council of Scientific and Industrial Research, Government of India.



**Figure.** Comparison of PFGE patterns of *NotI*-digested chromosomes of *Vibrio cholerae* O1 isolates from Kuwait with those of isolates obtained from various years (indicated by last 4 digits) from Kolkata, India. The digested chromosomes were separated on CHEF MAPPER (Bio-Rad, Hercules, CA, USA) and dendrogram constructed and analyzed by Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE, pulsed-field gel electrophoresis.

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## Correction: Vol. 18, No. 2

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DOI: <http://dx.doi.org/10.3201/eid2209.C12209>

**To the Editor:** In our earlier assessment of the etiologic role of human bocavirus 1 (HBoV1) among 109 constitutionally healthy children, seroconversion measured by a standard IgG enzyme immunoassay (EIA) was the prime marker of acute infection, and signs and symptoms during sampling intervals were interpreted from hand-written questionnaires and study nurse notes (1). In that study, we found that acute HBoV1 infection was associated with upper respiratory tract illness (URTI) and acute otitis media. However, after discovery of 3 new human bocaviruses (HBoV2–4), we used a competitive second-generation IgG EIA to differentiate between type-specific and cross-reacting IgG responses and reassessed the etiologic associations of each virus in the same pediatric cohort. We found only a weak correlation of HBoV1

infection with acute otitis media and no correlation with URTI (2).

There are 2 reasons for this discrepancy. First, the competitive second-generation EIA generated a different set of HBoV1-infected children. A total of 27 children initially interpreted as having HBoV1 seroconversions had, on reassessment, HBoV2 or HBoV3 seroconversions (2). Second, after improved reevaluation of clinical symptoms of the entire replenished cohort by 3 of the co-authors, we found that URTI symptoms of 38 children were classified differently. Despite tight sampling, intervals of 3 and 6 months might still have been too long for clinical assessment for young children with numerous respiratory infections per year. The authors regret any inconvenience to readers.

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## Correction: Vol. 22, No. 8

The name of Project Portinari, Rio de Janeiro, was misspelled in our August issue. The error has been corrected online ([http://wwwnc.cdc.gov/EID/article/22/8/AC-2208\\_article](http://wwwnc.cdc.gov/EID/article/22/8/AC-2208_article)).

## April 2015: Emerging Viruses

### Including:

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- Population Structure and Antimicrobial Resistance of Invasive Serotype IV Group B Streptococcus, Toronto, Ontario, Canada
- Norovirus Genotype Profiles Associated with Foodborne Transmission, 1999–2012
- Sequence Variability and Geographic Distribution of Lassa Virus, Sierra Leone
- Deaths Associated with Respiratory Syncytial and Influenza Viruses among Persons  $\geq 5$  Years of Age in HIV-Prevalent Area, South Africa, 1998–2009
- Highly Pathogenic Avian Influenza A(H5N1) Virus Infection among Workers at Live Bird Markets, Bangladesh, 2009–2010
- Increased Risk for Group B Streptococcus Sepsis in Young Infants Exposed to HIV, Soweto, South Africa, 2004–2008
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Giovanni Paolo Panini (1691–1765), *Alexander the Great Cutting the Gordian Knot* (ca. 1718–1719). Oil on canvas, 28 7/8 in × 23 1/2 in/ 73.3 cm × 59.7 cm. Public domain digital image courtesy of The Walters Art Museum, 600 N Charles St, Baltimore, Maryland, USA.

## The New Incurable Wound

Byron Breedlove and Paul M. Arguin

According to ancient mythology, the peasant Gordius, who married the fertility goddess Cybele, became king of Phrygia. He then dedicated his chariot to Zeus in the city Gordium and fastened it to a column with a large, complicated knot that became known as the Gordian knot. An oracle predicted that the future king of Asia would be the only person who could disentangle this knot.

Many individuals who traveled to Gordium attempted to untie the knot and thereby lay their claim to the throne, but their attempts proved futile. Then the Greek conqueror,

Alexander the Great, whose actual name was Alexander III of Macedon, visited the city in 333 BCE. He, too, was perplexed as he studied the knot, searching for its hidden ends. Whether prompted by impatience or insight, Alexander unexpectedly unsheathed his sword and sliced through the strands of rope, thereby severing and removing the knot. He subsequently conquered Asia, fulfilling the prophecy. He founded more than 70 cities and created a vast empire across three continents before his death in Babylon in June 323 BCE.

Alexander's bold, unexpected resolution gave rise to the oft-repeated saying, "cutting the Gordian knot." That saying—now ubiquitously and inevitably linked to the shopworn notion of "thinking outside the box"—continues,

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however, to help codify thorny conundrums in multiple disciplines, including law, commerce, technology, education, economics, warfare, medicine, and health.

