

EMERGING INFECTIOUS DISEASES®



Respiratory Infections

June 2014



Frederic Sackler Remington (1861–1909) *Unkilled (possibly The Cigarette a.k.a. Around the Campfire)* (ca. 1908–1909)
Oil on canvas, (30 x 27 inches/ 76.2 cm x 68.58 cm) Frederic Remington Art Museum, Ogdensburg, New York

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EMERGING INFECTIOUS DISEASES

June 2014



On the Cover

Frederic Sackrider Remington
(1861–1909)

Untitled
(possibly *The Cigarette*
a.k.a. *Around the Campfire*)
(ca. 1908–1909) Oil on canvas.
(30 × 27 inches/76.2 cm × 68.58 cm)

Frederic Remington Art Museum,
Ogdensburg, New York

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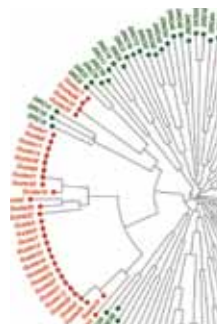
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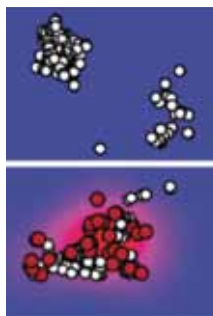
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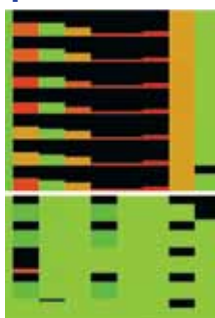
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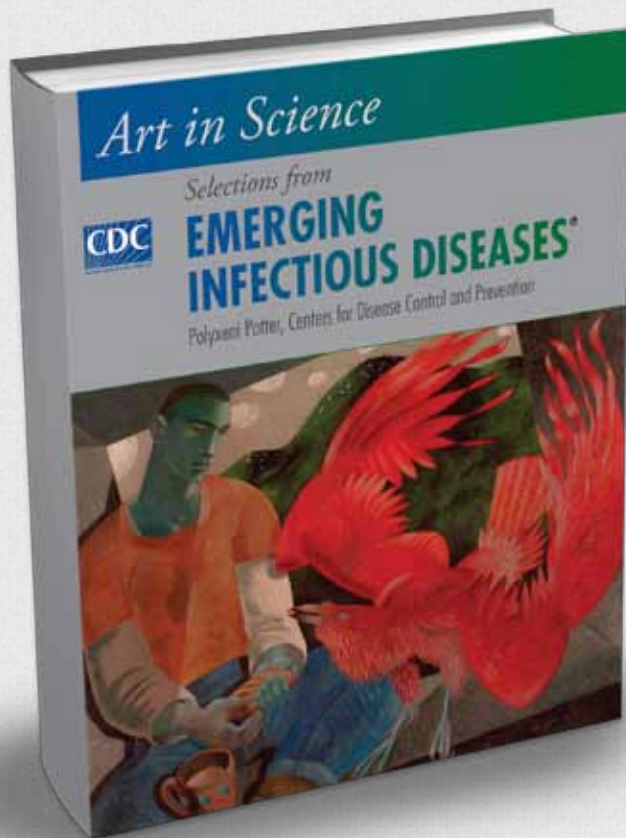
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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.



Adverse Pregnancy Outcomes and *Coxiella burnetii* Antibodies in Pregnant Women, Denmark

Stine Yde Nielsen, Kåre Mølbak, Tine Brink Henriksen, Karen Angeliki Krogfelt, Carsten Schade Larsen, and Steen Villumsen

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Learning Objectives

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

- Describe adverse outcomes of pregnancies with positive or increasing titers against *C. burnetii*, based on a case series from Denmark
- Distinguish the causal association between adverse pregnancy outcomes and *C. burnetii* infection
- Assess the overall risk for a Q fever–associated adverse pregnancy outcome.

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Authors

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A high risk for obstetric complications has been reported among women infected with *Coxiella burnetii*, the causative agent of Q fever, but recent studies have failed to confirm these findings. We reviewed national data collected in Denmark during 2007–2011 and found 19 pregnancies in 12

women during which the mother had a positive or equivocal test for antibodies to *C. burnetii* (IgM phase I and II titers ≥ 64 , IgG phase I and II titers ≥ 128). Of these 12 women, 4 experienced obstetric complications (miscarriage, preterm delivery, infant small for gestational age, oligohydramnion, fetal growth restriction, or perinatal death); these complications occurred in 9 pregnancies (47% of the 19 total pregnancies identified). Our findings suggest an association between Q fever and adverse pregnancy outcomes, but complications were identified in only 9 pregnancies during the study's 5-year period, indicating that the overall risk is low.

Author affiliations: Aarhus University Hospital, Aarhus, Denmark (S.Y.Nielsen, T.B. Henriksen, C.S. Larsen); and Statens Serum Institut, Copenhagen, Denmark (K. Mølbak, K.A. Krogfelt, S. Villumsen)

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Q fever is a zoonotic infection caused by *Coxiella burnetii*. Findings of adverse pregnancy outcome in infected women, high seroprevalence in animal studies, and large human outbreaks have placed increasing focus on Q fever in several European countries, including Denmark (1–4). In ruminants, infection with *C. burnetii* is associated with high numbers of bacteria in the placenta, and the infection is known to cause abortion, retained placenta, endometritis, and infertility (5,6). Humans are infected with *C. burnetii* predominantly by inhalation of contaminated aerosols, and persons who have contact with livestock are at highest risk for exposure (7). Among pregnant women, $\geq 90\%$ of those who show antibodies for *C. burnetii* that suggest recent infection may remain asymptomatic (8). Case series from France have associated symptomatic and asymptomatic *C. burnetii* infection during pregnancy with obstetric complications, including miscarriage, preterm delivery, and fetal death (9–11). In contrast, population studies from northern Europe have not found an association between *C. burnetii* and adverse pregnancy outcomes (12–15).

Cattle are the main reservoir for *C. burnetii* in Denmark. A recent study of the seroprevalence of *C. burnetii* in cattle found that bulk-tank milk samples tested positive for *C. burnetii* at 59 of 100 randomly selected farms (16). In addition, the reported prevalence of antibodies to *C. burnetii* among veterinarians in Denmark ranges from 36% to 47% (1,2,17). These findings show that exposure to *C. burnetii* is common in this country in the animal reservoir and in those who are occupationally exposed to livestock or who live in rural areas with livestock contact. However, the risk for and implications of infection with *C. burnetii* among pregnant women have not been exhaustively described (12,15). Because of this, and because findings from the case series in France conflict with results from population-based studies from the Netherlands and Denmark, we reviewed national data from Aarhus University Hospital, Aalborg University Hospital, Hospital of Southwest Jutland, Viborg Regional Hospital, Regional Hospital West Jutland, and Hilleroed Hospital in Denmark to identify women who had elevated antibodies to *C. burnetii* during pregnancy. We evaluated the course of infection, effects of treatment with cotrimoxazole (trimethoprim/sulfamethoxazole), and pregnancy outcomes for these women.

Materials and Methods

Every resident in Denmark is provided with a unique civil registration number that enables individual-level linkage between national registries. Data from health records at obstetric and infectious disease departments were thereby linked to civil registration numbers from women (18–45 years of age) who had positive or equivocal tests at the Statens Serum Institut for antibodies to *C. burnetii* during 2007–2011. Using these data, we identified pregnant

women who could be included in the study on the basis of positive serologic test results for *C. burnetii* and availability of titers from throughout pregnancy to enable evaluation of infection in paired samples.

Detection of Antibodies against *C. burnetii*

In Denmark, *C. burnetii* serologic testing is performed only at the Statens Serum Institut by indirect immunofluorescence assay (IFA; Focus Diagnostics, Cypress, CA, USA), according to the manufacturer's instructions. *C. burnetii* expresses 2 antigens, phase I and phase II. During active infection, phase II IgG and IgM are elevated; these results may remain positive for months to years. In acute Q fever, primarily antibodies against phase II antigens are raised, and these titers are higher than for antibodies against phase I antigens; IgM antibodies appear first. In chronic forms of the disease, antibodies against phase I antigens are elevated.

A local cutoff value adjusted to the population of Denmark has defined negative, equivocal, and positive titers (18); we included patients with equivocal and positive titers in our study. A sample was considered IFA-positive when IgM for phase I or phase II titers was ≥ 64 or IgG for any of the phases was ≥ 128 . A 4-fold increase in titers between 2 paired samples was defined as diagnostic for recent or acute *C. burnetii* infection.

PCR Analysis

DNA from urine samples was subjected to a Chelex 100-based DNA extraction method as described (19). DNA from placenta and bone marrow samples was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA from the cream layer of fresh breast milk samples was extracted by using a previously described protocol that included washing with phosphate-buffered saline and subsequent extraction with the DNeasy Blood and Tissue Kit (20). PCR was conducted with primers targeting the multi-copy gene *IS1111* as described (21).

Testing Indications and Pregnancy Outcomes

The indication for Q fever testing for most of the women was exposure to livestock. Two of the women were tested in a subsequent pregnancy because of a previous adverse pregnancy outcome; none were tested because of symptoms. Adverse pregnancy outcomes were defined as miscarriage, preterm delivery, single fetal death with a surviving co-twin, infant small for gestational age, oligohydramnion, fetal growth restriction, and perinatal death.

Results

We identified 12 women with equivocal and positive antibody titers for *C. burnetii* infection who underwent 19

pregnancies during the 5-year study period. All women were farmers or veterinarians and resided in rural areas of Denmark. Obstetric complications were recorded in 9 (47%) of the 19 pregnancies (Table 1). None of the women

were found to be IgM positive for pathogens regarded as classic causes of infection of the developing fetus during pregnancy; these pathogens included *Toxoplasma gondii*, parvovirus B19, rubella virus, cytomegalovirus, and herpes

Table 1. Patient characteristics and pregnancy outcomes in 12 women who had positive *Coxiella burnetii* titers during pregnancy, Denmark*

| Pt and preg no. | Patient data | | | | | Fetal gestational age at delivery, wk + d | Baby birth weight, g | Clinical outcome of pregnancy | <i>C. burnetii</i> test results |
|-----------------|--------------|--------|---|--------------------------------------|-----------------------|---|----------------------|---|--|
| | Age, y | Parity | Symptoms | Animal contact | Treatment during preg | | | | |
| Pt 1 | 33 | 2 | Fever and cough first weeks of pregnancy | Yes, cattle, vet | Yes, from wk 15 | 38 + 6 | 3,570 | Healthy baby | PCR urine wk 10 pos; bone marrow biopsy wk 15 neg; PCR breast milk, amniotic fluid, placenta neg |
| Pt 2† | 40 | 1 | Dry cough for weeks just before first pregnancy | Yes, cattle, vet | No | 8 wk | NA | Miscarriage | NA |
| Pt 3 | 30 | 1 | No | Yes, cattle, vet | Yes, from wk 10 | 39 + 1 | 3,500 | Healthy baby | PCR placenta neg |
| Pt 4‡ | 34 | 1 | No | Yes, cattle, vet | No | 39 + 0 | 3,030 | Single fetal death around wk 8; surviving twin with healthy outcome | PCR breast milk, placenta neg |
| Pt 5§ | | | | | | | | | |
| Preg 1 | 32 | 1 | No | Yes, cattle, vet | No | 40 + 3 | 3,000 | Dysmature baby | NA |
| Preg 2 | 33 | 2 | No | | No | 38 + 0 | 2,360 | Cesarean, IUGR (23% from wk 28) and oligo | |
| Pt 6 | 26 | 1 | NA | Yes, cattle, farmer | No | 39 | 3,720 | Healthy baby | PCR placenta neg |
| Pt 7¶ | 32 | 1 | No | Yes, cattle, vet | Yes, from wk 10 | 41 | 3,210 | Acute caesarean due to uterine rupture | PCR placenta neg |
| Pt 8 | | | | | | | | | |
| Preg 1 | 24 | 0 | No | Yes, cattle, assisting female farmer | No | 27 + 2 | NA | IUGR, oligo/malformations; baby died few hours postpartum | NA |
| Preg 2 | 26 | 1 | No | farmer | Yes, from wk 22 | 30 | 1,570 | Preterm baby | PCR placenta neg |
| Pt 9 | | | | | | | | | |
| Preg 1 | 30 | 0 | No | Yes, cattle, vet | From wk 20 | 39 + 4 | 3,790 | Healthy baby | PCR placenta, breast milk neg |
| Preg 2 | 33 | 1 | No | | No | 40 + 2 | 4,170 | Healthy baby | NA |
| Pt 10 | | | | | | | | | |
| Preg 1 | 30 | 0 | 1 mo dry cough at start of preg, short episode of fever | Yes, cattle, vet | Yes, from wk 10 | 39 + 6 | 3,420 | Healthy baby | PCR placenta, breast milk neg |
| Preg 2 | 33 | 1 | No | | No | 41 + 2 | 3,400 | Healthy baby | NA |
| Pt 11 | 30 | 1 | No | Yes, cattle, vet | No | 40 | 4,230 | Healthy baby | PCR placenta neg |
| Pt 12 | 31 | 1 | No | Yes, cattle, vet | Yes, from wk 22 | 39 + 5 | 3,570 | Healthy baby | PCR placenta neg |

*See Table 2 for specific titers. Pt, patient; preg, pregnancy; neg, negative; vet, veterinarian; NA, not applicable; IUGR, intrauterine growth restriction; oligo, oligohydramnion.

†Within 2 years, 3 spontaneous abortions and 1 extrauterine pregnancy.

‡Q fever in 2006 (not pregnant).

§Acute Q fever in 2006 (not pregnant), treated with 3 wk doxycycline.

¶Two spontaneous abortions before this pregnancy.

SYNOPSIS

simplex virus. However, not all women were exhaustively tested during pregnancy. For all women tested during pregnancy, *C. burnetii* serologic test results were available from no later than pregnancy week 13 (Table 2).

Two patients (1 and 10) reported dry cough and short episodes of fever; both had antibody titers during the first trimester consistent with acute infection. These patients were treated with cotrimoxazole beginning in gestational

Table 2. Results of testing for 12 women who had positive *Coxiella burnetii* titers during pregnancy, Denmark*

| Pt and preg | First sample | | | | | Last sample | | | | | Comments |
|-----------------|-------------------------|--------|-------|--------|-------|-------------------------------|--------|-------|--------|-------|---|
| | Date collected† | IgM II | IgM I | IgG II | IgG I | Date collected† | IgM II | IgM I | IgG II | IgG I | |
| Pt 1 | 7 wk | 2,048 | 8,000 | 1,024 | 512 | 38 wk | <64 | <64 | 256 | <128 | Negative titers 6 mo before this preg |
| Pt 2 | 8 wk, after miscarriage | 256 | <64 | 256 | 128 | 7 wk, after extrauterine preg | 512 | 128 | 512 | 512 | Maximum titers after second miscarriage: IgG phase II, 4,096; IgG phase I, 2,048; IgM phase I, 512; IgM phase II, 256 |
| Pt 3 | 3 wk | <64 | <64 | <128 | 512 | 33 wk | <64 | <64 | 128 | 1,024 | First titers taken 2 mo before this preg: IgG phase I, 1,024. Maximum titers in preg week 9: IgG phase I, 4,096; IgG phase II, 256‡ |
| Pt 4 | 10 wk | <64 | <64 | 1,024 | 512 | 1 day postpartum | <64 | <64 | 1,024 | 128 | First titers taken 3 mo before this preg identical to titers from preg week 10§ |
| Pt 5 Preg 1 | 9 wk | <64 | <64 | 256 | 512 | 38 wk | <64 | <64 | 256 | 256 | First titers taken 6 mo before this preg: IgM phase II, <64; IgM phase I, <64; IgG phase II, 512; IgG phase I, 1,024§ |
| Preg 2 | 12 wk | <64 | <64 | 128 | 1,024 | 31 wk | <64 | <64 | 512 | 1,024 | § |
| Pt 6 | 13 wk | <64 | <64 | 256 | <128 | At birth | <64 | <64 | 128 | <128 | No titers available before this preg. Maximum titers in preg: IgG phase II, 256‡ |
| Pt 7 | 8 wk | 128 | <64 | 4,096 | <128 | At birth | 64 | <64 | 512 | <128 | Titers positive 6 mo before this preg: IgM phase II, 128; IgM phase I, <64; IgG phase II, 2,048; IgG phase I, <128§ |
| Pt 8 Preg 1 | 12 wk | 128 | <64 | 512 | 256 | 26 wk | 256 | <64 | 1,024 | 256 | Maximum titers in this preg: IgG phase II, 2,048‡ |
| Preg 2 | 10 wk | <64 | <64 | 256 | 128 | 26 wk | <64 | <64 | 256 | 256 | Maximum titers in this preg: IgG phase I, 1,024; IgG phase II, 1,024‡ |
| Pt 9 Preg 1 | 10 wk | <64 | <64 | 256 | <128 | 39 wk | 64 | <64 | 128 | <128 | Maximum titers in this preg: phase II IgG, 1,024‡ |
| Preg 2 | 12 wk | <64 | <64 | 256 | <128 | 36 wk | <64 | <64 | 256 | <128 | § |
| Pt 10 Preg 1 | 8 wk | 512 | <64 | 4,096 | <128 | 36 wk | <64 | <64 | 256 | <128 | No titers available before this preg§ |
| Preg 2 | 9 wk | <64 | <64 | 128 | <128 | 14 wk | <64 | <64 | <128 | <128 | § |
| Pt 11 | 7 wk | <64 | <64 | 128 | 128 | 26 wk | 256 | 256 | 512 | <128 | Titers positive 1 mo before this preg: IgM phase II, <64; IgM phase I, <64; IgG phase II, 1,024; IgG phase I, <128§ |
| Pt 12 | 10 wk | <64 | <64 | 2,048 | <128 | 37 wk | <64 | <64 | 1,024 | 1,024 | Negative titers during preg 2 years earlier. Maximum titers in this preg: IgG phase II, 2,048 |

*See Table 1 for specific patient and outcome data. Pt, patient; preg, pregnancy; vet, veterinarian; NA, not applicable; IUGR, intrauterine growth restriction.

†Gestational week.

‡Remaining titers have not risen above/beyond values in beginning/end of pregnancy.

§No further rise of titers in this pregnancy; indicates that titers had not risen beyond values at beginning/end of pregnancy.

week 15 or 10, respectively; patient 1 was treated throughout pregnancy and patient 10 until gestational week 39. Patient 1 had a PCR-positive urine sample in gestational week 10, but results of PCR on a bone marrow biopsy from gestational week 15 and PCR on amniotic fluid and placenta were negative. For patient 10, no *C. burnetii* DNA was detected by PCR from placental tissue or breast milk; for her second pregnancy, serologic test results for *C. burnetii* were negative, PCR on placenta was not performed, and the pregnancy had a healthy outcome.

Patient 2 reported weeks of dry cough without fever during the weeks just before the first pregnancy that ended with miscarriage. She had 3 miscarriages and an extrauterine pregnancy within 2 years, and the titers that were found indicate that she was acutely infected weeks before the first miscarriage. Her antibody titers reached a maximum level after the second miscarriage. No embryo material was tested from any of her miscarriages.

Patients 3, 5, and 12 had serologic profiles with IgG phase I titer of 1,024 at the end of pregnancy; 2 of the patients were treated during pregnancy, but none had symptoms, received a diagnosis of endocarditis, or received long-term postpartum treatment. Patient 12 had a serologic profile indicating reactivation of *C. burnetii* infection, but her antibody titers had been negative in a previous pregnancy 2 years earlier.

Patient 4 seroconverted before her second pregnancy, during which she experienced a single fetal death around gestational week 8 and a surviving co-twin. She had a decrease in IgG phase I during pregnancy; the surviving twin was delivered healthy and at term.

Patient 5 was treated for acute Q fever before her second pregnancy. Her antibody titers were stable during the second pregnancy, and her baby was full-term but slightly small for gestational age. After a short interpregnancy interval, she had a significant increase in IgG phase II titers during her third pregnancy; because of fetal growth restriction and oligohydramnion noted during gestational week 28, she had a cesarean section during gestational week 38. The placentas from these 2 pregnancies were not tested.

Patient 8 had rising antibody titers during her first pregnancy, and because of bleeding and contractions, she had an acute cesarean section in week 27 and gave birth to a severely growth retarded and malformed infant who lived only a few hours. The fetus and placenta were not tested for *C. burnetii*, but results of testing for toxoplasmosis, cytomegalovirus, and parvovirus B19 were negative, as were results of genetic testing for neuromuscular diseases. Her titers decreased slightly postpartum, but during her second pregnancy, titers increased significantly, and treatment with cotrimoxazole was initiated around gestational week 22. In gestational week 30, she spontaneously went into labor and gave birth to a healthy baby. Treatment

was terminated immediately postpartum, and her antibody levels decreased, indicating that she was not chronically infected. Thus, patients 5 and 8 had serologic indication of a reactivation of infection with *C. burnetii*: a postpartum decline in antibody titers followed by a ≥ 4 -fold increase in titers during the next pregnancy.

Three of the remaining pregnancies (in patients 6, 9, and 11) had an uncomplicated course with a healthy pregnancy outcome. Patient 7 had an acute cesarean because of rupture of the uterus; she had had a cesarean in her first pregnancy.

In summary, 3 patients (1, 2, and 10) reported symptoms of acute Q fever. At least 1 (patient 8) appeared to have seroconverted without symptoms close to the beginning of her first pregnancy.

For 7 of the 19 pregnancies, treatment with cotrimoxazole was initiated in the patient; 6 of these pregnancies resulted in healthy, full-term babies, and no mention of severe side effects was found in the mothers' medical records. One of the women who had obstetric complications received treatment with cotrimoxazole (patient 8, in her second pregnancy). By comparison, among the 12 pregnancies in which no treatment was given, 8 resulted in obstetric complications. The effect of treatment with cotrimoxazole on complications was tested, but the difference was not significant ($p = 0.057$ by Fisher exact test; data not shown).

PCR was performed on placentas from 10 pregnancies and in 4 of these, breast milk was also tested; no results were positive. For 7 of the 10 pregnancies in which placentas were tested, the woman had received treatment with cotrimoxazole during pregnancy.

Discussion

Adverse pregnancy outcome was observed in 9 of 19 pregnancies among 4 of the 12 pregnant women in which equivocal or positive tests for *C. burnetii* antibodies were found. One woman had 3 miscarriages and an extrauterine pregnancy, 1 experienced preterm delivery, 1 had a single fetal death with a surviving co-twin, and 1 delivered a small-for-gestational-age baby. Oligohydramnion and fetal growth restriction were found in 2 pregnancies; 1 had a healthy outcome, but in the other, the baby died a few hours postpartum.