The depiction of Alexander's eureka moment on this month's cover was imagined by Giovanni Paolo Panini, "the most celebrated and popular view painter in eighteenth-century Rome," according to the National Gallery of Art. Panini not only excelled as a *vedutisti*, he was also an architect and a professor of perspective and optics at the French Academy in Rome. He was considered a master of perspective, and his vistas of Rome, which featured many of the city's antiquities, may have inspired creation of the Panini projection, a mathematical rule for constructing images with very wide fields of view, which was recently rediscovered and is now used in software for creating and viewing panoramic photographs.

Panini places Alexander in the center right of the bottom third of the painting, among a scattered group of onlookers. Some in the crowd, as well as a dog, watch with interest; others stand stiff and cross-armed—they have seen this act before. A child behind him holds his shield. The rows of columns and patterned floor add drama and perspective; the angled shadow cast by the balcony leads to Alexander, his sword glinting in the sunlight as he raises his left hand to warn away anyone who might step in for a closer look. A sculpture depicts Zeus perched on his stone throne, gripping his thunderbolt and peering directly at the viewer as if to say, "I knew this day would come."

The interrelated, complex issues that have joined to create the current public health crisis of antimicrobial drug resistance constitute a Gordian knot as well. The question of whether we could see the rise of a postantibiotic period of infectious diseases that could mirror conditions of the preantibiotic and prevaccine period is not theoretical. Another Alexander, Sir Alexander Fleming, noted while accepting the 1945 Nobel Prize awarded for his 1928 discovery of penicillin that "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body."

Howard Walter Florey, Ernst Boris Chain, and Norman Heatley subsequently recognized the potential of Fleming's discovery and developed an effective drug from penicillin. Since the 1940s, antibiotics have greatly reduced illness and death from infectious diseases. But their widespread, and often inappropriate, use has come at a price: the infectious organisms have adapted to the antibiotics, making the drugs less effective.

Current events confirm Fleming's prescience: bacterial infections incurable by antibiotics are now possible.

Researchers found that a high proportion of swine-pathogenic *Escherichia coli* in Japan are resistant to colistin and noted concern for "a risk for transmission of *mcr-1* from these strains to human-pathogenic bacteria." A recently published report describes a patient in the United States infected with *E. coli* containing the *mcr-1* resistance gene on a plasmid conferring resistance to colistin, the current antibiotic of last resort for treating patients with infections caused by some multidrug-resistant bacteria. Like Zeus, Fleming knew this day would come.

Some of the overlapping strands woven into the Gordian knot of antimicrobial resistance are myriad mutations and adaptations of various infectious organisms, lack of development of new antimicrobial agents, modern agricultural practice, and ineffective antibiotic stewardship. Tackling individual problems such as multidrug-resistant *Shigella* sp. infections, antibiotic overuse, or the transition of *Clostridium difficile* and *Staphylococcus aureus* from institutionally acquired to community-acquired infections is vital because an all-encompassing solution to the puzzle, such as that found by Alexander the Great, does not seem to be on our horizon.

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# EMERGING INFECTIOUS DISEASES®

## Upcoming Issue

*Including*

- Vaccine-Derived Polioviruses and Children with Primary Immunodeficiency, Iran, 1995–2014
- Community- and Healthcare-Associated *Clostridium difficile* Infections, Finland, 2008–2013
- Increase in Meningococcal Serogroup W Disease, Victoria, Australia, 2013–2015
- Persistence of Antibodies against Middle East Respiratory Syndrome Coronavirus
- Chikungunya Virus in Febrile Humans and *Aedes aegypti* Mosquitoes, Yucatan, Mexico
- Daily Reportable Disease Spatiotemporal Cluster Detection, New York, New York, USA, February 2014–September 2015
- Distinct Zika Virus Lineage in Salvador, Bahia, Brazil
- Sporotrichosis-Associated Hospitalizations, United States, 2000–2013
- Unmet Needs for a Rapid Diagnosis of Chikungunya Virus Infection
- Cerebral Syphilitic Gumma within 5 Months of Syphilis in HIV-Infected Patient
- *Culex pipiens* and *Aedes triseriatus* Mosquito Susceptibility to Zika Virus
- Synovial Tissue Infection with *Burkholderia fungorum*
- Sexual Transmission of Zika Virus and Duration of Detection in Semen, New Zealand, 2016
- Recombinant Enterovirus A71 Subgenogroup C1 Strains, Germany, 2015
- Outbreaks of Human *Salmonella* Infections Associated with Live Poultry, USA, 1990–2014
- Cat Scratch Disease in the United States, 2005–2013
- Effect of Geography on the Analysis of Coccidioidomycosis-Associated Death
- Resolution of a Chikungunya Outbreak in a Prospective Cohort, Cebu, Philippines, 2012–2014

Complete list of articles in the October issue at  
<http://www.cdc.gov/eid/upcoming.htm>

## Upcoming Infectious Disease Activities

October 9–13, 2016  
6th Annual  
Calicivirus Conference  
Savannah, GA, USA  
<https://www.faingroup.com/Calicivirus/>

October 28–30, 2016  
ID Week  
New Orleans, LA, USA  
<http://www.idweek.org/>

October 29–November 2, 2016  
American Public Health Association  
Denver, CO, USA  
<https://www.apha.org/events-and-meetings/annual/past-and-future-annual-meetings>

November 4–7, 2016  
IMED  
International Meeting on Emerging  
Diseases and Surveillance  
Vienna, Austria  
<http://imed.isid.org/>

November 13–17, 2016  
ASTMH  
American Society of Tropical  
Medicine and Hygiene  
Atlanta, GA, USA  
<https://www.astmh.org/>

November 29–December 2, 2016  
Institut Pasteur International Network  
Scientific Symposium  
Paris, France  
<http://www.pasteur-network-meeting2016.org/>

December 3–8, 2016  
ASLM  
African Society for Laboratory Medicine  
Cape Town, South Africa  
<http://aslm2016.org/>

April 22–27, 2017  
ECCMID  
European Congress of Clinical  
Microbiology and Infectious Diseases  
Vienna, Austria  
<http://www.eccmid.org/>

March 29–31, 2017  
SHEA  
Society for Healthcare  
Epidemiology of America  
St Louis, MO, USA  
<http://www.shea-online.org/>

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Announcements may be posted on the journal Web page only, depending on the event date.

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### Article Title

## Treatment Outcomes for Patients with Extensively Drug-Resistant Tuberculosis, KwaZulu-Natal and Eastern Cape Provinces, South Africa

### CME Questions

- 1. Your patient is a 37-year-old woman with extensively drug-resistant tuberculosis (XDR TB). According to the retrospective cohort study by Kvasnovsky and colleagues, which of the following statements about XDR TB treatment outcomes overall and XDR TB characteristics in subgroups based on HIV status is correct?**
- A. Overall, outcomes were favorable in one third of patients
  - B. HIV status subgroups did not differ significantly in percentage with smear-positive disease
  - C. Cavitory disease was less frequent in HIV-negative patients than in other groups
  - D. HIV-negative as well as HIV-positive patients had approximately 1 year of previous TB treatment before starting XDR TB treatment
- 2. According to the retrospective cohort study by Kvasnovsky and colleagues, which of the following factors is most likely a predictor of favorable XDR TB treatment outcomes?**
- A. Negative sputum microscopy result for acid-fast bacilli at treatment start
  - B. Negative HIV status
  - C. Among HIV-positive patients, initiation of antiretroviral therapy before initiation of XDR TB treatment
  - D. Weight more than 40 kg
- 3. According to the retrospective cohort study by Kvasnovsky and colleagues, which of the following statements about predictors of unfavorable XDR TB treatment outcomes is correct?**
- A. In multivariate analysis, HIV positivity was not a predictor of unfavorable outcome
  - B. HIV positivity was the strongest predictor of unfavorable outcome
  - C. For weight less than 50 kg, adjusted hazard ratio for unfavorable outcome was 1.56 (95% CI 1.23–1.98)
  - D. Culture conversion after 8 months was a significant predictor of unfavorable outcome

### Activity Evaluation

<b>1. The activity supported the learning objectives.</b>					
Strongly Disagree					Strongly Agree
1	2	3	4	5	
<b>2. The material was organized clearly for learning to occur.</b>					
Strongly Disagree					Strongly Agree
1	2	3	4	5	
<b>3. The content learned from this activity will impact my practice.</b>					
Strongly Disagree					Strongly Agree
1	2	3	4	5	
<b>4. The activity was presented objectively and free of commercial bias.</b>					
Strongly Disagree					Strongly Agree
1	2	3	4	5	

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### Article Title

## Use of Testing for West Nile Virus and Other Arboviruses

### CME Questions

**1. You are seeing a 31-year-old woman in the emergency department who presented with 2 days of fever, confusion, and headache. You suspect an acute infection of the central nervous system. What should you consider regarding the epidemiology and clinical presentation of West Nile neuroinvasive disease (WNND)?**

- A. West Nile virus (WNV) is the most common arboviral disease in the United States
- B. All infections with WNV are associated with symptoms, which can be severe
- C. There are no endemic areas of WNV infection in the United States
- D. WNND is known to promote encephalitis, but not meningitis

**2. Which of the following statements regarding testing in cases of potential WNND in the current study sample is most accurate?**

- A. WNV testing was conducted in 92% of patients
- B. WNV testing was performed in 58% of patients
- C. WNV was the most common virus causing central nervous system infection
- D. A positive result was found in 11% of patients who tested for WNV