The observed complication rate of 47% may seem high, but the causal relationship of this finding may not be clear. For example, none of the tested placentas were examined by histopathology, and all of the 10 placentas tested by PCR were negative for *C. burnetii*. Furthermore, not all of the women were thoroughly tested for other infections. One possible explanation for the lack of findings related to the placenta in 7 of the cases could be the patient's treatment with cotrimoxazole or focal placental infection. Nonetheless, we did observe adverse pregnancy outcome

in 8 (67%) of 12 pregnancies in which the women were not treated with cotrimoxazole, which supports the beneficial effects of treatment.

Among the cases we reviewed, none of the 4 breast milk samples tested by PCR were positive for *C. burnetii*. This bacterium has been found in human milk (22,23), but the implications for the breastfed child are unclear. Because of the lack of evidence, breastfeeding has been deemed safe according to the obstetric guidelines in Denmark for the treatment of *C. burnetii*-seropositive pregnant women and their newborns.

Two studies, in the Netherlands (24) and Canada (25), have suggested a low rate of placental *C. burnetii* infection in asymptomatic women and that obstetric complications in symptomatic cases may be explained by massive placental necrosis following a higher bacterial load in the placenta, systemic infection, or both. A study in France (26) found that untreated Q fever in 1 pregnancy may be reactivated in a subsequent pregnancy, a result found in 3 patients in our study (patients 5, 8, and 12).

The evidence for an adverse pregnancy outcome in humans in relation to Q fever mainly originates from case studies from France of referred, infected, pregnant patients, as well as pregnancies in which a diagnosis of Q fever was reached retrospectively, after an adverse outcome (9–11). Carcopino et al. reported clinical symptoms in 32 (60.4%) of 53 cases and a chronic serologic profile in more than half of patients and concluded that Q fever in pregnancy may cause severe complications (10). The Netherlands has recently experienced an unprecedented Q fever outbreak that has prompted 2 large studies in pregnant women. One study, a population-based study of 1,174 serum samples collected at the twelfth week of pregnancy, found no association between antibodies to *C. burnetii* and adverse pregnancy outcome among women living in the area with the highest Q fever incidence. The other study, a randomized, controlled trial, tested 1,229 pregnant women living in high-risk areas during the outbreak; 15% of the women were seropositive in both the intervention group and the control group, and no difference was found in obstetric complications (13). Hence, the findings from France were not reproduced in the Netherlands. Likewise, a recent study in Denmark assessed the association between presence of antibodies to *C. burnetii*, seroconversion, and pregnancy outcome and found that seropositivity was not associated with miscarriage, preterm birth, or low birthweight (15).

Differences in findings among these various studies may be explained in part by differences in study design. The indication for serologic testing is a crucial point; in our study, the indication for testing was exposure to livestock for most of the women. In contrast, the women in the case series in France were primarily tested because of pathologic

conditions during pregnancy or clinical symptoms (e.g., fever, hepatitis) or retrospectively because of an adverse pregnancy outcome. Angelakis et al. (11) found that 17 of 30 *C. burnetii* seropositive pregnant women were asymptomatic; only 2 of these had an uncomplicated pregnancy, but no placentitis or isolation of *C. burnetii* was found in 14 available biopsy specimens. These authors suggested that the different rates of obstetric complications found in various geographic areas could be related to strain specificity, potentially because of differences in plasmid types.

All the women in this case series had contact with livestock in Denmark, and it is reasonable to assume that these women were occupationally exposed to endemically occurring *C. burnetii* infections among cattle. However, the observations by Angelakis et al. suggest that strains of *C. burnetii* in Denmark, and possibly cattle in general, might be less virulent than that seen when the infection is acquired from other animal reservoirs (e.g., goats). In France, goats and sheep have been the main source of *C. burnetii* infection. The recent outbreak in the Netherlands was detected shortly after a large number of dairy milk farms had changed from cattle to goats as production animals. In Denmark, goat and sheep farms are rare, and despite high clinical awareness during the past 7 years, no reports have described a microbiologically verified outbreak of Q fever in humans or a case of chronic Q fever that was definitely acquired in Denmark. However, a large percentage of dairy cattle in Denmark shed *C. burnetii*, and a high prevalence of antibodies has been found among pregnant women who had exposure to cattle (16,17). These observations could, in part, be an explanation for the discrepancies in rates of serious adverse pregnancy outcomes among studies from different countries.

In conclusion, we evaluated risks and implications in 19 pregnancies with positive or rising titers against *C. burnetii* from Denmark, a country that has high seroprevalence of *C. burnetii* but low prevalence of clinical Q fever and for which cattle are the primary bacterial reservoir. In this case series, almost half of the women had obstetric complications, which is comparable to previous case series. We found complications in 8 out of 12 untreated pregnancies; 7 pregnant women received long-term treatment with cotrimoxazole. In this study, serologic signs of Q fever were associated with adverse pregnancy outcome. However, in none of the cases could we identify a definite causal relationship between *C. burnetii* seropositivity and adverse pregnancy outcome. Because only 9 cases of adverse pregnancy outcome were found over 5 years, despite increased awareness among the relevant risk groups, and because community studies in Denmark and the Netherlands have failed to confirm this association, the overall risk for a Q fever-associated adverse pregnancy outcome in Denmark is likely to be low.

Dr Nielsen is a physician in the Department of Clinical Microbiology, Aarhus University Hospital, and a postdoctoral scholar at the Department of Occupational Medicine, Hospital Region West, Herning, Denmark. Her research interests include infections, obstetrics, pediatrics, and microbiology.

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Address for correspondence: Stine Yde Nielsen, Department of Clinical Microbiology, Aarhus University Hospital, Brendstrupgaardsvej 100, 8200 Aarhus N, Denmark; email: stineyde@dadlnet.dk

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Genetic Evidence of Importation of Drug-Resistant *Plasmodium falciparum* to Guatemala from the Democratic Republic of the Congo

Jaymin C. Patel, Steve M. Taylor, Patricia C. Juliao, Christian M. Parobek, Mark Janko, Luis Demetrio Gonzalez, Lucia Ortiz, Norma Padilla, Antoinette K. Tshefu, Michael Emch, Venkatachalam Udhayakumar, Kim Lindblade, and Steven R. Meshnick

Imported malaria threatens control and elimination efforts in countries that have low rates of transmission. In 2010, an outbreak of *Plasmodium falciparum* malaria was reported among United Nations peacekeeping soldiers from Guatemala who had recently returned from the Democratic Republic of the Congo (DRC). Epidemiologic evidence suggested that the soldiers were infected in the DRC, but local transmission could not be ruled out in all cases. We used population genetic analyses of neutral microsatellites to determine the outbreak source. Genetic relatedness was compared among parasites found in samples from the soldiers and parasite populations collected in the DRC and Guatemala; parasites identified in the soldiers were more closely related to those from the DRC. A phylogenetic clustering analysis confirms this identification with >99.9% confidence. Thus, results support the hypothesis that the soldiers likely imported malaria from the DRC. This study demonstrates the utility of molecular genotyping in outbreak investigations.

Imported malaria threatens control and elimination efforts in countries that report low malaria transmission rates (1–3). In Central America, malaria transmission decreased by >50% during 2000–2010 (4); in 2010, the Guatemala Ministerio de Salud Pública y Asistencia Social reported

31 confirmed cases of malaria, all caused by the species *Plasmodium falciparum* (5). Central America is unusual compared with other areas in which malaria is endemic because chloroquine remains an effective treatment option for *P. falciparum* infection there, but not in other parts of the world (6–8); the introduction of parasites harboring chloroquine-resistant genotypes could fuel a resurgence of clinical illness and transmission.

In 2010, an outbreak of malaria was reported among 12 soldiers from Guatemala shortly after they returned from a United Nations (UN) peacekeeping mission in the Democratic Republic of the Congo (DRC). Of the 12, 8 also reported visiting ≥ 1 area in Guatemala in which malaria is endemic when they returned but before the outbreak was identified. An outbreak investigation was undertaken after 1 of the infected soldiers died; laboratory tests of blood from this patient identified chloroquine-resistant and -sensitive strains of *P. falciparum*.

The epidemiologic evidence suggested that the soldiers were infected while stationed in the DRC (9). Because the local acquisition of chloroquine-resistant parasites in Guatemala could necessitate a change in local treatment practices, it was vital to determine the origin of the soldiers' infections.

Molecular markers have been used to assess the genetic relatedness of malarial parasites from different geographic regions (10,11). Accordingly, if the soldiers acquired *P. falciparum* in the DRC during their stay, the genotypes of the parasites isolated from the soldiers' samples would be more closely related to parasites from the DRC than to parasites from Guatemala. To test this hypothesis, we used molecular methods from the field of population genetics to determine the source of the malaria outbreak among the soldiers who returned to Guatemala after being stationed in the DRC.

Author affiliations: University of North Carolina, Chapel Hill, North Carolina, USA (J.C. Patel, S.M. Taylor, C.M. Parobek, M. Janko, M. Emch, S.R. Meshnick); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (P.C. Juliao, V. Udhayakumar, K. Lindblade); Military Medical Center, Guatemala City, Guatemala (L.D. Gonzalez); Universidad de Valle de Guatemala, Guatemala City (L. Ortiz, N. Padilla); and University of Kinshasa, Kinshasa, Democratic Republic of the Congo (A.K. Tshefu)

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Methods

Study Participants

We included *P. falciparum* parasites from 3 distinct populations: 1) soldiers from Guatemala returning from the DRC with malaria; 2) adult residents of the DRC; and 3) residents of Guatemala (adults and children). The initial outbreak investigation in Guatemala received appropriate human subject review by the Universidad del Valle de Guatemala (Guatemala City, Guatemala) and the US Centers for Disease Control and Prevention (Atlanta, GA, USA) and was qualified as public health practice because its purpose was to identify and treat malaria cases among military personnel returning from the DRC. Samples from these soldiers were anonymized, and the investigations reported in this publication were reviewed and approved by the same institutions. Samples from the DRC were obtained during the 2007 Demographic Health Survey (DHS), which was approved by the review boards of Macro International (Calverton, MD, USA), the University of Kinshasa School of Public Health (Kinshasa, DRC), and the University of North Carolina (Chapel Hill, NC, USA). Samples collected from a previous malaria surveillance study (conducted during 1998–2000) that was originally approved by the Universidad del Valle de Guatemala human subjects review board were used to determine population structure of the parasite population of Guatemala.

In August of 2013, Guatemala sent its 13th mission to the DRC since it began sending troops in 2000 (12). In January 2010, 144 soldiers from Guatemala and 6 civilian support staff were deployed to the DRC as part of a United Nations peacekeeping mission. Upon return to Guatemala in October 2010, 12 soldiers were found to be infected with *P. falciparum* by using active and passive case detection; the infections were confirmed by using nested PCR. Of the 12 soldiers, 5 reported clinical symptoms and the other 7 were asymptomatic; date of onset of symptoms for the 5 soldiers ranged from October 12, 2010 (5 days before leaving the DRC) to November 7, 2010 (3 weeks after their arrival in Guatemala) (9). The first soldier in whom malaria was diagnosed died and was found to have been infected with parasites that had chloroquine-sensitive and chloroquine-resistant genotypes (9). Samples from all 12 soldiers were included in the analysis reported here.

To compare the parasites found in the DRC with those that infected the returning soldiers, we analyzed *P. falciparum* parasites from 74 participants in the national 2007 DRC DHS. The parent study and ancillary studies have been described in detail (13–16). For the analysis reported here, we selected 7 clusters, all of which included >10 *P. falciparum*-positive persons, as part of a study to quantify gene flow in *P. falciparum* strains within the DRC. Three outbreak clusters (81, 88, and 183) were chosen because

they were located on or near the Congo River, a principal route of human transportation (17); 2 clusters (164 and 211) were chosen because they were not on the river but were approximately the same distance apart from the 3 river sites as the river sites were from each other; and 2 clusters (29 and 203) were chosen because they were far away from the other clusters (Patel et al., unpub data) (Figure 1). We also included 40 *P. falciparum* specimens collected from previous surveillance studies conducted in Guatemala during 1998–2000 for comparison of genetic profiles of the local parasite population.

Microsatellite Analysis

Genomic DNA (gDNA) from the DRC samples was isolated from dried blood spots as described (13). We used the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's recommendations to extract gDNA from the soldiers and from samples from persons who were indigenous to Guatemala. All samples were sealed securely and stored at -20°C.

Eight neutral microsatellites were selected on chromosomes 2 (C2M33, C2M34, C2M29, C2M27) and 3 (C3M40, C3M88, C3M39, and C3M69) to assess whether the parasites identified in the soldiers were related to those found in the DRC. PCR primer sequences and cycling conditions for samples from the soldiers and other persons in Guatemala were adapted from earlier studies (18). For the samples from the DRC, a slightly different PCR technique was used. Specimens were initially amplified in single-round PCR protocols similar to those for samples from the soldiers and from persons in Guatemala; for those that failed to return PCR products, we used a heminested strategy wherein a newly designed additional external primer was used in a primary amplification, and then performed the standard round of amplification (19,20). Primer sequences are provided in the Table; their PCR cycling parameters have been described elsewhere (18,21). All PCR products were separated by capillary electrophoresis by using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). The alleles were scored by using GeneMapper software, version 3.7 (Applied Biosystems). Alleles were binned to the nearest 2 or 3 nucleotides in length depending on the size of the repeat unit. To distinguish alleles from background noise in multiple infections, we recorded peaks if they were >1/3 of the maximum peak level and exceeded 100 fluorescence units.

Data Analyses

To determine the source of the parasites found in the soldiers, we treated them as a discrete population and calculated the relatedness (β -diversity) between this parasite population and those identified in persons in the DRC and Guatemala. Genetic relatedness between the 3 populations



Figure 1. Location of the major cities, rivers, and the 7 Demographic Health Survey clusters (203, 81, 88, 183, 211, 164, and 29) within the Democratic Republic of the Congo (DRC) included in study of malaria outbreak among soldiers from Guatemala who had been stationed in DRC.

was calculated by using the Nei standard genetic distance (G_{ST}) and the Slatkin R_{ST} (22). G_{ST} is based on the infinite alleles model, which assumes that genetic differences arise through mutations and genetic drift, and was calculated in GenAlEx v6.4 (23) by using the following formula

$$Nei G_{ST} = -\ln \frac{\sum_{i=1}^k p_{ix} p_{iy}}{\sqrt{(\sum_{i=1}^k p_{ix}^2)(\sum_{i=1}^k p_{iy}^2)}}$$

in which p_{ix} and p_{iy} are the frequencies of the i th allele in populations x and y . Slatkin R_{ST} assumes that microsatellites evolve according to the stepwise mutation model in which novel alleles are created either by deletion or addition of a single repeated unit of microsatellite that has equal probability $\mu/2$ in both directions (22). R_{ST} was calculated in SPAGeDi v1.3 (24) by using the following formula

$$R_{ST} = \frac{(S - S_w)}{S}$$

in which S is the average squared difference in allele size between all pairs of alleles and S_w is the average sum of squares of the differences in allele size within each subpopulation. Pairwise R_{ST} comparisons were calculated by using ANOVA (a nested analysis of variance approach). Principal coordinate analysis was performed to quantify the variation between the parasite populations from the soldiers and persons in the DRC and Guatemala by using results of the pairwise R_{ST} comparisons. Principal coordinate analysis plots were generated by using GenAlEx v6.4 (23). We tested for associations between the neutral microsatellite markers on chromosomes 2 and 3 by using an exact test of linkage disequilibrium that had 10,000 Monte Carlo steps in Arelquin v3.1 (25). After applying Bonferroni correction for multiple comparisons, we examined p values for significance.

Because we were studying parasite populations and not hosts, each individual host could contribute ≥ 1 parasite variant to the population. Since the indices above are calculated locus by locus, haplotype construction was unnecessary. However, because haplotypes are required for entering data into GenAlEx and SPAGeDi, we created “virtual”

Table: Neutral microsatellite loci and primer sequences used for PCR amplification of *Plasmodium falciparum* malaria genes to identify origin of drug-resistant genotype, Guatemala

| Locus name | Chromosome | Primer | Primer sequence (5'→3') | Tag | Expected product size, bp |
|------------|------------|------------|---------------------------|-----|---------------------------|
| C2M33 | 2 | Forward | CATTGCAAAATATATATTCTCC | FAM | 193 |
| | | Reverse | GTGATTTGTACAATGTACATA | | |
| | | Heminested | ATTGCGTAAATAACACATCTGCA | | |
| C3M88* | 3 | Forward | CAAAAATGAAAATGAAAAGG | HEX | 150 |
| | | Reverse | TAAAGGGTGCGCATATCAAT | | |
| | | Heminested | GTTATTCAAAAAGGACGAAACAAG | | |
| C3M69 | 3 | Forward | AATAGGAACAAATCATATTG | HEX | 173 |
| | | Reverse | AGATATCCAGGTAATAAAAAG | | |
| | | Heminested | TTTATGAACACCCCTCATGTCAC | | |
| C2M29 | 2 | Forward | GTGAATAACGGAAAAGGATA | FAM | 141 |
| | | Reverse | AAGATCAAATACCAGGTGA | | |
| | | Heminested | TTAAGAAAACAATCAGAAGCGATG | | |
| C2M27 | 2 | Forward | CTTTTAATCACTACCATGTTG | HEX | 117 |
| | | Reverse | ATAATTTAATTGAGGATACCT | | |
| | | Heminested | TTGTATGTATCACTTTTTTCATTAC | | |
| C3M40 | 3 | Forward | GGGTAAAGAAAACACACAAA | FAM | 128 |
| | | Reverse | AATGTGTATATTACTAGAAGC | | |
| | | Heminested | TCCGAATATGGAATGTCGAAAG | | |
| C3M39 | 3 | Forward | CAAGAAGATAGGGATGATAAC | FAM | 159 |
| | | Reverse | TATTAATTGGTCTTCACCCG | | |
| | | Heminested | GGAGGAACGTAAAGAAGATATTG | | |
| C2M34 | 2 | Forward | TCCCTTTTAAAATAGAAGAAA | FAM | 260 |
| | | Reverse | GATTATATGAAAGGATACATG | | |
| | | Heminested | TTCACCTTTGTAATTAGAACATATC | | |

haplotypes for mixed genotypes. If ≥ 2 alleles were detected on 1 locus, distinct haplotypes were created that differed from each other only at the locus with multiple alleles. If multiple loci contained 2 alleles, 2 distinct haplotypes were created; alleles were randomly assorted for each locus. The same was done if multiple loci contained 3 alleles. If ≥ 1 loci had 2 alleles and ≥ 1 loci had 3 alleles, we created 2 full haplotypes using random assortment as above and a third haplotype that was missing data on loci that had 2 alleles. To assess whether the arrangement of these virtual haplotypes affected the analyses, we recalculated all indices of genetic relatedness using a different assortment of virtual haplotypes.

In addition to these analyses, to investigate the geographic clustering of the parasite populations, we calculated genetic distances and created a neighbor-joining phylogenetic tree (26) based on the Cavalli-Sforza and Edwards chord distance model (27) in Populations v.1.2.31 (28) and visualized it in the *ape* package for R (29). Further, we used Fast UniFrac, which is a frequently used measure of genetic differentiation between pathogen populations, to group the parasite populations with precision derived from permutation (30). To determine whether the observed population splits were caused by chance alone, we used 1,000 permutations. For these analyses, we used isolates for which the majority ($>50\%$) of the microsatellite markers were characterized.

Results

Initially, 8 microsatellite loci were characterized by capillary electrophoresis. One locus was censored in all analyses

because of poor amplification success ($<50\%$). For the remaining 7 loci, 71%–85% of the samples were successfully amplified. Seven infected soldiers and 8 infected persons from the DRC were excluded from further analyses because we were unable to amplify the majority of the microsatellites in specimens collected from them. Final analysis was conducted on samples from 5 infected soldiers, 74 infected persons from the DRC, and 40 infected persons from Guatemala.

Similar to the organisms causing infections documented in persons in the DRC, most of the organisms associated with the soldiers' infections contained polyclonal antibodies. Of the remaining 5 infected soldiers, samples from 3 (60%) contained ≥ 1 locus that had 3 genetically distinct alleles. This is similar to the distribution in the DRC, where strains from 47.3% ($n = 35$) of the subjects had ≥ 2 alleles in ≥ 1 locus. All the samples from persons living in Guatemala contained a single genotype. No significant linkage disequilibrium was observed between the neutral microsatellite markers used in all 3 study populations (data not shown).

To identify the source of the infections, we measured the genetic relatedness between parasite populations obtained from the soldiers, from the DRC residents, and from residents of Guatemala. Pairwise $R_{ST}/(1-R_{ST})$ and G_{ST} comparisons were calculated; values of ≤ 0 signify virtual identity whereas increasing values signify increasing divergence. Overall, parasites in samples from the soldiers were much more closely related to the parasites from the DRC ($(R_{ST}/(1-R_{ST}) = 0.204$ and $G_{ST} = 0.278$) than to parasites from Guatemala ($(R_{ST}/(1-R_{ST}) = 2.138$ and

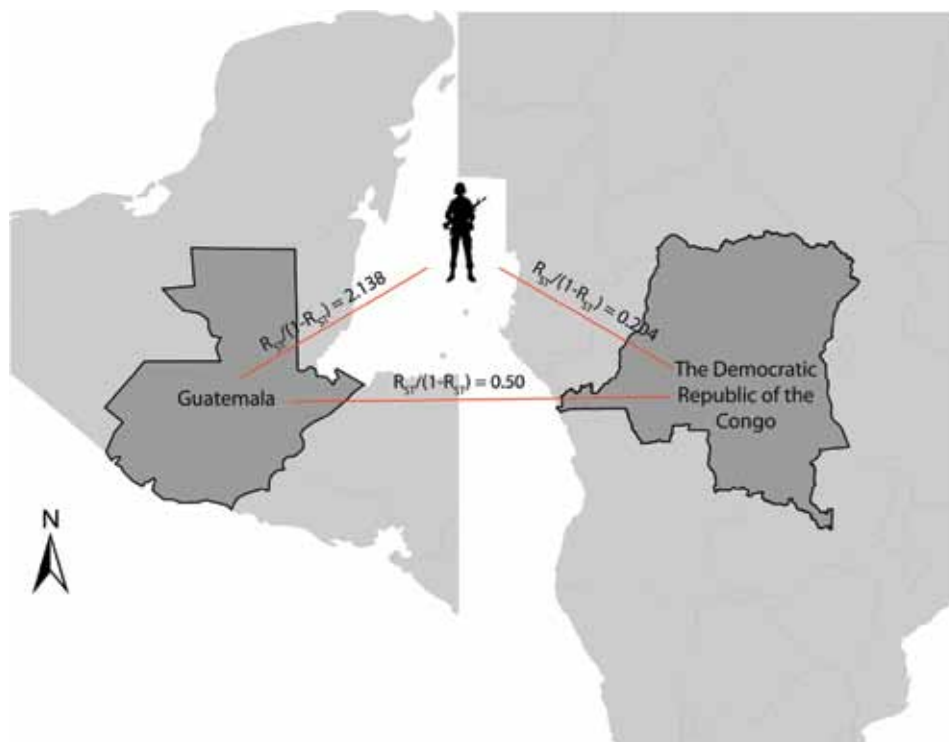


Figure 2. Genetic relatedness (pairwise R_{ST} comparisons) among *Plasmodium falciparum* identified in samples from the soldiers from Guatemala, persons in the Democratic Republic of the Congo, and persons in Guatemala.

$G_{ST} = 2.028$) (Figure 2). Both metrics (R_{ST} and G_{ST}) indicated that parasites identified in the soldiers were more closely related to those found in the DRC than in those from Guatemala. However, pairwise R_{ST} may be more appropriate because of the high mutation rates in microsatellites (19,22). When we used a different assortment of virtual haplotypes, genetic relatedness between the different parasite populations remained the same (data not shown).

The phylogenetic analyses also revealed a stark clustering effect between the 3 parasite populations. Visual inspection of the neighbor-joining tree (Figure 3) showed that the parasites found in the soldiers were part of the parasite population from the DRC and the parasites found in Guatemala were distinct from the other 2 populations. The ecologic clustering algorithm, after 1,000 jackknife permutations, also clustered the parasites from the soldiers with the parasite population in the DRC while they remained distinct from the parasites from Guatemala (Figure 4). The predicted differences between the parasites from Guatemala and those from the DRC and soldiers returning to Guatemala were statistically significant (>99.9% confidence).

We further attempted to examine the genetic relatedness of parasites, comparing those from each DRC cluster, the soldiers, and Guatemala. We observed that the parasites identified in samples from soldiers were more closely related to every selected DHS cluster within the DRC than to parasites from the samples from persons

living in Guatemala ($R_{ST}/(1 - R_{ST})$ range = 0.065–0.202) (data not shown).

Discussion

Molecular tools have become valuable in tracking the source of infectious agents in outbreak investigations (31,32). This study, which was based on the use of population genetic analyses of microsatellite data, supports previous epidemiologic findings that an outbreak of *P. falciparum* malaria in soldiers from Guatemala, who returned after their peacekeeping mission in the DRC in 2010, was caused by an imported parasite population from the DRC (9). This study further validates the use of molecular epidemiologic tools in malaria outbreak investigations.

Genetic relatedness among 3 parasite populations was first assessed by pairwise R_{ST} and G_{ST} . Parasites from the soldiers were more closely related to those from the DRC than those from Guatemala (Figure 2). These results were corroborated by neighbor-joining phylogenetic analyses and by Fast Unifrac, which showed with >99.9% confidence that the parasites from the soldiers were part of the parasite population from the DRC and distinct from the native parasites from Guatemala (Figures 3, 4). An alternate hypothesis is that there was a prior introduction of DRC parasites into Guatemala; support for this hypothesis is provided by our neighbor-joining phylogenetic tree, which includes a subset of DRC isolates that clustered with the parasites from Guatemala (Figure 3), and that finding

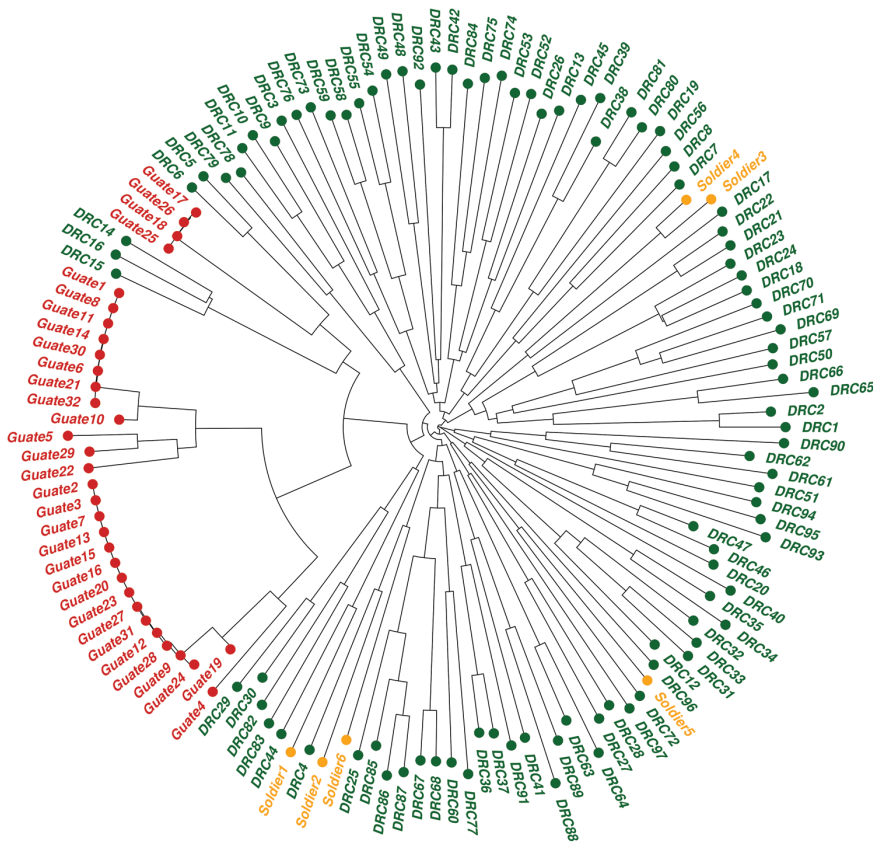


Figure 3. Neighbor-joining tree of 3 *Plasmodium falciparum* populations. Prefixes of genomes indicate parasite origins: Green text indicates parasite populations from the Democratic Republic of the Congo (DRC); orange indicates parasite populations detected in soldiers who were returning from the DRC to Guatemala; red indicates parasite populations from Guatemala.

suggests that the soldiers were infected after returning to Guatemala. However, this subset of DRC parasites was substantially different from the dominant clones in circulation in Guatemala, and the ecologic clustering algorithm consistently clustered parasites separately (Figures 3, 4). Additionally, given the high degree of heterogeneity within DRC parasites, it is reasonable to expect some overlap between a subset of parasites from the DRC and other parasite populations. Overall, the results from the molecular analyses, as well as the epidemiologic investigation (9) indicate that the source of the parasites among the soldiers from Guatemala was most likely the DRC. The results support the clinical information obtained from the soldiers. None of the soldiers adhered to malaria prophylaxis or used insecticide-treated bed nets as recommended during their time in the DRC. Lack of adherence to preventive measures has been identified as a risk factor for malaria infection among travelers to malaria-endemic countries (33). Further, the results are consistent with the travel history of the soldiers and the hypothesis from the epidemiologic investigation that the soldiers acquired malaria while traveling through the northern DRC and that the source of the outbreak was in the DRC and not in Guatemala (9).

Countries in Central America are experiencing low levels of malaria incidence, and several of them are

taking steps toward its elimination (4,34). In June 2013, the Council of Health Ministers from Central America and the Dominican Republic called for malaria elimination in the region by 2020 (35). Although chloroquine-resistant *P. falciparum* strains are widespread, Central America is one of the few regions in the world where chloroquine remains an effective treatment option for locally acquired malaria (6–8). Importation of chloroquine-resistant strains could lead to increased malaria-related illness and deaths, even though the local *Anopheles* spp. vector population may be refractory to foreign *Plasmodium* strains (36,37). One of the 12 soldiers, who died after returning from the peacekeeping mission, was found to have been infected with parasites of the *P. falciparum* chloroquine-resistance transporter (*pfert*) genotype CVIET, but it was not clear whether this patient acquired resistant parasites from the DRC or locally (9). It is unlikely that the patient acquired chloroquine-resistant parasites locally because these parasites are absent in Central America (7,8).

If chloroquine-resistant strains circulated in Guatemala, chloroquine drug pressure would positively select them and ultimately render chloroquine ineffective. Conversely, chloroquine resistance has been widespread in the DRC since at least 2002, which is supported by the report that the hallmark K76T mutation has been found in 93%

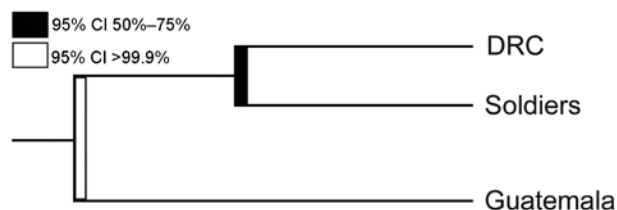


Figure 4. Phylogenetic tree showing predicted clustering between *Plasmodium falciparum* populations from the Democratic Republic of Congo (DRC), soldiers returning to Guatemala from the DRC, and Guatemala. The predicted split between parasites identified in samples taken in Guatemala and parasites from DRC among soldiers was significant (95% CI >99.9%) (black bar); the predicted split between parasites from DRC and returning soldiers was not significant (95% CI 50%–70%) (white bar). Computed by using Fast UniFrac (30) with jackknifing and 1,000 permutations.

of Congolese specimens (38). The current data provide evidence that this soldier acquired chloroquine-resistant *P. falciparum* in the DRC. Indeed, the microsatellite data suggested that this soldier had multiple strains. Given increasing globalization, international travel, and high frequency of military peacekeeping missions from Central America to Africa, persons returning from a malaria-endemic country who have malarial symptoms should be suspected of harboring chloroquine-resistant strains. These circumstances also provide impetus for countries such as Guatemala to make artemisinin-based combination therapies and other appropriate treatment available to treat imported multidrug-resistant malaria cases.

This study had several limitations. First, we included samples from only 7 DHS clusters. Including more clusters, especially those near Bunia, Isiro, and Dungo, which the soldiers reported having traveled through, would have helped in triangulating the source of infection with a higher resolution. Also, the local samples collected in Guatemala included in the study were collected during different years (1998–2000) from the outbreak. Because of the low number of malaria cases reported annually in Guatemala (31 total cases reported in 2010), it was not feasible to include samples from persons in Guatemala that were collected closer to the time of the outbreak. Further, of the 12 patients identified during the outbreak investigation, we were able to successfully characterize microsatellite profiles for *P. falciparum* parasites in only 5 patients who had microscopically detectable parasitemia levels. Microsatellites could not be amplified in samples obtained from any of the 7 patients who had asymptomatic *P. falciparum* infection. A possible explanation for this failure could be the potential degradation of gDNA between the time of extraction and time at which the microsatellite markers were characterized. However, the results of the study were likely unaffected as shown by the stark similarity between parasites

from the soldiers and persons in the DRC and substantial divergence between parasites from the soldiers and persons in Guatemala.

Molecular epidemiology and population genetic tools have been used successfully to identify geographic areas where outbreaks of poliomyelitis originate, leading to focused intensification of public health efforts to reduce polio-related illness and future outbreaks (39,40). Here we have demonstrated the use of molecular tools to conclusively identify the source of a parasite population during an outbreak investigation. Because of the extreme minimal diversity of the parasites from Guatemala, we were able to triangulate the source of the outbreak with high statistical significance. However, if each of the 3 parasite populations were as highly diverse as those in the DRC, we would require a greater number of samples and would use other genotyping methods which characterize parasite subpopulations on a finer scale, such as next-generation deep-sequencing methods to sequence polymorphic targets or whole genomes. There is also a clear need for capacity strengthening of sample collection, storage, and extraction techniques to support genotyping in countries that report low malaria transmission rates. These molecular tools could help strengthen existing surveillance efforts to prevent future outbreaks and the reintroduction of malaria in countries working toward malaria elimination.

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Mr Patel is a doctoral student in epidemiology at the University of North Carolina at Chapel Hill. His primary research interests include the use of molecular markers to study the geographic and temporal spread of malaria and the development of field appropriate molecular diagnostic tools for malaria.

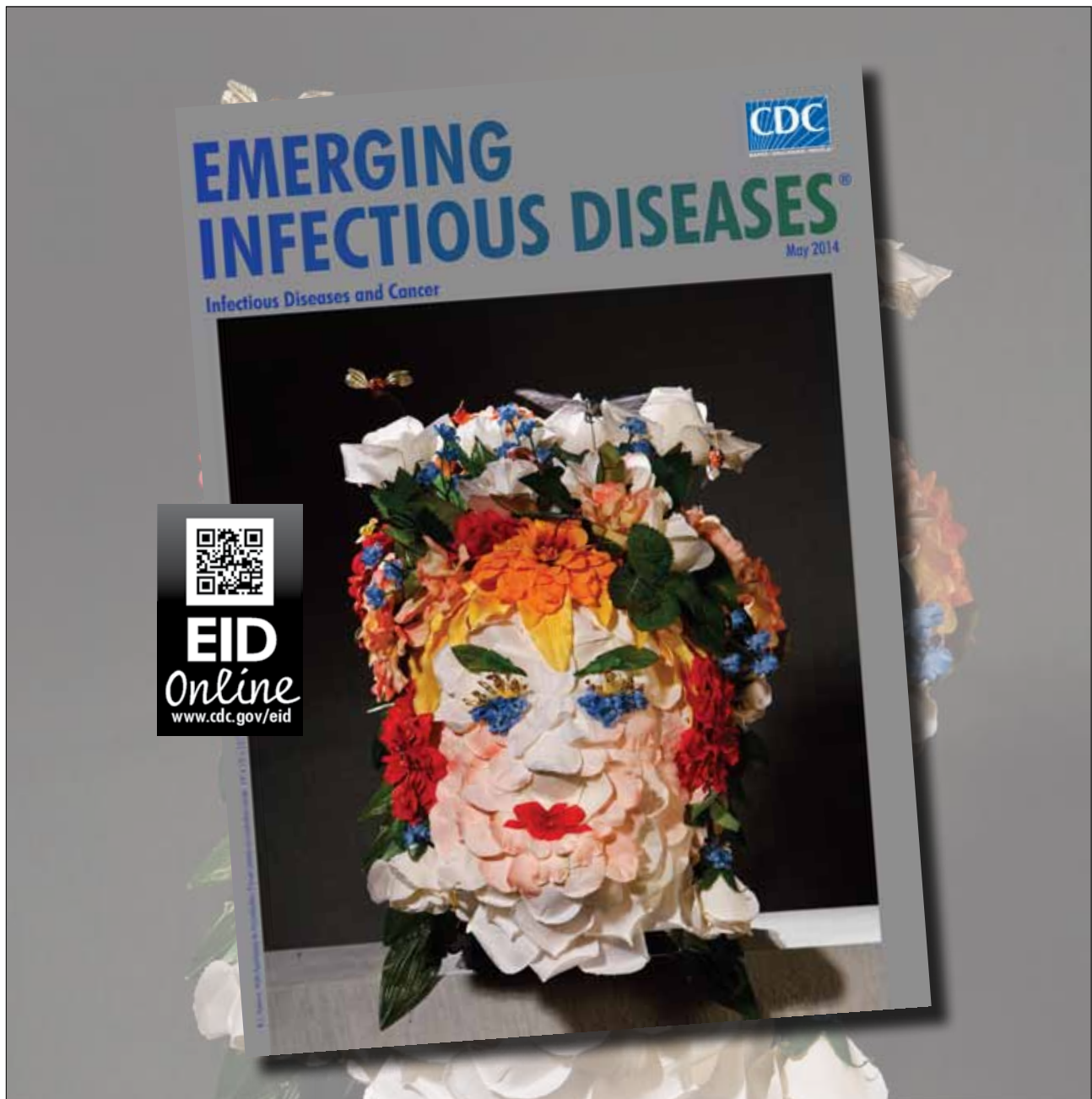
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Address for correspondence: Jaymin C. Patel, 135 Dauer Drive, 3206 Michael Hooker Research Building, UNC–Chapel Hill, Chapel Hill, NC 27599, USA; email: jaymin86@email.unc.edu



Short-Term Malaria Reduction by Single-Dose Azithromycin during Mass Drug Administration for Trachoma, Tanzania

Stephen E. Schachterle,¹ George Mtove, Joshua P. Levens, Emily Clemens, Lirong Shi, Amrita Raj, J. Stephen Dumler, Beatriz Munoz, Shelia West, and David J. Sullivan

Single-dose mass drug administration of azithromycin (AZT) is underway to eliminate trachoma worldwide. Studies in Ethiopia showed a reduction in all-cause childhood deaths after administration. To examine the effect of single-dose AZ MDA on prevalent malaria infections in a large prospective cohort of children and parents in Dodoma Province, Tanzania, we quantified the temporal prevalence of malaria parasitemia by real-time PCR for 6 months after single-dose AZT. In the first month after treatment but not in subsequent months, *Plasmodium falciparum* infections were reduced by 73% (95% CI 43%–89%) in treatment versus control villages and differences remained significant ($p = 0.00497$) in multivariate models with village-level random effects. Genetic sequencing of *P. falciparum* ribosomal L4 protein showed no mutations associated with AZT resistance. AZT mass drug administration caused a transient, 1-month antimalarial effect without selecting for *P. falciparum* ribosomal L4 resistance mutations in a region with a 10-year history of treating trachoma with this drug.

Malaria can be treated or prevented with the broad-spectrum antimicrobial drugs tetracycline or azithromycin (AZT) (1). In vitro, AZT interferes with malarial parasite replication by targeting the unique apicoplast organelle of the parasite (1). AZT inhibits malarial parasite growth 10-fold every 48 hours, and the pharmacokinetics of AZT predict that it remains at concentrations high enough to limit parasite growth for >1 week (2). AZT might

interfere with transmission by exoerythrocytic inhibition of parasite liver stages in humans and mice (3) and by interference with ookinete and sporozoite production in mosquitoes (4). Monotherapy with AZT is not typically used to treat malaria. However AZT is highly effective against *Chlamydia trachomatis*, the causative agent of trachoma, which causes blindness (5,6). Persons with malaria who live in trachoma-endemic regions may undergo repeated AZT therapy as part of the World Health Organization-sponsored global trachoma eradication program (7,8).

Data regarding the effects of AZ mass drug administration (MDA) on malaria are limited. In a cluster randomized trial in The Gambia, AZ MDA given in 3 doses (20 mg/kg) 7 days apart reduced malaria rates by half when measured at 1 time point in children 5–14 years of age (9). More recently, a 49% reduction in the odds of death (95% CI 29%–90%) was reported for children 1–9 years of age in an AZ MDA treatment group compared with controls in Ethiopia (10,11). Porco et al. suggested that reductions in malaria prevalence associated with AZT MDA might have contributed to observed decreases in overall deaths (10).

For malaria treatment, a randomized clinical trial that compared AZT/artesunate with artemether/lumefantrine in Muheza, Tanzania, reported that the odds of treatment failure were 5 times greater (95% CI 3.3–11.4) in the group that received AZT/artesunate (12). The authors postulated that the AZT/artesunate showed treatment failure because MDA for trachoma in Tanzania could have led to localized *Plasmodium* spp. resistance to AZT (12). *Plasmodium* spp. drug resistance to AZT has not been documented in the field. However, in vitro selection for AZT resistance identified a G76V mutation among conserved active site amino acids at position 71–79 in *P. falciparum* apicoplast-encoded

Author affiliations: Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA (S.E. Schachterle, L. Shi, A. Raj, D.J. Sullivan); National Institute for Medical Research, Ubwari, Tanzania (G. Mtove); and Johns Hopkins University School of Medicine, Baltimore (J.P. Levens, E. Clemens, J.S. Dumler, B. Munoz, S. West)

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¹Current affiliation: Pfizer, Inc., New York, New York, USA.

ribosomal protein L4 (*PfRpl4*) (PFC10_API0043) (1). A Cochrane meta-analysis report stated that “azithromycin’s future for the treatment of malaria does not look promising” (13) and cited studies in which AZT, although well tolerated, was inferior to tetracycline for malaria prophylaxis (14–17).

In the current study, we evaluated malaria prevalence in a cohort of 2,053 children and adults in central Tanzania to examine the effect of single dose AZ MDA on prevalent malaria infections. We also searched for *PfRpl4* mutations that might confer *P. falciparum* AZT resistance.

Methods

Study Site and Sampling

Study participants were from 8 rural agricultural villages in Dodoma Province, central Tanzania (18–20). The study period was January 12, 2009–July 21, 2009 and was coincidental with a period in which rainfall was <60% of the average amount (21,22). AZT was offered to all the residents of the treatment villages, including residents not part of the follow-up investigation. Four treatment villages were selected on the basis of trachoma prevalence >10% in children 1–9 years of age (trachoma prevalence 12%, 18%, 18%, and 14%), rather than by randomization (18,19), which was consistent with World Health Organization AZT MDA guidelines for trachoma control (4). Four control villages with lower trachoma rates (8%–10%) were chosen on the basis of geographic proximity.

A complete census was conducted in the villages > In each village, 130 families with children <5 years of age were randomly selected for follow-up prevalence blood sampling; 1 child and 1 adult were randomly selected from each family. Sample size estimations were based on prestudy estimates of a malaria prevalence of 10% in children <5 years of age in the Dodoma region (23). Follow-up fingerprick blood sampling was performed at baseline and at 1, 3, 4, and 6 months later. Children were divided into 3 subgroups that were sampled once at weeks 2 (group 1), 6 (group 2), and 8 (group 3). Contemporaneous to the monthly blood sampling, staff members from villages conducted an active surveillance program in which children were visited weekly and screened for axillary temperature and questionnaires were answered regarding fever, diarrhea, and respiratory disease (18,19).

Blood samples were collected on ProteinSaver903 (GE Healthcare, Pittsburgh, PA, USA) for analysis by real-time PCR for all participants at each scheduled monthly visit (20). When a fever (temperature $\geq 37.5^{\circ}\text{C}$) in adults and children was observed or when a caregiver reported a history of fevers in children during the weekly surveillance, a rapid diagnostic test (RDT) (Paracheck-Pf, Orchid Biomedical Systems, Goa, India) and thick and thin blood films were prepared. RDTs were performed by trained study staff and interpreted

in the field at the time of sampling. Two experienced microscopists at the Amani Laboratory in Muheza, Tanzania, read the slides blinded to the PCR or RDT results. Discordant results were read by a third microscopist. Persons with positive results by RDT and all febrile children <5 years of age were treated with artemether/lumefantrine according to national guidelines and removed from later analysis.

Information on bednet ownership was obtained through standardized participant interviews. Latitude, longitude, and altitude of home locations were measured by using the GPSMAP 76 unit (Garmin, Olathe, KS, USA).

Quantitative PCR

DNA extraction and real-time PCR are described elsewhere (20). Five 3-mm diameter punches, equivalent to 25 mL of whole blood, were removed from the filter papers. DNA was extracted by using a commercial 96-well kit (Promega, Fitchburg, WI, USA). The DNA was concentrated by glycogen acetate and acetate/ethanol precipitation and low-speed ($3,000 \times g$) centrifugation for 30 min.