**3. Which of the following factors was most significantly associated with more frequent testing for WNV in cases of possible WNND in the current study?**

- A. Presence of focal neurologic abnormalities
- B. Lower comorbid illness score
- C. Presence of seizures
- D. Absence of nuchal rigidity

**4. Which of the following findings from cerebrospinal fluid (CSF) was most associated with testing for WNV in cases of possible WNND in the current study?**

- A. Higher CSF pleocytosis
- B. Normal CSF glucose levels
- C. CSF lymphocytic pleocytosis
- D. Elevated CSF protein levels

### Activity Evaluation

**1. The activity supported the learning objectives.**

Strongly Disagree

1

2

3

4

Strongly Agree

5

**2. The material was organized clearly for learning to occur.**

Strongly Disagree

1

2

3

4

Strongly Agree

5

**3. The content learned from this activity will impact my practice.**

Strongly Disagree

1

2

3

4

Strongly Agree

5

**4. The activity was presented objectively and free of commercial bias.**

Strongly Disagree

1

2

3

4

Strongly Agree

5

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### Article Title

## Fluoroquinolone-Resistant and Extended-Spectrum $\beta$ -Lactamase-Producing *Escherichia coli* infections in Patients with Pyelonephritis, United States

### CME Questions

1. You are seeing a 37-year-old woman with a 5-day history of fever, dysuria, and flank pain. You suspect that the patient has pyelonephritis. What should you consider the Infectious Diseases Society of America (IDSA) 2010 recommendations regarding the treatment of uncomplicated pyelonephritis as you recommend treatment for this patient?

- A. Fluoroquinolones are not recommended as initial treatment for pyelonephritis
- B. Ceftriaxone or gentamicin may be added to initial treatment
- C. Trimethoprim/sulfamethoxazole is a good choice for patients with extended-spectrum  $\beta$ -lactamase (ESBL) bacterial infections
- D. Fluoroquinolones should be avoided only if the local resistance rate is in excess of 5%

2. The patient's initial urine culture grows *Escherichia coli*. What were the approximate rates of fluoroquinolone resistance and ESBL-producing *E. coli* among patients with uncomplicated pyelonephritis in the current study?

- A. Less than 1% for both
- B. An estimated 6% of isolates were resistant to fluoroquinolones; nearly 3% were ESBL producers
- C. Rate of fluoroquinolone resistance among isolates was 15%; nearly half were ESBL producers
- D. Nearly 40% of isolates were resistant to fluoroquinolones; 1% were ESBL producers

3. Which of the following variables was not a significant risk factor for *E. coli* fluoroquinolone resistance in the current study?

- A. Residence in a long-term care facility within 90 days
- B. Recent hospital admission
- C. Complicated vs. uncomplicated pyelonephritis
- D. Previous urinary tract infection with fluoroquinolone- or ceftriaxone-resistant organism

4. Which of the following variables was the strongest risk factor for ESBL-producing *E. coli* in the current study?

- A. Fluoroquinolone resistance of *E. coli*
- B. Age older than 65 years
- C. Residence in a long-term care facility within 90 days
- D. Intravenous antibiotic administration within the past 30 days

### Activity Evaluation

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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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# EMERGING INFECTIOUS DISEASES®

## JOURNAL BACKGROUND AND GOALS

### What are “emerging” infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as “emerging.” These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

### Why an “Emerging” Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC’s efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC’s efforts against the threat of emerging infections. However, even as it addresses CDC’s interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

### What are the goals of Emerging Infectious Diseases?

- 1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal
  - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
  - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
  - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.
- 2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal
  - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
  - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
  - ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.



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**Emerging Infectious Diseases** is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit <http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm>.

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**Manuscript Preparation.** For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

**Title Page.** Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

**Keywords.** Use terms as listed in the National Library of Medicine Medical Subject Headings index ([www.ncbi.nlm.nih.gov/mesh](http://www.ncbi.nlm.nih.gov/mesh)).

**Text.** Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

**Biographical Sketch.** Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

**References.** Follow Uniform Requirements ([www.icmje.org/index.html](http://www.icmje.org/index.html)). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

**Tables.** Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of bold-face. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

**Figures.** Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

**Videos.** Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

## Types of Articles

**Perspectives.** Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

**Synopses.** Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section

comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Research.** Articles should not exceed 3,500 words and 50 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 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

**Dispatches.** Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

**Another Dimension.** Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

**Research Letters Reporting Cases, Outbreaks, or Original Research.** Starting with the January 2017 volume, EID will publish letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should contain no more than 850 words (including the abstract) and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

**Letters Commenting on Articles.** Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

**Commentaries.** Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

**Books, Other Media.** Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

**Conference Summaries.** Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

**Online Reports.** Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

**Photo Quiz.** The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

**Etymologia.** Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

**Announcements.** We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to [eideditor@cdc.gov](mailto:eideditor@cdc.gov).

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