Multiplex real-time PCR was used to amplify the 18S *P. falciparum* ribosomal gene with a Cy5-labeled probe (20). Samples were processed in duplicate on manually loaded 384-well plates. A 40-cycle standard PCR protocol was used in a CFX 384 real-time PCR Detection System Thermocycler (BioRad, Hercules, CA, USA). Baseline relative fluorescence units (RFUs) were readjusted by using Bio-Rad CFX manager software. The real-time PCR system also detected *Borrelia* spp. reported by Reller et al. (24). The sensitivity and specificity of the real-time PCR versus that of RDT or microscopy were reported by Schachterle et al. (20). The real-time PCR detected 1–100 parasites/mL and showed the highest sensitivity in latent class analysis on febrile study patients. For the monthly surveys, microscopic analysis was not performed for the 8,711 samples for which real-time PCR was conducted. The RFU cutoff used was greater than that for >50 negative blood samples from control patients at Johns Hopkins University. The cutoff of 650 RFUs for the last cycle of the highest real-time PCR replicate had a specificity of 100% for samples from the control group at Johns Hopkins University and a specificity of 94% for samples from febrile patients in Tanzania; microscopy was used as a reference method (20).

Single-Nucleotide Polymorphism Analysis of *PfRpl4* Gene for AZT Resistance

Blood samples from treatment and control villages that had higher parasite densities by real-time PCR and microscopy were subjected to PCR amplification and whole gene fragment cloning into the pCR2.1 plasmid. Amplification and cloning were followed by full *PfRpl4* gene DNA sequencing of multiple bacterial clones for each patient and examination of mutations anywhere in this gene (1).

Statistical Analysis

Data were analyzed from the perspective of an intention to treat in which participants from AZT MDA-treated villages who refused treatment were classified with those who accepted AZT as members of treatment villages. Univariate ratios were used to compare prevalence proportions in treated versus untreated villages. CIs for ratios that compared proportions of prevalent infection in treated versus untreated villages were estimated by using 2-sided exact binomial tests.

Maps were used to display geospatial patterns in infection prevalence and malaria clustering over time (Google Maps, Mountain View, CA, USA, and Quantum Geographic Information System Open Source Geospatial Foundation Project (www.qgis.org/en/site/). Geographic coordinates of *P. falciparum*-positive and -negative blood samples were projected into kilometers, and locations were smoothed by using quadratic kernel intensity estimation. Kernel intensity estimators predict spatial intensity at unsampled points from nearby sampled points by using a quadratic function to heavily weight the nearest measured points and de-emphasize points sampled at greater distances. Weighted integrals were used to estimate intensity smoothing parameters for quadratic functions, assuming a 2-dimensional (i.e., spatial), stationary, isotropic point pattern process. Spatial odds ratios (ratio of kernel intensities from positive blood samples to kernel intensities from negative blood samples) (25), were determined for *P. falciparum*.

To examine the effect of AZT MDA while adjusting for malaria clusters observed on maps, we used multivariate logistic models with random effects to compare odds of prevalent malaria infection for AZT MDA treatment villages with odds for control villages. Random intercepts were fit to account for the multilevel or hierarchical design that nested persons within villages. This model enabled accurate statistical inference by adjusting CIs and p values for *P. falciparum* clusters in villages, and this inference was apparent in prevalence maps (25). Residual spatial autocorrelation was assessed by using variograms, and residual temporal autocorrelation was eliminated by restricting models to 1 sampling interval (26). Multiple models that examined the effect of AZT MDA on prevalent malaria were fit to control for confounding by other drivers of malaria prevalence and to address the robustness of standard errors to potential spatial residual autocorrelation. Altitude and self-reported bednet ownership were held constant to control for confounding in the multivariate model. A priori, we believed any AZT MDA effect would be most perceptible between month 1 and baseline.

To distinguish between the effect of AZT on prevalence and incident infections, we performed a sensitivity analysis for only *P. falciparum* infections that had been preceded by a negative *P. falciparum* test result in the previous sampling interval. These data also underwent a

second sensitivity analysis that included participants treated with artemether/lumefantrine who had been removed from the previous analyses.

Data management and analysis were conducted by using R statistical software (27). R with the SPLANCS package was used for kernel intensity estimation (28), and the LME4 package was used for the random effects model (29). All R code is available upon request.

Results

Characteristics of Study Population

The study site in the Kongwa District of Tanzania included 8 villages and 12,898 persons. Four villages were chosen on the basis of increased prevalence of trachoma (>10%) in children 1–9 years of age. Excluded were 34 of 66 villages that had received AZT MDA in the previous year. In the 4 villages that received AZT MDA, 6,252 persons received AZT and 642 did not receive AZT. A census in untreated control villages identified 5,991 persons. These villages were chosen because of geographic proximity to treatment villages. In each village, pairs of a parent and a child <5 years of age were randomly chosen for inclusion (1,045 persons in the AZT MDA treatment group and 1,008 persons in the control group for follow-up) (Figure 1). Blood samples for real-time PCR testing were collected from participants at baseline and at months 1, 3, 4, and 6

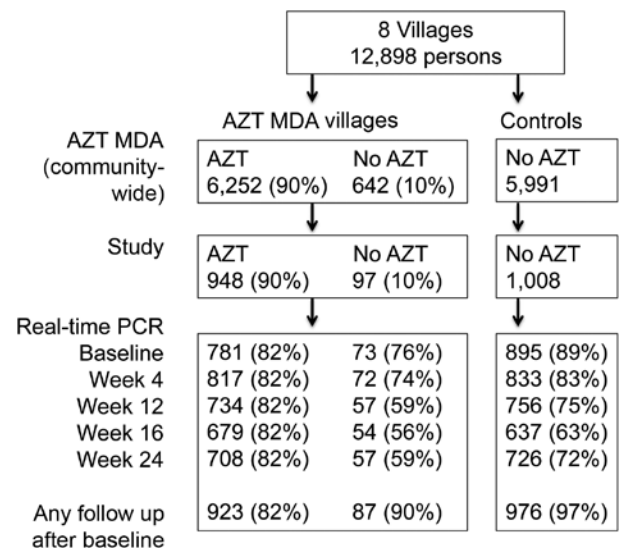


Figure 1. Flowchart of participants in study of short-term malaria reduction by single-dose azithromycin (AZT) during mass drug administration (MDA) for trachoma, Tanzania, January 12–July 21, 2009. AZT MDA (village-wide) and study panels show that 90% of persons who were intended to receive AZT received this drug. Total study participants with $\approx 1,000$ in each group, shown in the study panel, contributed samples that are shown in the real-time PCR panel at each sampling time. Percentages in the real-time PCR panel used values from the study panel as the denominator.

(4,437 patient-time samples from AZT MDA treatment villages and 4,274 patient-time samples from control villages). At any follow-up period after baseline, 1,010 (97%) study participants in AZT MDA villages who received AZT and 976 (97%) study controls were sampled for real-time PCR at least once. At baseline, treatment and control villages reported antimalarial drug use, latrine access, education (based on the highest level of education attained by the father), and home elevations. However, self-reported bednet ownership and fever histories were higher in treatment villages than in control villages ($p < 0.05$) (Table 1).

Univariate Analysis by Time

Overall, the proportion of *P. falciparum*-infected participants was highest (6%) at baseline. Infections decreased sharply in treated villages and gradually in control villages throughout follow-up period (Figure 2). At the baseline evaluation, 6% (53/854) of participants from AZT MDA treatment villages were positive for *P. falciparum* compared with 6% (54/894) of participants from AZT MDA control villages. No differences in odds of infection were observed between control and treatment villages (odds ratio 1.03, 95% CI 0.68–1.55) (Figure 3, Table 2).

By month 1, *P. falciparum* prevalence in AZT MDA treatment villages decreased to 2% (14/851) and *P. falciparum* prevalence in AZT MDA control villages decreased to 5% (37/779). The odds ratio for AZ MDA treated villages compared with control villages was 0.34 (95% CI 0.17–0.64), which is consistent with a 66% reduction in the odds of *P. falciparum* infection in AZT MDA treatment participants compared with AZT MDA control participants. Beyond month 1, the association between AZT MDA and reduced prevalence of malaria infection decreased. However, overall rates of *P. falciparum* infection decreased below levels for which reliable inferences could be made.

A village level comparison of changes in malaria prevalence for AZT MDA treatment villages versus prevalence in control villages indicated that 1 village in each group had no change in malaria prevalence, and 3 AZ MDA treatment villages had a significant ($p < 0.05$, by Fisher exact test) decrease in malaria prevalence ($p = 0.489$, $p = 0.0002$, and $p = 0.0005$) and 2 in the control group had significant ($p = 0.0008$ and $p = 0.001$) increases. Village 5 in the control group had a significant ($p = 0.0001$) decrease (Figure 4;

online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/6/13-1302-Techapp1.pdf).

In a subgroup of 200 children examined for malaria infection at weeks 2, 6, and 8, the malaria prevalence of 2%–5% was lower than the 10% expected (23). Thus, reliable prevalence comparisons were not relevant. During the study, febrile patients were tested by RDT and microscopy. During the critical period of 5–42 days after AZT MDA, 46 and 48 children in the treatment and control villages, respectively, were evaluated for fever. Among febrile children, only 5 children from treatment villages and 3 children from control villages were positive by real-time PCR.

Multivariate Analysis

At month 1, reductions in *P. falciparum* malaria prevalence were observed by logistic regression models that controlled for differences in self-reported bednet ownership and home altitude (Table 3) and included village level random effects. The odds of *P. falciparum* infection were 63% (95% CI 28%–81%) but less in treated villages than in control villages after adjusting for bednet ownership and home altitude in a model with village-level random intercepts. Because self-reported history of fever, malaria medication, latrine access, and education of the head of the household were not associated with malaria prevalence and did not appreciably alter the interpretations of the multivariate models, they were not included in the reported estimates. As was evident in univariate analysis, no differences in malaria rates existed at baseline, and the association between AZT MDA and reduced malaria rates decreased after the first month of follow up.

Spatial information on home locations enabled a thorough examination of residual spatial autocorrelation. A total of 51% (19/37) of *P. falciparum* infections at month 1 occurred in control village 8 (Figure 4), which raised concerns that residual spatial autocorrelation artificially decreased CIs from the reported models despite use of the random effects model. However, a variogram of standardized residuals from the model suggested that residual spatial autocorrelation was appropriately controlled by the village level random effects. The data were reanalyzed with incident infections that had been preceded by a negative real-time PCR result for *P. falciparum*; results did not change appreciably. We also observed an age-independent

Table 1. Comparison between azithromycin MDA treatment and control participants at baseline, Tanzania, January 12–July 21, 2009*

| Variable | MDA treatment group | MDA control group |
|--|---------------------|-------------------|
| Bednet ownership, yes/not† | 457/725 (63) | 328/789 (42) |
| History of fever, yes/not† | 26/813 (3) | 10/869 (1) |
| Malaria medication, yes/no | 157/814 (19) | 161/869 (19) |
| Latrine access, yes/no | 297/981 (30) | 276/971 (28) |
| Education of head of household, yes/no | 4.2 (3.41) | 3.2 (3.46) |
| Altitude, m | 1,206 (32.89) | 1,200 (45.99) |

*Values are no. positive/no. tested (%) for categorical measures or mean (SD) for continuous measures. MDA, mass drug administration.

† $p < 0.05$ by χ^2 test.

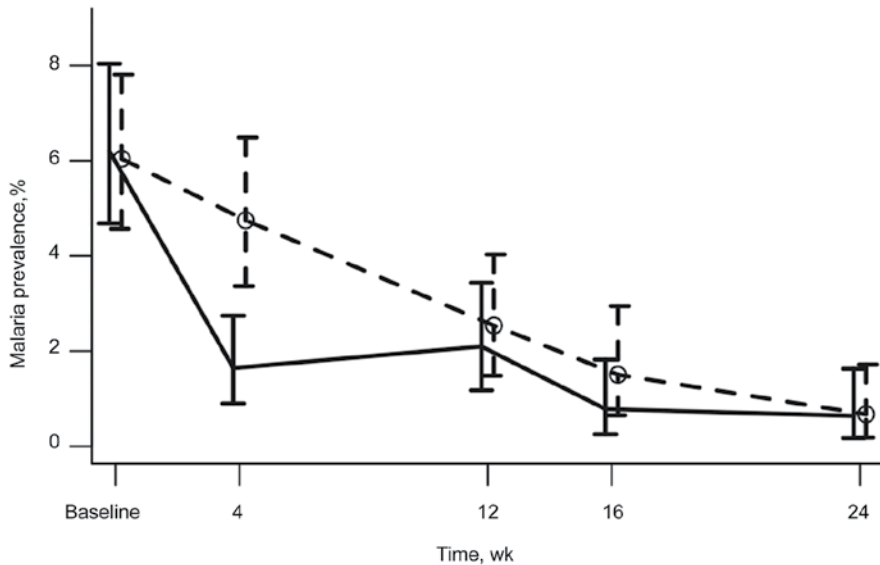


Figure 2. Effect of azithromycin (AZT) mass drug administration (MDA) in treatment and control villages by time in study of short-term malaria reduction by single-dose AZT during MDA for trachoma, Tanzania. January 12–July 21, 2009. Proportions of real-time PCR prevalent *Plasmodium falciparum* infections are shown in participants from treatment villages (solid line) and control villages (dashed line and circles). Error bars indicate 95% CIs from exact binomial tests.

decrease in malaria prevalence at month 1 for persons 1–10 years of age (univariate analysis) and for persons >10 years of age (univariate analysis and multivariate analysis) (online Technical Appendix Table 2).

Analysis of *PfRpl4* Mutations Associated with Azithromycin Resistance

Sequencing of full-length *P. falciparum* ribosomal L4 protein was performed for samples from 12 patients. We did not find evidence of single-nucleotide polymorphisms

(SNPs) conferring AZT resistance in amino acid region 71–79 (1). A synonymous SNP at position K36 was found in all bacterial plasmid clones from samples of 1 patient and in a *P. falciparum*-positive control patient who was not from Tanzania. A single plasmid clone from a sample of participant from an AZT MDA control village contained a nonsynonymous SNP in the active site of the *PfRpl4* gene at A78S. However, 17 other plasmid clones from the same blood sample did not contain the mutation, which suggested the aberrant sequence resulted from a Taq

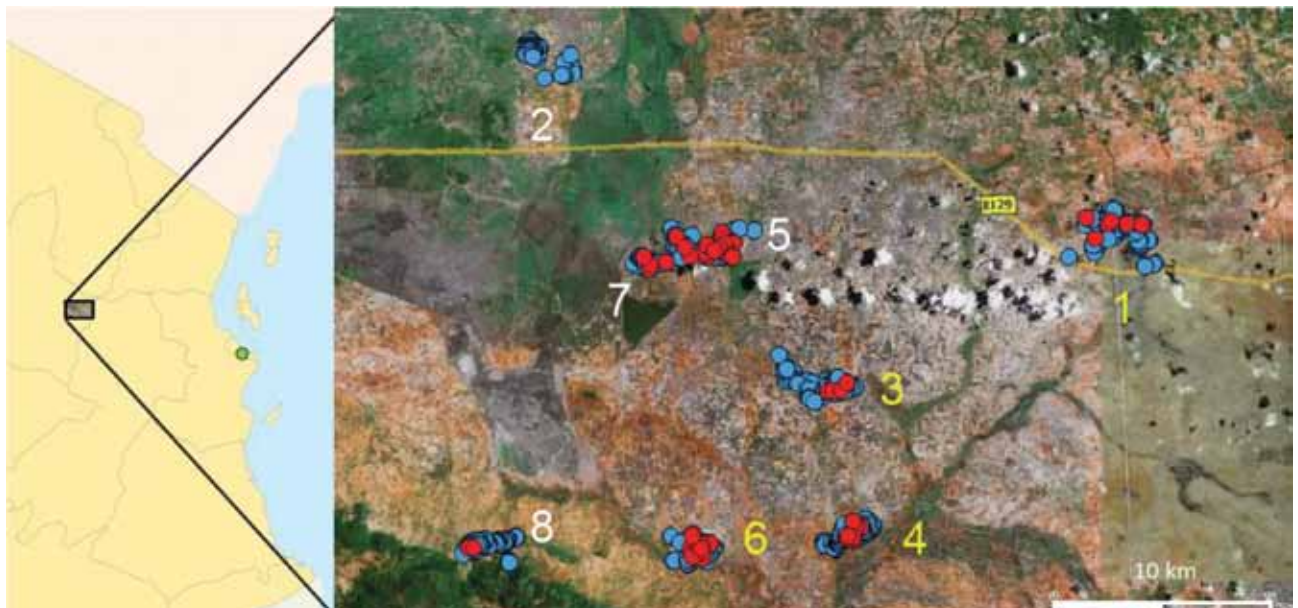


Figure 3. Study villages and location of malaria cases at baseline, Tanzania, January 12–July 21, 2009. Study site included 8 rural villages in central Tanzania, Dodoma Province. Four azithromycin (AZ) treatment villages in southeastern Tanzania are indicated by yellow numbers 1, 3, 4, and 6. Four nearby control villages in northwestern Tanzania are indicated by white numbers 2, 5, 7, and 8. The background map was extracted from Google Earth (Google, Mountain View, CA, USA). Households are indicated by blue dots, malaria cases at baseline are indicated by red dots, and Dar es Salaam (capital) is indicated by the green dot.

Table 2. Univariate association between AZT MDA and *Plasmodium falciparum* malaria prevalence, Tanzania, January 12–July 21, 2009*

| Time | No. negative | No. positive | OR (95% CI) |
|----------|--------------|--------------|-------------------|
| Baseline | | | |
| AZT– | 840 | 54 | 1.03 (0.68–1.55) |
| AZT+ | 801 | 53 | |
| Month 1 | | | |
| AZT– | 742 | 37 | 0.34 (0.17–0.64)† |
| AZT+ | 837 | 14 | |
| Month 3 | | | |
| AZT– | 653 | 17 | 0.83 (0.38–1.77) |
| AZT+ | 700 | 15 | |
| Month 4 | | | |
| AZT– | 523 | 8 | 0.52 (0.13–1.81) |
| AZT+ | 632 | 5 | |
| Month 6 | | | |
| AZT– | 589 | 4 | 0.95 (0.18–5.12) |
| AZT+ | 621 | 4 | |

*AZT, azithromycin; MDA, mass drug administration; OR, odds ratio.
†p<0.001.

polymerase error during plasmid cloning of the *Pf*RpL4 gene. Moreover, restriction enzyme *Hind*III, which was specific for the nonsynonymous SNP at A78S, did not digest multiple PCR amplicons from the original patient blood sample, which further suggested that the SNP on the *Pf*RpL4 gene was present at a low concentration or may have been an artifact of the Taq polymerase amplification. A78S has not been identified in other mutant bacteria associated with AZT resistance (1). Eighteen other nonsynonymous mutations were found in samples from participants from AZT MDA treatment villages, but these mutations were not in conserved amino acid regions for *Pf*RpL4 associated with AZT resistance in bacteria or the selected *P. falciparum* (except for an I39S were single occurrences among multiple clones from a single patient). Four synonymous SNPs were also detected (Table 4).

Discussion

AZT MDA was associated with a minimum reduction of 66% in odds of *P. falciparum* infection compared with odds of reduction for controls in the first month after drug administration, as shown by univariate and multivariate models on an individual level. Beyond 1 month, prevalence in treatment and control groups decreased, and there was no difference in prevalence between treatment and control groups. In addition, no mutations were detected in samples from treated persons who had parasitemias after drug treatment in the only gene associated with in vitro selection of AZT resistance. However, these results show more modest effects than those of other studies of AZT MDA, which reported large reductions in malaria (9) and that AZT MDA protection against malaria contributed to broader decreases in illness and death (10).

Because our data were analyzed from the perspective of an intention to treat, participants who did not receive AZT but lived in villages that received AZT MDA were classified with those who received AZT. The intention-to-treat analysis could have biased reported results toward the null because persons in AZT MDA treatment villages who benefited from AZT might have been more likely to become infected (online Technical Appendix Tables 3, 4). Because intention-to-treat analysis would probably bias results toward the null, we believe that it was a conservative analytic assumption.

An additional potential confounder is that all febrile children <5 years of age and adults who were positive for *P. falciparum* by RDT were treated according to national and Integrated Management of Childhood Illness guidelines with artemether/lumefantrine, which has a lingering 4-week prophylactic effect on parasitemia. However, inclusion of artemether/lumefantrine-treated participants did

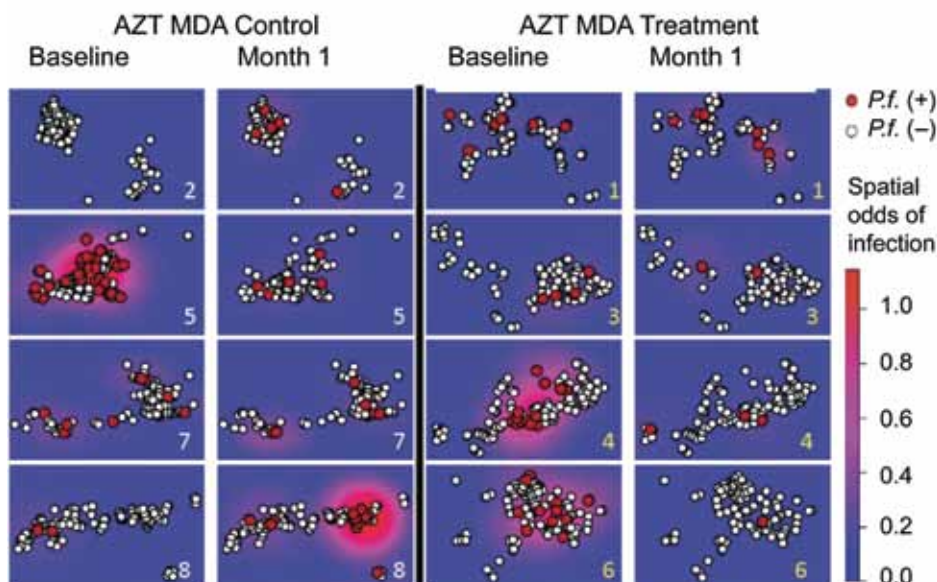


Figure 4. Effect of azithromycin (AZT) mass drug administration (MDA) in treatment and control villages over space and time, Tanzania, January 12–July 21, 2009. AZT MDA control villages (2, 5, 7, and 8) are shown on the left, and AZT MDA treatment villages (1, 3, 4, and 6) are shown on the right. Survey periods at baseline and month 1 are shown within treatment and control groups. Baseline infection and prevalent infections at month 1 (red circles) and negative test results (white circles) are shown. Spatial odds of *Plasmodium falciparum* (*Pf.*) infection are shown in areas of high odds of infection (red) and areas of low odds of infection (blue). Ln kernel smoothers were used to show spatial odds.

Table 3. Multivariate association between AZ MDA and *Plasmodium falciparum* prevalence with village-level random effects, Tanzania, January 12–July 21, 2009*

| Time | AZT MDA treatment, yes/no | Bednets, yes/no | Altitude, m |
|----------|---------------------------|---------------------|--------------------|
| Baseline | 1.16 (0.725–1.849) | 0.75 (0.483–1.158) | 0.99 (0.984–0.996) |
| Month 1 | 0.37 (0.188–0.743)† | 0.78 (0.418–1.439) | 1.01 (1.002–1.017) |
| Month 3 | 0.70 (0.922–1.64) | 1.55 (0.662–3.65) | 1.00 (0.992–1.012) |
| Month 4 | 0.62 (0.078–4.919) | 0.40 (0.095–1.673) | 1.02 (0.997–1.044) |
| Month 6 | 0.50 (0.074–3.347) | 1.54 (0.235–10.094) | 0.99 (0.966–1.016) |

*Values are odds ratio (95% CI). AZT, azithromycin; MDA, mass drug administration.
†p<0.005.

not affect the relevant proportions of malaria infection at month 1 (online Technical Appendix Table 5).

AZT treatment occurred at the village level, which might have exacerbated similarities in malaria risk among participants in the same village and created village-level clustering (25). Clustering within villages can bias results if it is not addressed in the statistical models (30). Village-level random effects were used to adjust variance estimates for within-village clustering and provide valid multilevel statistical inferences, given the clustering inherent in the study design (20,31). We argue that a relevant comparison is between the proportion of AZT MDA-treated and control villages, rather than a grouping of 4 villages in each AZT MDA intervention and control villages. Grouping findings into only 8 units on a village level does not produce relevant results and was not the design of the study.

AZT MDA was conducted according to the WHO SAFE (surgery, antibiotics, facial cleanliness, and environmental changes) trachoma control strategy, and assigning treatment to villages by randomization would have

been unethical because of the established effectiveness of this strategy in the study region. AZT MDA was examined from an observational perspective with the longitudinal cohort. We sought to minimize lack of comparability by selecting villages from a similar geographic region and by controlling for bednet ownership and altitude in multivariate analysis. We believe that residual confounding and bias are minimal because similar *P. falciparum* infection prevalence rates were observed at baseline and at months 3–6 after the effect of the AZT MDA had decreased.

In Ethiopia, Porco et al. reported a 49% (95% CI 10%–71%) decrease in odds of death in a randomized trial of AZ MDA villages compared with control villages that received a single dose of AZ (20 mg/kg to ≤1 g) (10). AZT protection from *P. falciparum* infection might have contributed to the overall decrease in deaths. Although the effect of AZT MDA on prevalent infections was strong, most infections observed were asymptomatic. The study in Ethiopia also had 3 dosing strategies (annual, biannual, and quarterly), which resulted in two thirds of study participants receiving more annual doses than used in the

Table 4. Resistance markers for cloned sequences of *Plasmodium falciparum* ribosomal *Pf*Rp14 protein gene, Tanzania, January 12–July 21, 2009*

| Patient ID | Time from azithromycin treatment, d | Parasites/μL by light microscopy | Parasites/μL by real-time PCR | RDT result | No. plasmid clones with WT sequence | Clones with synonymous SNP (no.) | Clones with nonsynonymous SNP (no.) | No. plasmids sequenced |
|------------|-------------------------------------|----------------------------------|-------------------------------|------------|-------------------------------------|----------------------------------|--|------------------------|
| 1 | 30 | 31,200 | 5,378 | + | 7 | L112L (1) | 0 | 8 |
| 2 | 30 | ND | 3,177 | ND | 3 | 0 | 0 | 3 |
| 3 | 30 | ND | 224.9 | ND | 0 | K36K (4) | 0 | 4 |
| 4 | 67 | 151,000 | 126,506 | + | 2 | 0 | 0 | 2 |
| 5 | 69 | 74,400 | 55,850 | – | 1 | S85S (1) | 0 | 2 |
| 6 | 80 | 49,160 | 16,410 | + | 4 | 0 | I38V(1), N109S (1), N166D (1) | 7 |
| 7 | 82 | 1,840 | 679 | + | 1 | 0 | F114L (1) | 2 |
| 8 | 94 | 26,760 | 11,030 | – | 1 | 0 | T56I (1) | 2 |
| 9 | 127 | 0 | 51,075 | + | 3 | 0 | 0 | 3 |
| 10 | 132 | ND | 22,774 | ND | 0 | 0 | I39S (1), S65N (1) | 4 |
| 11 | NT | 0 | 69,718 | + | 13 | 0 | A78S (1), †N134D (1), K100R and I126T (1), N166S (1), Y20H, K100%, and I139T (1) | 18 |
| 12 | NT | 11,160 | 2,495 | + | 2 | 0 | 0 | 2 |
| Control | NT | | | ND | 2 | 0 | 0 | 2 |
| Control | NT | | | ND | 2 | 0 | 0 | 2 |

*ID, identification; RDT, rapid diagnostic test; WT, wild type; SNP, single-nucleotide polymorphism; +, positive; ND, not done; –, negative; NT, not treated.
†An A78S nonsynonymous SNP in region associated with azithromycin resistance that was not present in other clones.

study in Tanzania. Our data provide only weak evidence that a reduction in malaria infection contributed to the reduction in deaths reported in Ethiopia.

At the study site, we did not find evidence of *P. falciparum* AZT resistance markers on a gene previously implicated in AZT resistance in vitro. We also observed a short-lived but major reduction in malaria. AZT MDA does not produce *Plasmodium* spp. drug resistance probably because other drugs with different mechanisms of action are used to treat malaria. This study might provide an optimistic note for AZ MDA planners and those living in areas in developing nations to which malaria and trachoma are endemic.

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Dr Schachterle is an associate director at Pfizer, Inc., New York, NY. His primary research interest is spatial analysis of infectious diseases.


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Address for correspondence: David J. Sullivan, Department of Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe St, Rm E5628, Baltimore, MD 21205, USA; email: dsulliva@jhsph.edu

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Rapid Spread and Diversification of Respiratory Syncytial Virus Genotype ON1, Kenya

Charles N. Agoti, James R. Otieno, Caroline W. Gitahi, Patricia A. Cane, and D. James Nokes

Respiratory syncytial virus genotype ON1, which is characterized by a 72-nt duplication in the attachment protein gene, has been detected in >10 countries since first identified in Ontario, Canada, in 2010. We describe 2 waves of genotype ON1 infections among children admitted to a rural hospital in Kenya during 2012. Phylogenetic analysis of attachment protein gene sequences showed multiple introductions of genotype ON1; variants distinct from the original Canadian viruses predominated in both infection waves. The genotype ON1 dominated over the other group A genotypes during the second wave, and some first wave ON1 variants reappeared in the second wave. An analysis of global genotype ON1 sequences determined that this genotype has become considerably diversified and has acquired signature coding mutations within immunogenic regions, and its most recent common ancestor dates to ≈2008–2009. Surveillance of genotype ON1 contributes to an understanding of the mechanisms of rapid emergence of respiratory viruses.

Human respiratory syncytial virus (RSV) is the major viral cause of bronchiolitis and pneumonia in infants and also a major cause of severe respiratory illness in the elderly (1). RSV infection usually occurs in annual epidemics, and the virus can re-infect persons throughout life. RSV isolates fall into 2 groups, A and B, and each group includes multiple genotypes. RSV epidemics are often caused by several variants of ≥1 RSV genotypes, and the dominant genotype is usually replaced each year (2). RSV's most variable protein, the attachment (G) glycoprotein, is also a target of protective antibody responses, and analysis of its encoding genome portion shows continuous accumulation

of genetic changes leading to antigenic drift (3,4). However, as a nonsegmented, single-stranded RNA virus, RSV does not show the abrupt antigenic changes that are sometimes seen in influenza A viruses. The abrupt changes in influenza A viruses commonly arise when genome segments reassort, sometimes acquiring new surface protein genes from animal sources, leading to antigenic shift as was seen in the recent influenza A(H1N1) pandemic strain (5). Nevertheless, twice in recent years, a distinct new genotype of RSV has arisen as a result of duplication within the G gene. The first of these new genotypes was detected in 1999 when 3 group B viruses with a 60-nt duplication in the C-terminal region of the G gene, which encodes strain-specific epitopes (4), were isolated in Buenos Aires, Argentina (6). This genotype was also observed in a retrospective analysis of RSV samples from 1998 to 1999 in Madrid, Spain (7). This novel genotype spread rapidly and by 2003 was being detected around the world; by 2006, it had become the predominant group B genotype (7,8).

In December 2010, a novel RSV group A genotype, ON1, with a 72-nt duplication in the C-terminal region of the G gene, was detected in Ontario, Canada (9). This genotype was also detected in Malaysia, India, and South Korea at the end of 2011 (10–12) and in Germany, Italy, South Africa, Japan, China, and Kenya in 2012 (13–15) (GenBank, unpub. data). The emergence and spread of these new genotypes, which can be readily tracked by G gene sequencing, provide an opportunity to re-examine 1) the interconnectedness of RSV epidemics at various levels (e.g., global, country, and community levels), 2) the spatial–temporal scale of the spread of variants, and 3) the pace and nature of associated genetic changes. Such examinations have the potential to bring new insights regarding how RSV persists to cause recurrent epidemics in human populations.

We conducted a detailed analysis of G gene variability of the ON1 genotype viruses detected among children inpatients at a hospital in rural Kenya in 2012. Two RSV epidemics were observed during the year, and a wave of genotype

Author Affiliations: Kenya Medical Research Institute–Wellcome Trust Research Programme, Kilifi, Kenya (C.N. Agoti, J.R. Otieno, C.W. Gitahi, D.J. Nokes); Public Health England, London, UK (P.A. Cane); and University of Warwick and WIDER, Coventry, UK (D.J. Nokes)

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ON1 cases occurred in each. We compare the phylogenetic relationship between the ON1 viruses detected in Kenya and ON1 viruses worldwide during a similar period.

Materials and Methods

Study Location and Participants

The study specimens were obtained from children <5 years of age who had been admitted with severe pneumonia to Kilifi District Hospital (KDH), Kenya, during 2012. All children were enrolled as part of an ongoing study, initiated in 2002, of the epidemiology and disease of RSV-associated pneumonia in case-patients (16–18). KDH, located in the coastal town of Kilifi, north of Mombasa, serves a rural (predominantly) and semiurban community. In this setting, epidemics of RSV disease occur on an annual basis, beginning in late October or early November of each year and continuing through June, July, or August of the next year (18).

Clinical Samples and Laboratory Methods

Since 2002, nasal wash or nasopharyngeal swab specimens have been collected from all children enrolled in the pneumonia study. The samples are tested for the presence of RSV antigen and/or nucleic acid by using the indirect fluorescence antibody test and real-time reverse transcription PCR, respectively (19). G gene sequencing is routinely undertaken on all samples from the KDH study site that have RSV-positive test results (16,20) (J.R. Otieno and colleagues, unpub. data). The ON1 genotype was first detected by this surveillance in February 2012. This report focuses on RSV A specimens collected during 2012.

Viral RNA extraction, reverse transcription, PCR amplification, and sequencing of the G gene were undertaken as described (16,20,21). The specimens were collected after informed consent was given by a parent or guardian for each child. The Kenya National Ethics Review body approved the study protocols.

Sequence Alignments and Comparison Dataset

Consensus G gene sequences for RSV A were initially aligned in MAFFT v6.884b (22) and trimmed in BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Viruses possessing the ON1 72-nt duplication were readily identifiable from the alignments. Sequences for Kilifi viruses corresponded to the terminal 702 nt and 630 nt in the G ectodomain regions of ON1 and non-ON1 viruses, respectively. A comparison dataset of ON1 genotype sequences deposited into GenBank was downloaded, collated, aligned with the Kilifi ON1 sequences, and used to derive a global phylogenetic tree. Because some sequences were limited in length, the final worldwide ON1 alignment was trimmed to include only the C-terminal G gene region over the terminal 333 nt.

Phylogenetic Analysis

Phylogenetic trees were constructed in MEGA5.2.1 (23) by using the maximum likelihood method under the general time-reversible model of evolution. The robustness of the phylogenetic clusters was evaluated by bootstrapping with 1,000 iterations. Viruses were considered to be the same variant if they were identical in nucleotide sequence over the region we sequenced. Variants were grouped into a similar lineage if they shared signature-coding mutations. The Kilifi sequences reported here are deposited in GenBank under accession numbers KF587911–KF588014.

Evolutionary Analysis

The rate of evolution of the ON1 G gene and the date of the most recent common ancestor (MRCA) of the viruses collected to date were estimated by 2 independent methods: 1) by using regression of the root-to-tip distances from the maximum-likelihood tree in Path-O-gen (<http://tree.bio.ed.ac.uk/software/pathogen/>) and 2) by using the BEAST v1.74 analysis package (<https://code.google.com/p/beast-mcmc/>), which uses the Bayesian Markov chain Monte Carlo approach (24). The analysis included only sequences for which the exact date of sampling was provided. Furthermore, to reduce the bias of oversampling from any 1 location, we included only viruses with unique nucleotide sequences in the C-terminus region in the analysis. The final data subset comprised 65 sequences from 7 countries.

The BEAST analysis was run through 50 million steps, with sampling every 2,500 steps, under the HKY model of evolution and the Bayesian skyride population growth model. Once the analysis was complete, run convergence was confirmed by using the Tracer v1.5 program (<http://tree.bio.ed.ac.uk/software/tracer/>); trees were summarized in TreeAnnotator and visualized in FigTree v1.40 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

During January 1–December 31, 2012, a total of 873 children who were admitted to KDH were eligible for the RSV surveillance study. Nasal wash or nasopharyngeal swab specimens were obtained from 834 of the children and tested for RSV. Of the 834 samples, 240 (28.8%) were RSV positive: 123 (51.3%) were group A infections, 114 (47.5%) were group B infections, and 3 (1.3%) were A/B co-infections. Of the 126 combined group A and group A/B viruses, 104 (82.5%) were successfully sequenced in the G gene ectodomain region, and of these, 77 (74.0%) possessed the ON1 genotype 72-nt duplication. The numbers of RSV A, B, and ON1 cases detected each month at KDH during 2012 are shown in Table 1. The number of cases detected each week is shown in Figure 1, panel A.

Table 1. Occurrence of RSV group A and B viruses and of genotype ON1 in Kilifi, Kenya, 2012*

| Month | No. RSV strains detected | | | | RSV group A sequencing result | | |
|------------------------|--------------------------|------------|-------------------|------------|-------------------------------|-----------|---------------|
| | Group A | Group B | Co-infected (A+B) | Total | No. (%) ON1 strains† | Non-ON1 | Not sequenced |
| First wave | | | | | | | |
| Jan | 0 | 18 | 0 | 18 | 0 | 0 | 0 |
| Feb | 6 | 23 | 0 | 29 | 1 (20.0) | 4 | 1 |
| Mar | 15 | 26 | 1 | 42 | 8 (53.3) | 7 | 1 |
| Apr | 14 | 16 | 1 | 31 | 5 (41.7) | 7 | 3 |
| May | 4 | 6 | 0 | 10 | 3 (100.0) | 0 | 1 |
| Jun | 11 | 2 | 0 | 13 | 9 (81.8) | 2 | 0 |
| Jul | 3 | 0 | 0 | 3 | 3 (100.0) | 0 | 0 |
| Aug | 2 | 0 | 0 | 2 | 2 (100.0) | 0 | 0 |
| Non-wave period | | | | | | | |
| Sep | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Second wave | | | | | | | |
| Oct | 7 | 1 | 0 | 8 | 4 (66.7) | 2 | 1 |
| Nov | 38 | 16 | 1 | 55 | 31 (91.2) | 3 | 5 |
| Dec‡ | 23 | 5 | 0 | 28 | 11 (84.6) | 2 | 10 |
| Total | 123 | 114 | 3 | 240 | 77 (74.0) | 27 | 21 |

*The calendar year 2012 spans parts of 2 epidemics: the 2011–12 epidemic, which occurred during October 2011–August 2012 (peak March 2012), and the 2012–13 epidemic, which occurred during October 2012–July 2013 (peak November 2012). Group B RSVs predominated during the 2011–12 epidemic (65.0%), and group A RSVs predominated during the 2012–13 epidemic (79.28%). RSV, respiratory syncytial virus.

†Percentages represent the proportion of ON1 viruses among RSV A sequenced samples from each month.

‡Because of local logistics problems, samples were not collected from all the subjects that were eligible in December.

Two Waves of RSV Genotype ON1 Infections

The ON1 genotype was first detected in Kilifi in February 2012, which was about the middle of the 2011–12 RSV epidemic. The ON1 viruses continued to be detected along with the other group A viruses through June 2012; in July and August, ON1 was the only RSV A genotype

detected, marking the end of the first wave (Table 1). Overall, however, the full 2011–12 RSV epidemic was dominated by group B viruses (65.0%), which co-circulated with the group A genotypes during the epidemic (Table 1, including footnotes; Figure 1, panel A). During the first 8 months of 2012, a total of 31 ON1 infections were

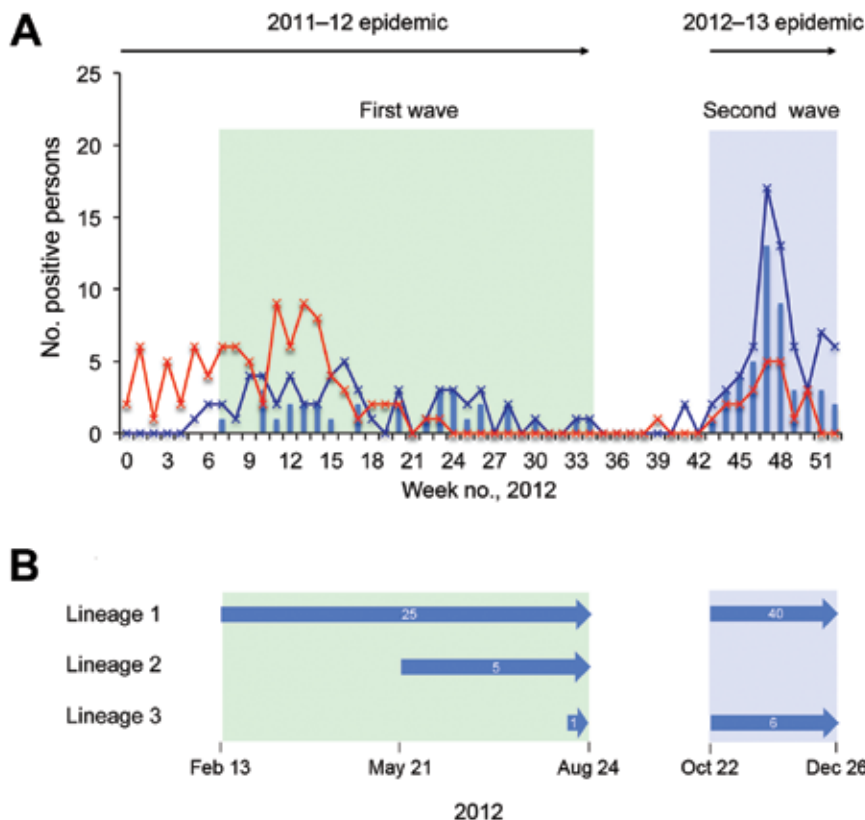


Figure 1. A) Number of persons positive for respiratory syncytial virus (RSV) genotype ON1 during 2 infection waves, Kilifi, Kenya, 2012. Blue line indicates cases of RSV group A infection; red line indicates cases of RSV group B infection; bars indicate number of ON1 case-patients admitted to Kilifi District Hospital during January 1–December 31, 2012. The first ON1 infection wave (green shading) overlapped with a 2011–12 RSV epidemic, and the second ON1 infection wave (blue shading) overlapped with a 2012–13 RSV epidemic (see arrows above graph). B) Lifespans of the main ON1 lineages observed at Kilifi. Numbers inside arrows indicate the number of sequences for the lineage named at the far left.

detected, representing 20.9% (31/148) of the overall RSV diagnoses and 60.8% (31/51) of the group A diagnoses with known ON1 status (Table 1).

The second wave of ON1 infections in Kilifi started in October 2012 at the beginning of the 2012–13 RSV epidemic, and detection continued up to the last month covered by the surveillance reported here (December 2012) (Table 1). During those 3 months, more cases of group A (75.6%, 68/90) than group B RSV were detected, and the ON1 genotype constituted the majority of the RSV A viruses (86.8%, 46/53) among those successfully sequenced (Table 1). Overall, more ON1 cases were recorded each week during the second infection wave than during the first wave (Figure 1, panel A), and the genotype appeared to

predominate the other RSV A genotypes during the second wave (Table 1).

Genetic Variability of ON1 Viruses from the 2 Infection Waves

Of the 77 sequenced ON1 viruses, 25 unique nucleotide sequences were identified across the 702 nt-long G gene region: 8 were found only in the first infection wave, 14 were found only in the second wave, and 3 were found in both waves. Phylogenetic analysis of the G gene region of these ON1 genotype Kilifi viruses identified 3 main lineages circulating in Kilifi. The 3 lineages comprised multiple phylogenetic clusters and several singleton sequences (Figure 1, panel B; Figure 2, panel A). Lineage 1 was the

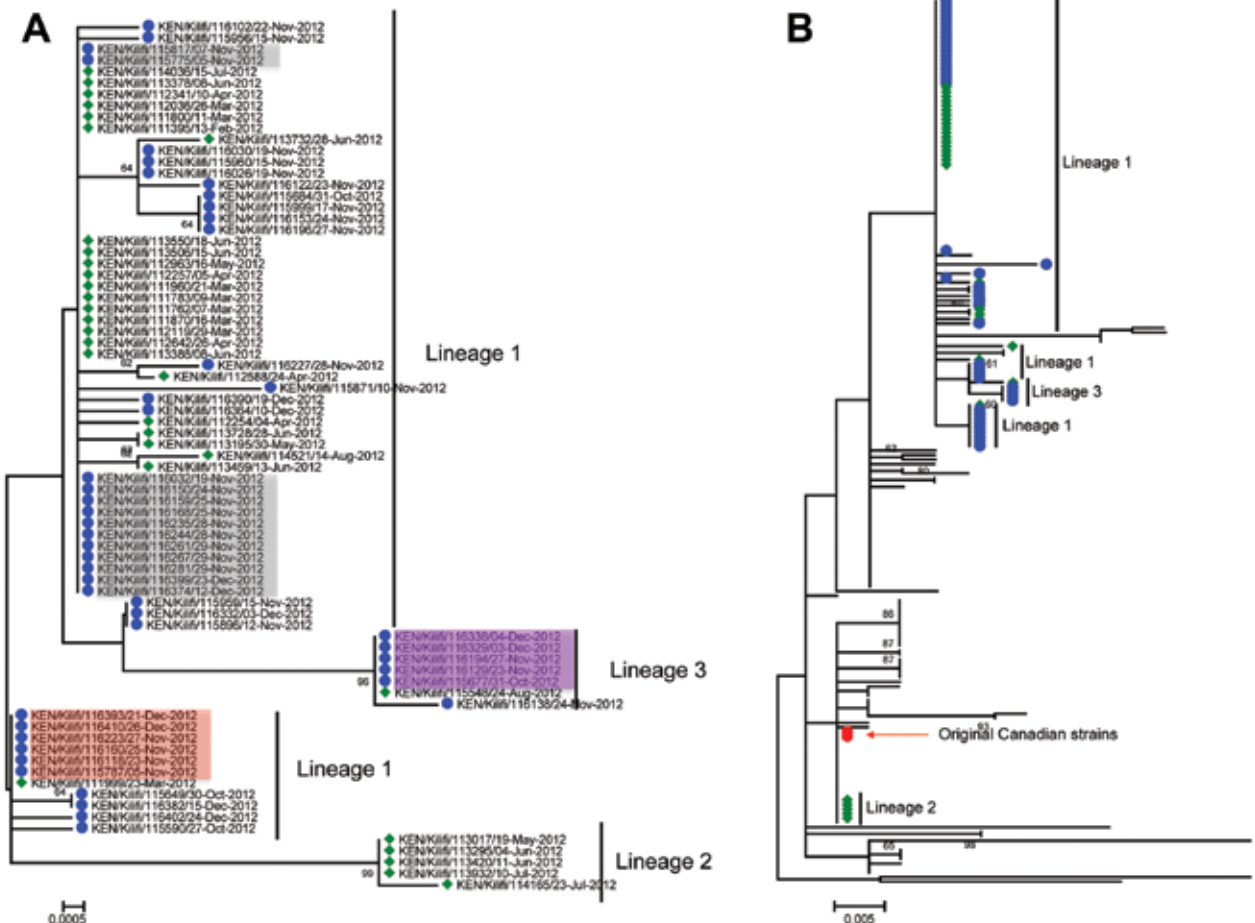


Figure 2. Phylogeny of respiratory syncytial virus genotype ON1 viruses detected globally and from Kilifi, Kenya. A) Maximum-likelihood, nucleotide-based phylogenetic tree showing the evolutionary relationships of the 77 Kilifi ON1 viruses across the sequenced portion (702 nt-long) of the attachment (G) protein gene. The taxon nomenclature on the tree is as follows: A 3-letter code representing country of isolation/(location within country of isolation, if provided)/GenBank accession number (or identification for Kilifi sequences)/date of isolation. Kilifi viruses identified during the first infection wave are preceded by a green diamond; those identified during the second infection wave are preceded by a blue circle. Highlighted names (i.e., red, gray, and purple, indicating viruses by taxa) had sequences identical to those for viruses from the first ON1 infection wave. B) Maximum-likelihood, nucleotide-based phylogenetic tree showing the evolutionary backbone structure of 118 global ON1 viruses sequences, together with the 77 Kilifi ON1 viruses sequences across the sequenced portion (333 nt long) of the G third C-terminus region. The positions of the Kilifi viruses from the first infection wave are indicated by a green diamond, those from the second wave are indicated by a blue circle; the red circle indicates the position of the original Canadian ON1 viruses. On both trees, only bootstrap support values >60 are shown on the branches. Scale bars indicate nucleotide substitutions per site.

first to be identified and included most (84.4%, 65/77) of the Kilifi ON1 viruses. The sequences of this lineage did not fall into a single cluster, but they were closely related and shared signature mutations (Table 2; online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/6/13-1438-Techapp1.pdf). Lineages 2 (5 viruses) and 3 (7 viruses) were assigned from the 2 phylogenetic clusters that were prominent from the rest of the Kilifi ON1 sequences and on well-supported branches (bootstrap >90%). These lineages most likely represented 2 independent introductions of the ON1 genotype into the Kilifi community during the first infection wave. It is also possible that lineage 1 was introduced multiple times, which would explain its sequence diversity (shown by the multiple small clusters and the singleton sequences). Figure 2, panel B, shows how the 3 Kilifi lineages fit into the global picture on phylogenetic analysis of the C-terminal third region of all ON1 sequences detected throughout the world to date (see detail below).

Persistence of ON1 Variants between Infection Waves

G nucleotide sequences for 24 of 46 ON1 viruses sequenced from the second infection wave were identical to sequences of 3 variants from the first wave (Figures 1, panel B; Figure 2, panel A), suggesting possible sustained transmission of these variants in the community through the interepidemic trough. Two of these first-wave variants

were from lineage 1, and the third was from lineage 3. Phylogenetic analysis of viruses from the first and second infection waves (Figure 2, panel A) showed that lineage 1 and 3 viruses were detected during both waves, but lineage 2 was detected only during the first wave (Figure 1, panel B). These persisting first-wave variants were initially detected on February 13, March 23, and August 24, 2012, and they were still being detected in December 2012 (during the second infection wave), the last month of the surveillance reported here.

Genetic Variability of ON1 Viruses Identified Globally

To evaluate the global genetic variability of the ON1 genotype, we combined the G gene sequences from the 77 ON1 viruses from Kenya with 118 ON1 G gene sequences in GenBank from 9 other countries; the GenBank sequences represented all ON1 sequences available as of September 8, 2013. The phylogenetic relationships of these combined ON1 sequences in the G gene are shown in Figure 2, panel B, and in Technical Appendix Figure 2 (taxon names provided). Sequences for 72 of the 77 ON1 viruses from Kenya (i.e., all lineage 1 and 3 sequences from the Kilifi data) fell into 1 branch of the global phylogeny. The bootstrap support value for the 72 sequences was low, indicating the presence of just a few unique substitutions. This branch also included 20 viruses from Japan, 2 viruses from

Table 2. Signature codon changes in circulating global RSV group A genotype ON1 viruses*

| Signature change† | First detected | No. viruses with mutation | No. viruses compared‡ | Detection location(s) | Comment(s) |
|-------------------|----------------|---------------------------|-----------------------|-------------------------------------|---|
| P88S | 2012 Nov | 3 | 7 | China | Occur within the mucin-like first hypervariable region; potentially O-glycosylated (4) |
| L115P | 2012 Aug | 10 | 84 | China, Kenya | Defines Kenyan RSV lineage 3; occurs within the mucin-like first hypervariable region; potentially O-glycosylated (4) |
| S128F | 2012 May | 5 | 84 | Kenya | Defines Kenya RSV lineage 2 |
| T136I | 2012 Feb | 54 | 111 | Kenya | Defines Kenyan RSV lineage 1 |
| P172A | 2012 Dec | 9 | 150 | Italy | None |
| P206Q | 2011 | 5 | 162 | India, Italy, Japan | None |
| T249N | 2012 Jan | 5 | 195 | Italy | None |
| L274P | 2011 | 131 | 195 | India, Italy, Germany, Japan, Kenya | Predicted to be positively selected; exhibits reversible amino acid substitutions (i.e., flip-flop pattern) (25); position N-glycosylated in some strains |
| H290Y | 2012 Feb | 5 | 195 | Italy | Predicted to be positively selected; exhibits flip-flop pattern (25) |
| G296S | 2012 Dec | 11 | 195 | Italy | Adjacent codon (i.e., 295) predicted to be positively selected (26) |
| L298P | 2011 | 133 | 195 | India, Italy, Germany, Japan, Kenya | Duplicated epitope; concurrent substitution with L274P in many sequences |
| Y304H | 2011 | 129 | 195 | India, Italy, Germany, Japan, Kenya | None |
| E308K | 2012 Feb | 13 | 195 | Italy, South Africa, China | None |
| L310P | 2011 | 96 | 195 | Italy, Kenya, Japan, India | Equivalent to L286P in non-ON1 genotypes; change previously observed in certain monoclonal antibody escape mutants (27) |

*RSV, respiratory syncytial virus.

†Only substitutions observed in >3 viruses are shown.

‡Numbers in this column indicate the number of sequences that span the given amino acid position; the number 195 represents all the datasets we collated, including the sequences for viruses from Kilifi, Kenya. The numbers differ because the nucleotide sequences found in GenBank for these viruses were of variable lengths.

Italy, and the 1 virus from India (Technical Appendix Figure 2). Of the 20 Japanese viruses within this branch, 12 were identical to 1 of the Kilifi variants that appeared in the first infection wave and persisted into the second wave. The ON1 Kilifi lineage that fell outside this main Kenyan branch (i.e., lineage 2) clustered with the original ON1 viruses from Canada together with ON1 viruses from Germany, Malaysia, and Italy (Figure 2, panel B).

The reconstruction of a phylogenetic tree combining all the global ON1 sequences, from which the duplicated region had been excised, and the non-ON1 sequences detected in Kilifi in 2012 showed that the global ON1 sequences, including those from Kilifi, form a monophyletic cluster away from sequences of non-ON1 viruses. This finding reaffirmed that the Kilifi viruses with the duplication (ON1) did not arise *de novo* locally (data not shown).

The global geographic locations for which ON1 sequences were available in GenBank as of September 8, 2013, and the number of sequences present by country

are shown in Figure 3, panels A and B. The temporal patterns for the detections of the ON1 viruses in Kilifi and in the GenBank dataset are consistent with 2 ON1 infection waves in 2012; the genotype was rare in 2011, despite first being detected in 2010 (Figure 3, panel C).

Signature Amino Acid Substitutions

Several nonsynonymous substitutions were predicted in the first and second hypervariable regions of the ON1 G protein. Signature coding mutations that were observed in ≥ 3 viruses are summarized in Table 2, and the amino acid alignment of the deduced G protein C-terminus region from the unique nucleotide sequences among the combined Kenyan ON1 and GenBank collated dataset is shown in Figure 4. Several of the changes within the second hypervariable G region had occurred on codon positions (relative to the prototype strain RSV A2) previously predicted to be positively selected: codons 225, 226, 246, 248, 249, 253, 256, 262, 265, 272, 274–276, 280, and 284–286 (25,28). Four

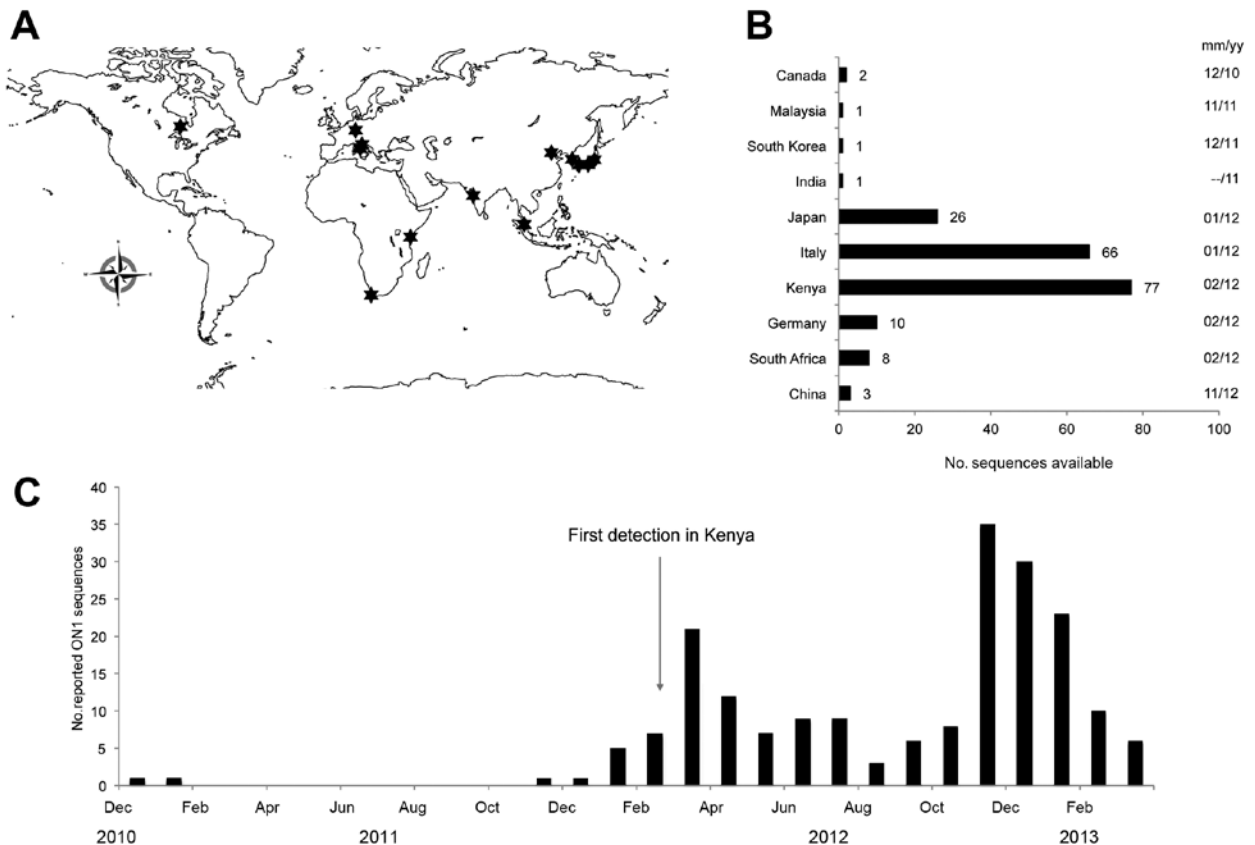


Figure 3. A) Geographic locations (indicated by stars) where respiratory syncytial virus genotype ON1 sequences had been detected and reported as of September 8, 2013. B) Number of ON1 sequences present in GenBank as of September 8, 2013, including sequences submitted for the viruses detected in Kilifi, Kenya, during 2012. The month and year that the first ON1 virus was reported for each country are given on the far right. C) Monthly reported detections of the ON1 viruses with sequences from the combined worldwide dataset. The month of first ON1 detection at Kilifi is indicated by an arrow. Note that the data presented in this figure have not been systematically collected to represent the geographic and temporal distribution of the ON1 genotype. Instead, the data are derived from sequence submissions to GenBank, with inherent sampling bias, that do not necessarily reflect the total number of cases from the different locations; the submissions do, however, indicate the rapid spread of the genotype.

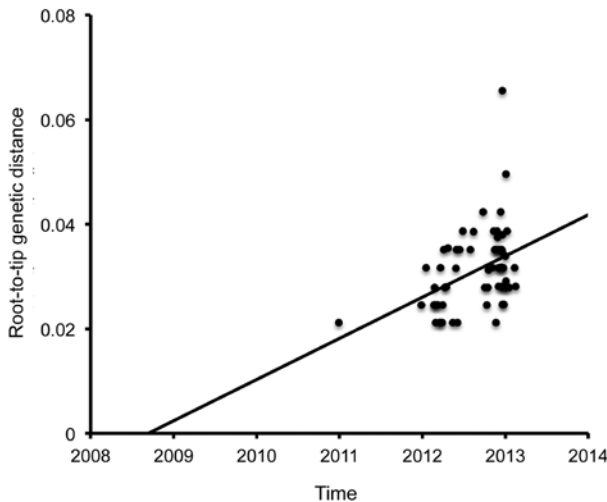


Figure 5. Most recent common ancestor analysis of the 65 respiratory syncytial virus genotype ON1 viruses in GenBank and the sequences for ON1 viruses detected in Kilifi, Kenya, during 2012. The analysis was done by root-to-tip regression of the genetic distances from the maximum-likelihood tree.

which also possesses a large nucleotide duplication, closely relate to findings regarding the BA genotype and confirm that novel RSV strains do spread rapidly and widely.

Of particular interest is the observation that within a few years of the generation of these new genotypes, variants have evolved with accumulated signature coding changes in regions of the G protein targeted by the neutralizing antibody response. For example, a previously identified putative epitope around codon 274 was duplicated in the ON1 genotype, and some of the emerging variants have shown amino acid changes in both copies of this epitope (Figure 4). This suggests that such ON1 variants were selected by the change on this particular epitope. Furthermore, in many ON1 variants, additional coding changes are observed at codons previously predicted to be positively selected (25,26,28), at potential *N*-glycosylation codons (4), and at positions previously reported in escape mutants from certain monoclonal antibodies (e.g., L310P, which in viruses without the duplication will be equivalent to L286P) (27). The P286L change has been associated with abrogation of reaction of peptides to convalescent-phase human serum (31). Thus it may be deduced that the amino acid changes already observed in the ON1 variants have led to profound differences in its antigenic profile.

The presence of the multiple ON1 genotype lineages in Kilifi, some occurring as multiple phylogenetic clusters or several distinct sequences, suggests that there have been multiple introductions of the genotype into the region. Some of these ON1 viruses have partial G gene sequences identical to the few ON1 genotype GenBank sequences available from around the world, indicating that

ON1 variants that arose soon after its emergence are also quickly spreading worldwide. We also detected identical ON1 G gene sequences for viruses from the 2 RSV infection waves in Kilifi, which could reflect continued local transmission of the first-wave viruses or further new introductions of identical viruses of the genotype into the community during the second wave.

MRCA analysis determined that ON1 first emerged in 2008 or 2009. This would suggest that the variant was circulating undetected before December 2010, and its first location of occurrence may remain unknown. This estimate of the time between first occurrence and detection is shorter than the ≈ 12 years proposed by Tsukagoshi et al. (13). Overall, by both the maximum likelihood and Bayesian methods, we estimated that this variant had a higher rate of evolution in its C-terminus region (point estimates of $>5.0 \times 10^{-3}$ nucleotide substitutions per site per year) than previously predicted for RSV A (3.382×10^{-3} [95% HPD interval 1.911×10^{-3} to 4.954×10^{-3}]) (32). This finding suggested an accelerated evolution rate in this variant early in its lifetime; however, our estimates have wide intervals, and it should be noted that viruses with G gene nucleotide sequences nearly identical to those of the original Canadian ON1 viruses were co-circulating with the diversified viruses at least up to late in 2012.

Our RSV ON1 genotype analyses were limited to the G ectodomain region (≈ 700 nt), and analyses for the global data were limited to the G C-terminus region (≈ 330 nt). Whole-genome comparison of 2 ON1 sequences currently available in GenBank (accession nos. JX627336/2011 [12] and KC731482/2011 [11]) showed up to 57 nt differences, a substantial proportion of which occurred beyond the G protein gene region (data not shown). Thus, to better understand the local and global molecular epidemiology and phylogeography of the ON1 genotype, whole-genome sequences must be compared. To that end, we are currently conducting whole-genome sequencing of the ON1 viruses from Kilifi.

The phylodynamics of these emergent RSV genotypes with large nucleotide duplications in the G gene (i.e., the BA and ON1) enable parallels to be drawn with pandemic influenza viruses arising from antigenic shift. The cause of the apparent enhanced biologic fitness of the BA and ON1 genotypes (whether virologic or immunologic) is not well understood. Since around 2005, the BA genotype has dominated all other group B genotypes. We cannot tell if the ON1 genotype will also eventually dominate other group A genotypes. However, during the preparation of this report, the detection of RSV genotype ON1 was reported in 3 more countries: Thailand (33), Latvia (34), and Cyprus (35). Thus the prevalence and geographic distribution of ON1 is rapidly changing. The monitoring of this change will lead to a better understanding

of the factors underlying the successful emergence of variant genotypes and help inform future methods for the control of RSV.

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Mr Agoti is a research assistant in the Viral Epidemiology and Control group, Kenya Medical Research Institute–Wellcome Trust Research Programme, and a PhD student. His primary interest is to inform epidemic control strategies by applying phylogenetic and bioinformatics tools to understand the evolution and origins of respiratory viruses and relationships between viral epidemics.

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Address for correspondence: Charles N. Agoti, KEMRI–Wellcome Trust Research Programme, PO Box 230, Kilifi 80108, Kenya; email: cnyaigoti@kemri-wellcome.org

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Bats as Reservoir Hosts of Human Bacterial Pathogen, *Bartonella mayotimonensis*

Ville Veikkolainen,¹ Eero J. Vesterinen,¹ Thomas M. Lilley, and Arto T. Pulliainen

A plethora of pathogenic viruses colonize bats. However, bat bacterial flora and its zoonotic threat remain ill defined. In a study initially conducted as a quantitative metagenomic analysis of the fecal bacterial flora of the Daubenton's bat in Finland, we unexpectedly detected DNA of several hemotrophic and ectoparasite-transmitted bacterial genera, including *Bartonella*. *Bartonella* spp. also were either detected or isolated from the peripheral blood of Daubenton's, northern, and whiskered bats and were detected in the ectoparasites of Daubenton's, northern, and Brandt's bats. The blood isolates belong to the *Candidatus*-status species *B. mayotimonensis*, a recently identified etiologic agent of endocarditis in humans, and a new *Bartonella* species (*B. naantaliensis* sp. nov.). Phylogenetic analysis of bat-colonizing *Bartonella* spp. throughout the world demonstrates a distinct *B. mayotimonensis* cluster in the Northern Hemisphere. The findings of this field study highlight bats as potent reservoirs of human bacterial pathogens.

The 1,100 species of bats (*I*) constitute ≈20% of known mammalian species and are outnumbered only by animals in the order Rodentia. Bats play a vital role in natural ecosystems in arthropod suppression, seed dispersal, and pollination. Modern-day economies also benefit from these voracious predators of crop and forest pests (2). However, bats have been implicated as reservoir hosts for viral human pathogens, such as paramyxoviruses (3) and rabies virus and related lyssaviruses (4). Compelling evidence also indicates that bats carry asymptotically some of the most deadly viruses, including Marburg (5) and Ebola (6) viruses. Whether bats carry clinically significant bacterial pathogens is unknown.

Author affiliations: University of Turku, Turku, Finland (V. Veikkolainen, E.J. Vesterinen, T.M. Lilley, A.T. Pulliainen); and University of Helsinki, Helsinki, Finland (A.T. Pulliainen)

The development of next-generation sequencing techniques has revolutionized biological science. It is now possible—and cost-friendly—to gain access to massive amounts of qualitative and quantitative sequencing data in a short time without a priori knowledge of the sequence (7). Most bacteria do not grow on laboratory media, and next-generation sequencing technologies have proven useful for studying bacterial species diversity and dynamics, even in complex systems like the gut (8). Our initial objective in 2010 and 2011 was to conduct a quantitative metagenomic analysis of the fecal bacterial flora of the Daubenton's bat (*Myotis daubentonii*) in Finland. Unexpectedly, we found that the fecal material contained DNA of several hemotrophic and ectoparasite-transmitted bacterial genera, such as *Bartonella*. This DNA may originate either from bleeding into the intestine or from the insect prey of the bats that includes the abundant bloodfeeding bat ectoparasites. Therefore, the study further focused on detecting and isolating *Bartonella* spp. from peripheral blood and ectoparasites of several bat species in Finland in 2012.

Materials and Methods

Bartonella spp. nucleotide sequences have been deposited in GenBank under accession nos. KF003115–KF003145. The metagenomic reads are stored at the National Center for Biotechnology Information Sequence Read Archive under BioProject SRP023235 (accession nos. experiment: SRX286839, run: SRR868695). We have described the detailed protocols, including bat sampling for peripheral blood, fecal droppings, and ectoparasites; metagenomic analysis of fecal DNA; isolation of *Bartonella* from peripheral blood; extraction of DNA from bat blood, ectoparasites, and *Bartonella* isolates; *Bartonella* and ectoparasite PCR analyses; transmission electron

microscopy; and nucleotide sequence and phylogenetic analyses in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/6/13-0956-Techapp1.pdf).

Results

Quantitative Metagenomic Analysis of DNA from Bat Feces

We obtained ≈200,000 high-quality sequences (average length 167 bp) from DNA sequencing of fecal material from a Daubenton's bat (online Technical Appendix Figure 1). Sequences (≥50 bp) were assigned on the basis of best E-value BLASTN scores (<http://blast.ncbi.nlm.nih.gov/blast.cgi>) in GenBank. The most abundant non-metazoan sequence matches were with bacteria. The genera *Leucostoc*, *Enterobacter*, *Lactococcus*, and *Chlamydia* dominated (Figure 1). Surprisingly, the fecal material also contained DNA of the ectoparasite-transmitted genera, such as the hemotrophic bartonellae (9). It was thought that this DNA originated either from bleeding into the intestine or from the insect prey of the bats that includes the abundant bloodfeeding bat ectoparasites. PCR verified the presence of *Bartonella* DNA in the bat fecal material. The transfer messenger RNA gene (*ssrA*) (10) could be amplified and was sequenced from the fecal material of 1 Daubenton's bat, 1 northern bat (*Eptesicus nilssonii*), and 1 Brandt's bat (*Myotis brandtii*) (no. 2771, no. 2788, and no. 2786, respectively; online Technical Appendix Table 1). The obtained 218-bp *ssrA* sequences were 100% identical. The closest matches in GenBank, with a similarity score of 94.8% (183/193 bp), were *B. tamiae* Th339 (GenBank accession no. JN029780) and Th307 strains (GenBank accession no. JN029778) isolated from 2 humans in Thailand (11).

Candidatus Status Species *B. mayotimonensis* and Novel *Bartonella* Species

Bats belonging to the 4 most prevalent bat species in Finland were captured in August and September 2012 at 3 locations in southwestern Finland (online Technical Appendix Table 1). Culturing of peripheral blood samples of 5 Daubenton's bats and 1 northern bat yielded distinct colonies. The isolates were identified as *Bartonella* spp. by sequencing a PCR-amplified 485-bp fragment containing the hypervariable regions V6–V8 of the 16S rRNA gene. Overall health of the bats as analyzed by body condition indexing was not affected by the *Bartonella* infection (online Technical Appendix Table 1).

The 16S rRNA gene sequences are highly conserved within the genus *Bartonella* and thus not robust in differentiating species (12). Therefore, we sequenced PCR-amplified fragments of the RNA polymerase β -subunit gene (*rpoB*), citrate synthase gene (*gltA*), filamenting temperature-sensitive mutant Z gene (*ftsZ*), VirB type IV secretion system VirB4

component gene (*virB4*), hypervariable region 2 of the 16S-23S rRNA intergenic spacer region (ISR), and *ssrA* (online Technical Appendix Table 2). Sequencing of *rpoB* was first conducted on all 28 clonal isolates. Three distinct *rpoB* alleles were identified (online Technical Appendix Table 1). The multilocus sequence analysis (MLSA) was completed on 1 *rpoB*-1 allele isolate (clone 3, bat no. 1157, referred to hereafter as 1157/3), 1 *rpoB*-2 allele isolate (clone 1, bat no. 2574, referred to hereafter as 2574/1), and 1 *rpoB*-3 allele isolate (clone 1, bat no. 1160, referred to hereafter as 1160/1). Thin-section transmission electron micrographs of these isolates are shown in the online Technical Appendix Figure 2. No major pili or fimbriae-like structures were detected on the surface of the rod-shaped bacteria.

Results of BLASTN homology searches performed in January 2013 are shown in online Technical Appendix Table 3. ISR is a robust species discriminatory marker within the genus *Bartonella* (13,14). ISR of the strain 2574/1 did not have any hits, whereas ISR of strains 1157/3 and 1160/1 had only 1 hit in GenBank *Candidatus* *B. mayotimonensis* (15), with high sequence similarity scores. Sequence analyses of the other MLSA markers (online Technical Appendix Table 3) further indicate that isolates 1157/3 and 1160/1 belong to the *Candidatus*-status species *B. mayotimonensis* and that strain 2574/1 belongs to a new *Bartonella* species. Indeed, the lowest pairwise genetic distance values with the concatenated *rpoB*, *gltA*, 16S rRNA, and *ftsZ* sequence fragments of the bat strains 1157/3 and 1160/1 in the genus *Bartonella* were 0.040 and 0.038, respectively, with *Candidatus* *B. mayotimonensis* (online Technical Appendix Table 4). Because the distance value 0.05 is the recommended cutoff value for species delineation (16), the bat isolates 1157/3 and 1160/1 classify as strains of the *Candidatus*-status species *B. mayotimonensis*. The bat strain 2574/1 belongs to a new *Bartonella* species because the lowest genetic distance value in the genus *Bartonella* was 0.070 with *B. washoensis*, above the 0.05 cutoff value (16).

Figure 2 shows the phylogenetic position of the bat *Bartonella* isolates based on comparisons of concatenated sequences of *rpoB*, *gltA*, 16S rRNA and *ftsZ*, available for *Candidatus* *B. mayotimonensis* (15) and all type strains of the *Bartonella* species (online Technical Appendix Table 5). The neighbor-joining and maximum-likelihood trees demonstrate that bat isolates 1157/3 and 1160/1 cluster with *Candidatus* *B. mayotimonensis* with high bootstrap values in a distinct phylogenetic position. The new *Bartonella* species (strain 2574/1) clearly diverges from the other bat isolates.

Bat Ectoparasite Flies and Fleas as Vectors for Transmitting *Bartonella*

We sequenced a PCR-amplified fragment of the mitochondrial cytochrome c oxidase subunit I (17) and also

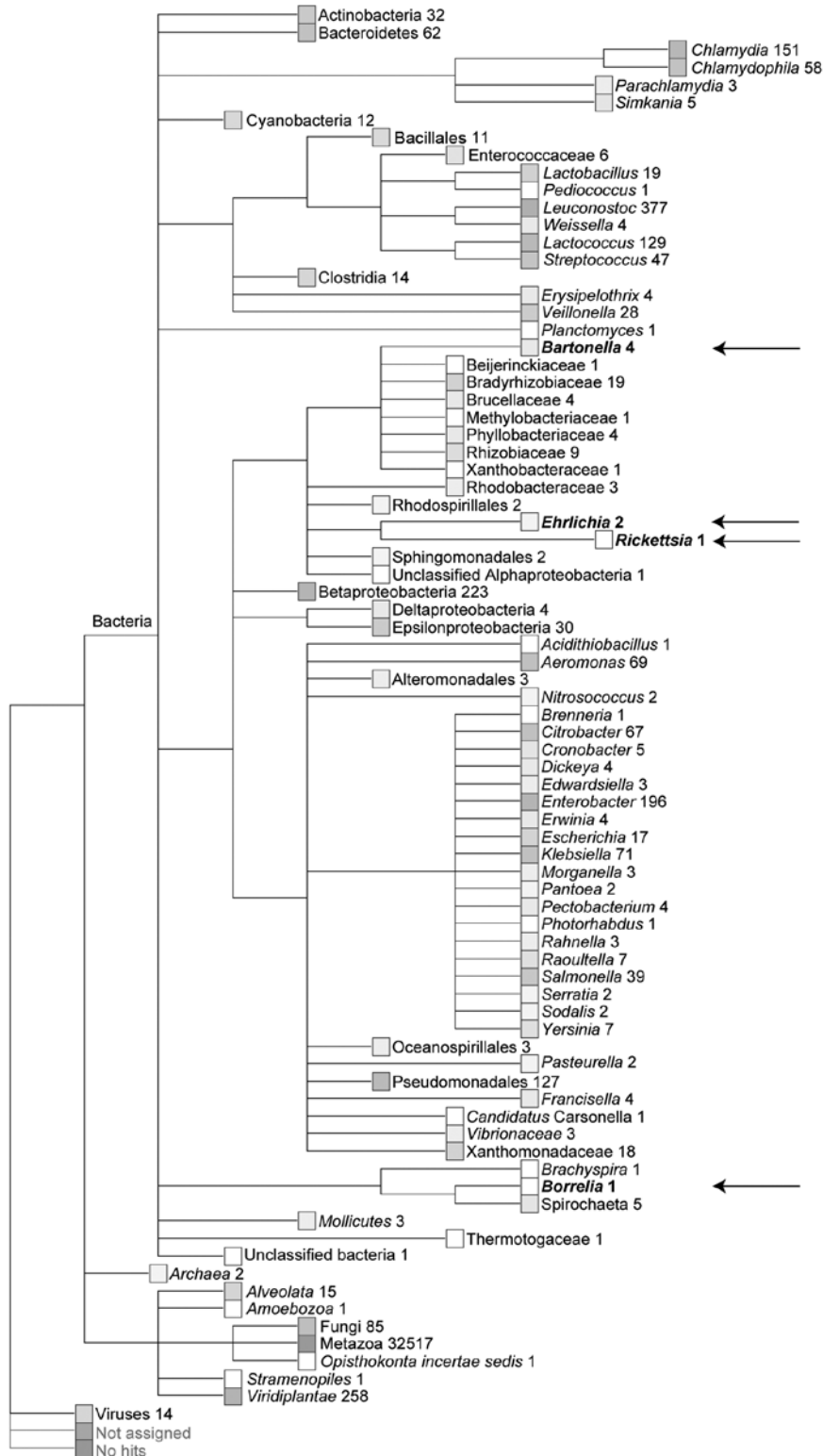


Figure 1. Quantitative metagenomic analysis of the fecal DNA of the Daubenton's bat. The sequences (≥ 50 bp) were assigned on the basis of best E-value BLASTN scores (<http://blast.ncbi.nlm.nih.gov/blast.cgi>) in GenBank. Numbers refer to the amount of sequences assigned to a given taxon. No hits refers to sequences that had no similarity to any sequences in GenBank. Not assigned refers to sequences that had similarity in GenBank but they could not be reliably assigned to any organism. Arrows mark the ectoparasite-transmitted bacterial genera, which unexpectedly were detected in the bat fecal DNA preparation.

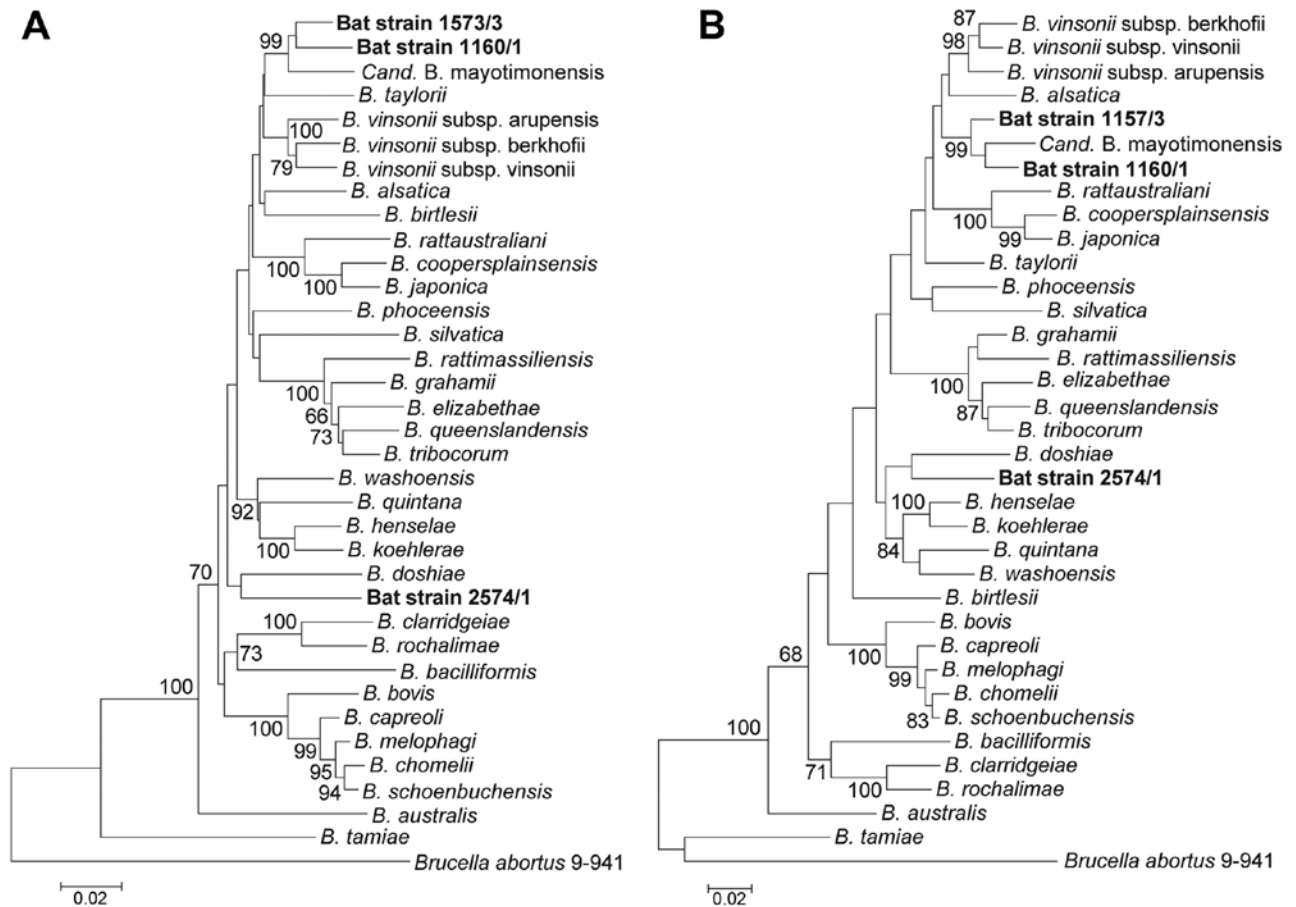


Figure 2. Phylogenetic positions of the bat blood isolates among members of the genus *Bartonella*. Neighbor-joining (A) and maximum-likelihood (B) trees are based on the alignment of concatenated sequences of 4 multilocus sequence analysis markers (*rpoB*, *gltA*, 16S rRNA, and *ftsZ*). Sequence information from the type strains of all known *Bartonella* species and from the *Candidatus* *B. mayotimonensis* human strain was included into the analysis (online Technical Appendix Table 5, wwwnc.cdc.gov/EID/article/20/6/13-0956-Techapp1.pdf). Numbers on branches indicate bootstrap support values derived from 1,000 tree replicas. Bootstrap values >60 are shown. Scale bars indicate nucleotide substitutions per site.

used visual inspection to identify the ectoparasites of 18 bats (online Technical Appendix Table 1). Ectoparasite DNA preparations of 2 fleas and 10 flies were analyzed with a PCR protocol targeting the *Bartonella rpoB*. The blood isolate *rpoB* alleles 1 and 2 were detected in samples from 1 flea and 2 flies, respectively (online Technical Appendix Table 1). In addition, 2 novel *rpoB* alleles were detected. The *rpoB*-5 allele detected in a fly sample is distantly related to the currently known *Bartonella rpoB* sequences. The highest BLASTN sequence identity score with the *rpoB*-4 allele detected in a flea sample, and from 1 blood DNA preparation of a culture-negative whiskered bat (no. 1156, online Technical Appendix Table 1), was 97.8% (397/406 bp) with the corresponding fragment (FJ376736) of *Candidatus* *B. mayotimonensis*. This is a higher value than with the *rpoB*-1 and *rpoB*-2 alleles. Moreover, a partial 338-bp *gltA* fragment could

be amplified from the *rpoB*-4-positive flea sample. The highest BLASTN sequence identity score with *Candidatus* *B. mayotimonensis* was 93.2% (315/338 bp), which is higher than with the isolates 1157/3 (92.0%, 311/338 bp) and 1160/1 (92.3%, 312/338 bp). The data further support the conclusion that bats are reservoir hosts of *B. mayotimonensis* and indicate that the bat flies and fleas transmit *Bartonella* spp. to new hosts.

Phylogenetic Analysis of *Bartonella* spp. that Colonize Bats Worldwide

A maximum composite likelihood-based neighbor-joining tree (Figure 3) was constructed on the basis of 253-bp *gltA* sequences obtained from *Bartonella* that infect bats in the United Kingdom (18), Kenya (19), Guatemala (20), Taiwan (21), and Peru (22). The 5 *Bartonella*-like bacteria detected in minced heart tissues in the United

Discussion

Bartonella spp. are facultative intracellular bacteria that typically cause long-lasting hemotropic bacteremia in their mammalian reservoir hosts, such as rodents (9). The relapsing bacteremia can last weeks, months, or even years, thereby favoring transmission by bloodfeeding arthropods. In recent years, increasing numbers of *Bartonella* spp. have been implicated as zoonotic human pathogens. A frequent symptom is endocarditis, usually suspected in cases in which conventional culture-based diagnostics fail. The most prevalent endocarditis-causing species are *B. quintana* (23,24) and *B. henselae* (25), but *B. elizabethae* (26), *B. alsatica* (27), *B. koehlerae* (28), *B. vinsonii* subsp. *berkhoffii* (29), and *B. vinsonii* subsp. *arupensis* (30) also have been detected or isolated. Recently, a new type of *Bartonella* was detected in a resected aortic valve tissue of a human endocarditis patient (15). A species name, *Candidatus B. mayotimonensis* was proposed because a pure microbiological culture was not obtained. The reservoir host in nature also remained elusive. As part of a study designed to characterize the microbiome of bats, bacteria that belong to the *Candidatus*-status species *B. mayotimonensis* were either detected or isolated from peripheral blood samples and the ectoparasites of bats. In addition, a new *Bartonella* species (strain 2574/1) was isolated from the blood and detected from the ectoparasites.

The ad hoc committee to reevaluate the species definition in bacteriology has proposed that descriptions of novel species could be based solely on gene sequence analyses (31). In the current study, 6 genes, including the robust *Bartonella* spp. discriminatory marker, the ISR, were used (13,14). It is remarkable that ISR of the 2574/1 isolate did not have any hits, whereas ISRs of 1157/3 and 1160/1 isolates had only 1 hit in GenBank, *Candidatus B. mayotimonensis*. If *gltA* shares <96.0% and *rpoB* <95.4% nt sequence similarity with those of the validated species, the newly encountered *Bartonella* strain can be considered a new species (32). According to these criteria, which were proposed in 2003 when half of the currently known species were known, the bat isolate 2574/1 is a new *Bartonella* species. The bat isolates 1157/3 and 1160/1 belong to the *Candidatus*-status species *B. mayotimonensis* on the basis of the *rpoB* sequences but would belong to a new *Bartonella* species on the basis of the *gltA* sequences. Because the species classification gave contradictory results, sequence analyses of other MLSA markers and phylogenetic analyses were performed. In addition, we used 4 concatenated MLSA markers to determine pairwise genetic distance values to the known members of the genus. The bat isolate 1157/3 and 1160/1 *ftsZ* sequences had a significantly higher sequence similarity with *ftsZ* of *Candidatus B. mayotimonensis* than with any other type strain sequence. The neighbor-joining and maximum-likelihood phylogenetic

trees with the concatenated *rpoB*, *gltA*, 16S rRNA and *ftsZ* sequences both demonstrated that the bat isolates 1157/3 and 1160/1 cluster with *Candidatus B. mayotimonensis* with high bootstrap values in a distinct phylogenetic position. Moreover, the genetic distance values demonstrate that the bat isolates 1157/3 and 1160/1 classify as strains of the *Candidatus*-status species *B. mayotimonensis*. We propose that the bat isolate 1160/1 is the type strain of *B. mayotimonensis*.

Findings of the study raised an interesting question: how could *Bartonella* spp., or any other hemotropic bacterium, be transmitted from the bat into the human host? Daubenton's bats prefer to roost in abandoned woodpecker cavities and bird boxes, whereas the other bat species are often found in the attics of houses in close proximity to humans. Given that *Bartonella* spp. are hemotropic, transmission through bat bite and saliva is not considered likely. Moreover, at Turku University Central Hospital, which is responsible for a population base of 500,000, only 2 or 3 patients per year are admitted with a bat bite (J. Oksi, pers. comm.). These numbers probably reflect the frequency of bat bites in most countries of the Northern Hemisphere. We propose that fecal droppings of blood-fed bat ectoparasites might transmit *Bartonella* spp. into the human host, assisted by superficial scratching or tissue trauma of the skin. The presence of viable bacteria in feces of body lice (*Pediculus humanus*) that have been feeding on *B. quintana*-infected rabbits is well documented (33,34). Similar observations have been reported for the feces of experimentally infected cat fleas (*Ctenocephalides felis*) (35,36). Most importantly, intradermal injection of feces from fleas that had fed on a *B. henselae*-infected cat led to bacteremia in a pathogen-free cat (37). Ectoparasite bite-mediated transmission is also possible, but the bat bugs (*Cimex* spp.) known to also feed on humans were not analyzed in the current study.

The reported metagenomic analysis of bat fecal material indicates that bats are reservoir hosts for several pathogenic bacterial genera. No comprehensive study has been published on the bacterial flora of bats in light of its zoonotic threat to humans. The major research focus has been on viruses, and several deadly viruses have been detected or isolated (3–6). One of the main conclusions from these studies is that bats tolerate their deadly companions relatively well, a feature that has been discussed in the context of long evolutionary history of bats (38). Bats are also highly mobile and long-lived, ideal as pathogen reservoirs. Metagenomics-driven approaches should be continued to assess the pathogenic potential of bacteria that colonize bats.

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Dr Veikkolainen is a postdoctoral research fellow of the Pulliainen laboratory. His research interests include the dynamics of bat microbiome and bat immunology, with special emphasis on the recognition and control of bacterial colonization.

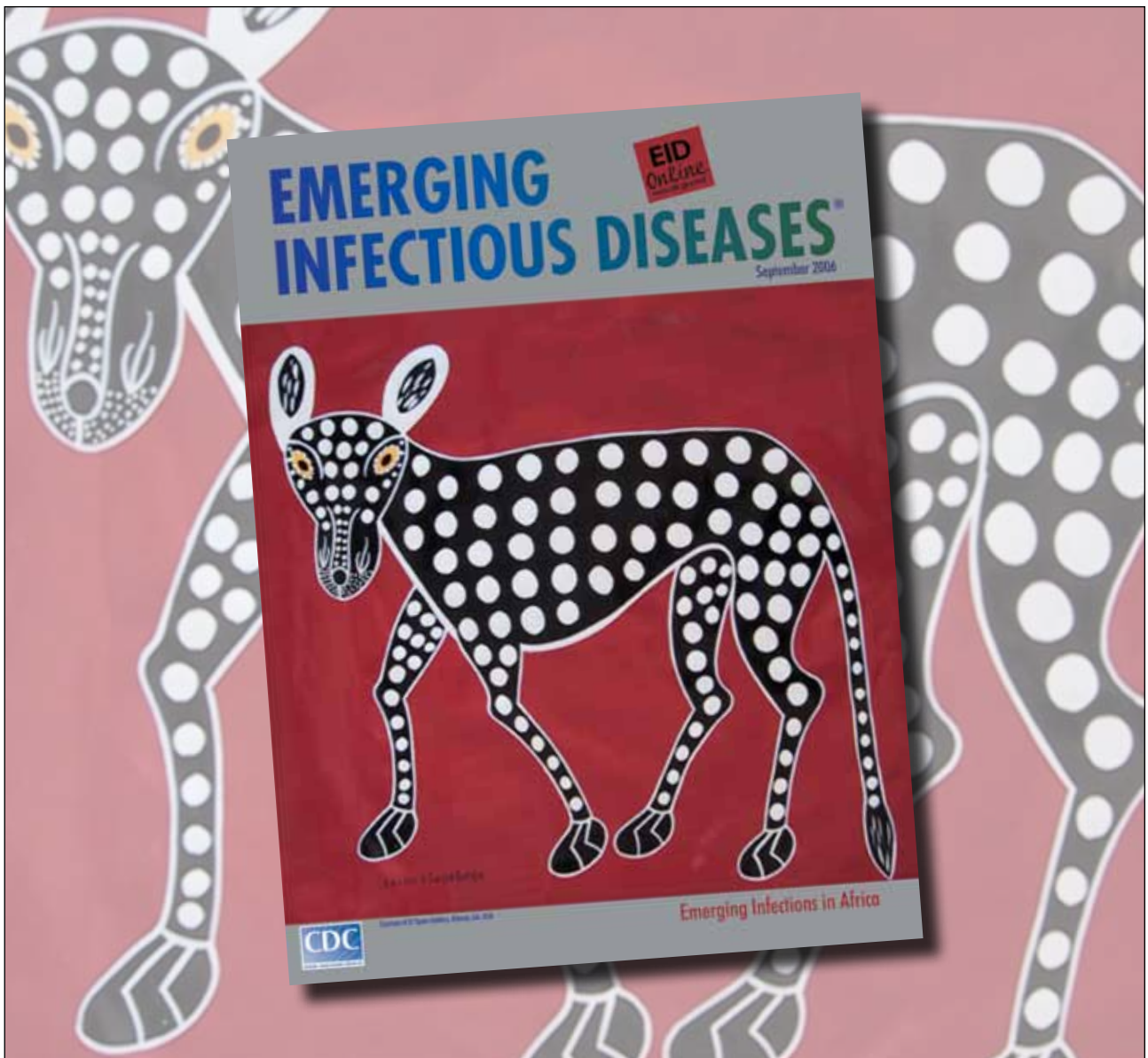
Mr Vesterinen is a PhD student at the Department of Biology, University of Turku. His research interests include food web dynamics and biological interactions, and he has established a laboratory dedicated to fecal analysis in the Laboratory of Genetics, University of Turku.

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Address for correspondence: Arto T. Pulliainen, Division of General Microbiology, Department of Biosciences, University of Helsinki, P.O. Box 56 (Viikinkaari 9), Biocenter 1, FI 00790, Helsinki, Finland, email: arto.pulliainen@helsinki.fi



Oral Fluid Testing for Pertussis, England and Wales, June 2007–August 2009

Helen Campbell, Gayatri Amirthalingam, Norman K. Fry, David Litt, Timothy G. Harrison, Karen Wagner, Natasha S. Crowcroft, and Elizabeth Miller

Existing pertussis surveillance systems tend to under-identify less severe cases among older children and adults. For routine follow-up of notified, nonconfirmed, clinically diagnosed pertussis cases, use of an oral fluid test was pilot tested in England and Wales during June 2007–August 2009. During that period, 1,852 cases of pertussis were confirmed by established laboratory methods and another 591 by oral fluid testing only. Although introduction of serologic testing in 2002 led to the greatest increase in ascertainment of pertussis, oral fluid testing increased laboratory ascertainment by 32% overall; maximal increase (124%) occurred among children 5–9 years of age. Patients whose pertussis was confirmed by oral fluid testing were least likely to be hospitalized, suggesting that milder community cases were being confirmed by this method. Oral fluid testing is an easily administered, noninvasive surveillance tool that could further our understanding of pertussis epidemiology and thereby contribute to decisions on vaccination strategies.

Existing surveillance systems for pertussis are incomplete and tend to be biased toward identifying severe cases in infants (1,2), as reflected by extremely high reported incidence for this age group (3–5). Underascertainment of cases in older patients is well recognized because of a combination of factors, including reduced likelihood that patients with milder symptoms will seek health care, underdiagnosis for patients who do seek health care, and underreporting (6–9). Although these persons usually experience milder disease, often without classic signs and symptoms, some become substantially ill (9) and can still infect vulnerable infants.

Author affiliations: Public Health England, Colindale, London, UK (H. Campbell, G. Amirthalingam, N.K. Fry, D. Litt, T.G. Harrison, K. Wagner, E. Miller); and Public Health Ontario, Toronto, Ontario, Canada (N.S. Crowcroft)

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Additional test methods that provide adequate sensitivity and specificity and that are acceptable to health professionals and patients could therefore improve ascertainment of pertussis and provide data that are more representative of disease within the population.

Since the early 1960s, *Bordetella pertussis* has been isolated from the nasopharynx by use of conventional microbiological techniques. However, culture requires that a specimen be collected early in the illness and might lack sensitivity because the organism is delicate and any delay in processing specimens can reduce the probability of isolation (10). Isolation of the organism is more difficult if the patient has been vaccinated, if the patient has received antimicrobial drugs, and if too much time has gone by since onset of cough. PCR testing for the presence of *B. pertussis* DNA in nasopharyngeal samples is more sensitive than culture because the organism does not need to be viable (11). PCR sensitivity, however, decreases substantially with increasing patient age and duration of symptoms (12). Serologic testing has been established as a diagnostic method complementary to PCR, and recommendations by European Union reference laboratories for such assays have been described (13). Serologic testing is used in at least 20 European countries (14) (including the United Kingdom since 2002), Japan, and Australia to diagnose infection in patients who have been coughing for at least 2 weeks, when culture and PCR are less likely to yield positive results (10) as has been demonstrated in certain studies (15). Serologic testing has been used predominantly for older children and adults who tend to seek care later (5).

The Health Protection Agency (HPA; which became Public Health England on April 1, 2013) Respiratory and Systemic Infection Laboratory (which became the Respiratory and Vaccine Preventable Reference Unit on April 1, 2013) developed an ELISA to detect IgG against pertussis

toxin in oral fluid (16). This test was intended to act as a surrogate for the serum antibody assay. Oral fluid sampling is appealing because collection is straightforward; it is non-invasive (oral fluid is collected from around the gum line by using an absorbent swab) and can be collected by the patient or parent/guardian in the home and mailed to the laboratory for testing. The oral fluid assay detects seropositivity with a sensitivity of 79.7% (95% CI 68.3%–88.4%) and a specificity of 96.6% (95% CI 91.5%–99.1%) (16). Thus, oral fluid titers of >70 arbitrary units have a positive predictive value of 76.2%–93.2% for pertussis among children with chronic cough when used as a surrogate for the serum ELISA, assuming disease prevalence of 12%–37% (which includes the lower and upper limits of disease prevalence shown by other studies) (17).

Similar oral fluid antibody tests have been developed by HPA as surrogates for serologic testing for measles, mumps, and rubella (18,19). Oral fluid testing of patients after their formal notification of clinically diagnosed measles, mumps, and rubella has been conducted in England and Wales since 1994; this test has been acceptable and is used to augment routine serologic diagnosis for these diseases (18). After completion of a successful small-scale study in 2 areas of England (20), it was decided to conduct a national pilot test for the use of oral fluid testing for pertussis as a similar surveillance tool to obtain laboratory confirmation of pertussis cases statutorily notified on the basis of clinical diagnosis. Oral fluid testing was chosen because of the ease of sample collection and the predicted increased patient compliance with use of a noninvasive testing method. All laboratory-confirmed cases of pertussis are coordinated on a national basis, and each is followed up by asking the Health Protection Units (HPUs) or patients' general practitioners to complete a detailed surveillance questionnaire.

The aim of this national oral fluid surveillance was to improve case ascertainment and representativeness, increase rates of confirmation of notified cases, and provide more detailed information on notified cases of pertussis (if confirmed) via the surveillance questionnaire. Such improvements would strengthen the evidence base for vaccination policy decision making. We compared the effects of oral fluid testing as a notification follow-up service over the 27 months that it was available with effects during a comparable 27 months before its availability.

Methods

During June 2007–August 2009, a new oral fluid testing service was pilot tested throughout England and Wales. This testing service was provided free of charge by the HPA Respiratory and Systemic Infection Laboratory and Immunisation Department, through the 25 local HPA HPUs. The aim of this service was to seek laboratory confirmation

of formally notified pertussis cases for which the patient had been coughing for at least 2 weeks but a diagnosis had not been confirmed by another available method (culture, PCR, or serologic testing).

By law, the Proper Officer of the local HPU should be notified of pertussis cases that are diagnosed by clinicians in hospitals or primary care settings within the geographic area of the HPU's responsibility. When a case was reported, if the diagnosis had not been confirmed by culture, PCR, or serologic testing, the HPU mailed oral fluid sampling kits either directly to the patients or to the parents/guardians of patients (the kit was suitable for use at home) or to their general practitioner. The sampling kit contained an ORACOL saliva collection swab (Malvern Medical Developments Ltd, Worcester, UK), instructions, and a simple laboratory form for completion by patients. Detailed instructions were included in each kit, and they described how to collect the sample by brushing the swab along the gum line for 2 minutes.

Swabs were returned directly to the Respiratory and Systemic Infection Laboratory for testing in preaddressed packaging with prepaid postage, which was also included in the kit. Oral fluid was eluted from each swab and tested for IgG against pertussis toxin by ELISA as previously described (16). For patients who had been coughing for at least 2 weeks, a titer of ≥ 70 arbitrary units was considered consistent with recent infection in the absence of pertussis vaccination within the previous 12 months; as with serologic testing, antibodies from recent vaccination with pertussis vaccine can potentially confound test results used to provide markers of recent infection (17). Thus, a positive result for those who had received a pertussis-containing vaccine within 1 year before specimen collection cannot be easily interpreted. A surveillance form, identical to that used for all laboratory-confirmed cases, was sent to the local HPU for collection of additional information, including the patient's vaccination history. We excluded completely from the dataset any patients whose oral fluid or serologic testing result was consistent with recent pertussis infection and who had received a pertussis-containing vaccine in the previous 12 months through this enhanced surveillance.

If primary testing (PCR, serologic testing, and oral fluid testing) was undertaken, information for all samples submitted for testing, regardless of test result, was available for analysis. PCR, with real-time assay, was offered for hospitalized infants throughout the study period (11). Serologic testing for the detection of IgG against pertussis toxin based on single high-titer serologic results, considered indicative of recent infection (21,22), was offered for patients who had been coughing for at least 2 weeks. PCR and serologic testing were not routinely offered by other laboratories in England and Wales at the time of this study. Culture of *B. pertussis* from patient samples was

undertaken in hospital diagnostic laboratories throughout England and Wales, and only positive results were reported to the Immunisation Department. These laboratories have been encouraged to submit putative *B. pertussis* isolates to the HPA Respiratory and Systemic Infection Laboratory for confirmation and national surveillance purposes. Therefore, data were available only for culture-confirmed (i.e., not culture-negative) cases, and a complete dataset of samples submitted for testing by culture in England and Wales was not available for analysis. For any given patient, >1 test sample might have been submitted. When data are shown by person, testing is presented in the following order: culture, PCR, serologic testing, oral fluid testing. Therefore, a patient with a positive culture and serologic testing result, for example, would be considered culture positive, and a patient if this was the only positive test result would be considered oral fluid positive.

In addition to analyzing laboratory-confirmed cases, we also analyzed pertussis notifications. Clinically notified cases were rendered anonymous and thus could not be linked to laboratory-confirmed cases for which full patient details were available. Routine follow-up (for epidemiologic data) of laboratory-confirmed cases of pertussis was in place throughout the study. Information about whether the patient was hospitalized was used to determine whether there was evidence that the profile of cases confirmed through oral fluid testing differed from that of cases confirmed through other established methods. By using logistic regression (Stata version 9, StataCorp, College Station, TX, USA), taking age and sex into account, we compared the risk for hospitalization (as an indicator of serious disease) by various test methods.

Results

During the oral fluid pilot testing period, 2,756 oral fluid kits were sent to HPUs, 2,587 clinical cases were reported, and 2,443 cases of pertussis were confirmed by at least 1 laboratory method (Figure 1). Of these confirmed cases, 751 were confirmed by oral fluid testing plus or minus other methods and 591 (24% of all cases) were confirmed by oral fluid testing only, which increased laboratory ascertainment of pertussis by 32% (591 confirmed by oral fluid only/1,852 overall [confirmed by other methods \pm oral fluid testing]), from 6% in <1 year to 124% in 5–9 years, assuming that these cases would not have been confirmed by other laboratory methods in the absence of oral fluid testing. During the pilot-testing period, 1,827 oral fluid samples were submitted and analyzed; thus, a high proportion (66%) of test kits distributed to HPUs resulted in samples being submitted for diagnostic testing.

During the pilot testing period, 29% (1,465) and 26% (196) of samples tested by serology and PCR, respectively, yielded positive results, and 41% (751) of the oral

fluid samples yielded positive results (Table 1). The largest number of oral fluid samples was submitted from patients ≥ 20 years of age. However, the highest proportion with positive results (61%) were children 10–14 years of age; the availability of oral fluid testing mostly increased the total percentage of confirmed cases in children 1–9 years of age; during the pilot testing period, 52% and 55% of laboratory-confirmed cases in children 1–4 and 5–9 years of age, respectively, were confirmed by oral fluid testing alone, as were 36% of cases in children 10–14 years of age, compared with 21% for patients ≥ 15 years of age (Table 2). Taking 2008 as a year when oral fluid data had been available for a complete year, combining cases confirmed by oral fluid testing only with cases confirmed by the established methods resulted in an increase in disease incidence from 1.3 to 2.4 cases/100,000 children 1–4 years of age; from 0.8 to 2.1 cases/100,000 children 5–9 years of age; from 4.3 to 6.5 cases/100,000 children 10–14 years of age; and from 1.2 to 1.5 cases/100,000 persons ≥ 15 years of age.

When the distribution of confirmed cases by test method was considered over a longer period, which encompassed the introduction of routine serologic testing by the Respiratory and Systemic Infection Laboratory in January 2002, it was clear that the introduction of serologic testing had the greatest overall effect on testing for and ascertainment of pertussis in England and Wales, and the discrepancy between notified and laboratory-confirmed cases was correspondingly reduced (Figure 2). The proportion of cases confirmed by serologic testing increased with increasing patient age (Table 2); 77% (1,012/1,307) of all cases in patients ≥ 15 years of age were confirmed by serologic testing during June 2007–August 2009, and 18% of cases were confirmed for those 6–11 months of age. A higher proportion of positive results among patients ≥ 1 year of age were obtained by oral fluid testing (40%) than by serologic testing (29%). When the 79.7% sensitivity of oral fluid versus serologic testing is corrected for, $\approx 50\%$ of the submitted oral fluid swab samples represented true cases of pertussis.

Patients with oral fluid-confirmed pertussis were least likely to be hospitalized in each age group ≥ 1 year of age (Figure 3). Overall, among those ≥ 1 year of age (among whom hospitalization was less frequent than among infants and oral fluid testing was more widely used), patients tested by serology and culture were 6 (95% CI 2.6–13.8) and 15 (95% CI 5.2–44.9) times more likely to be hospitalized than were those tested by oral fluid, when age and sex were taken into account. This finding suggests that milder cases of pertussis in the community were being confirmed through oral fluid surveillance.

Total numbers of samples submitted for PCR, serologic testing, and oral fluid testing were compared with notifications during the same period (Table 1). More patients had samples submitted for testing than were formally notified

at all ages, indicating substantial underreporting through the notification system despite the apparent improvement (Figure 2); notifications accounted for 56% of the number submitted for testing among those 1–14 years of age and 23% of those ≥ 15 years of age.

Discussion

The aim of this study was to improve case ascertainment and representativeness, increase rates of laboratory confirmation of notified cases, and provide more detailed information about confirmed cases of pertussis by surveillance questionnaire. Among oral fluid samples submitted during the pilot testing period, 751 (41%) had positive results, compared with 1,465 (29%) and 196 (26%) samples tested by serology and PCR, respectively. The highest proportion of positive samples (61%) was from children 10–14 years of age, and the availability of oral fluid testing most increased the total number of confirmed cases among children 1–9 years of age.

In the United Kingdom, pertussis vaccine is offered for infants at 2, 3, and 4 months of age; a booster dose 3 years after completion of the primary course is also offered. Since 1992, vaccine coverage by a child’s first birthday has been $\geq 90\%$, and since 2009–2010, receipt of the booster dose has been $\geq 85\%$. The primary aim of the pertussis immunization program is to minimize disease, hospitalization, and death among young infants. Despite these sustained high levels of coverage, increased pertussis activity occurred in England and Wales starting in October 2011, leading to declaration of a national outbreak in April 2012 (23,24). During this outbreak, the highest reported incidence of disease was among infants <6 months of age, followed by adolescents 10–14 years of age. In response to the continued increases in disease levels observed among young infants, the UK Departments of Health introduced a temporary program to offer pertussis vaccination to pregnant women; the program started in October 2012 and continued while disease levels remained high (25). This program passively

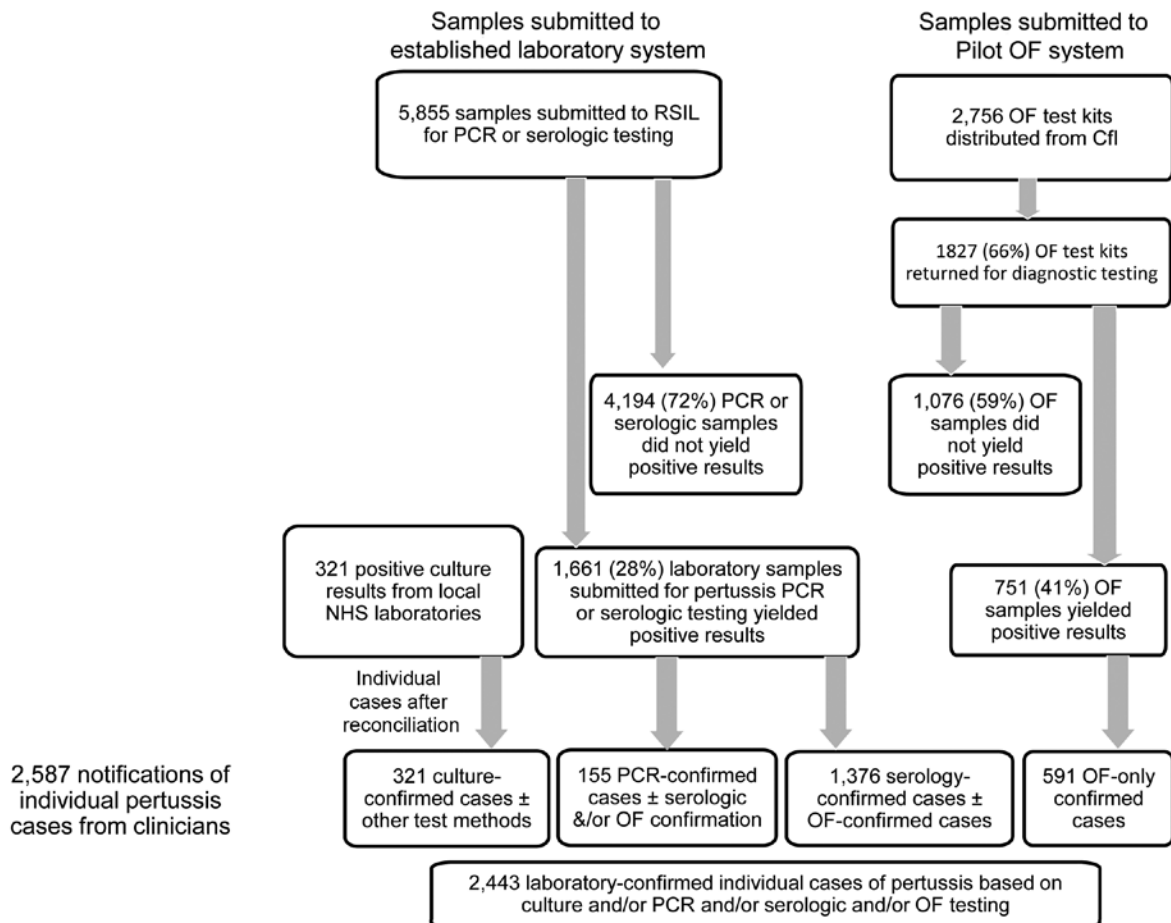


Figure 1. Distribution of notified cases of pertussis and samples that confirmed pertussis cases submitted to the Health Protection Agency (HPA; became Public Health England on April 1, 2013) Respiratory and Systemic Infection Laboratory (RSIL; became the Respiratory and Vaccine Preventable Reference Unit on April 1, 2013) and collated by the HPA Immunisation Department, June 2007–August 2009, England and Wales. OF, oral fluid; NHS, National Health Service; CfI, Centre for Infections.

Table 1. Distribution of samples received by RSIL for pertussis testing, England and Wales, June 2007–August 2009*

| Patient age, y | Test method, no. submitted/no. positive (% positive)† | | | | Notifications, no. |
|----------------|---|--------------|------------------|------------------|--------------------|
| | Oral fluid | PCR‡ | Serology | Total | |
| <1§ | 139/69 (50) | 682/187 (27) | 208/39 (19) | 1,029/295 (29) | 452 |
| 1–4 | 288/85 (30) | 41/5 (12) | 299/53 (18) | 628/143 (23) | 366 |
| 5–9 | 214/83 (39) | 6/1 (17) | 183/54 (30) | 403/138 (34) | 250 |
| 10–14 | 282/173 (61) | 13/3 (23) | 429/242 (56) | 724/418 (58) | 372 |
| ≥15 | 904/341 (38) | 19/0 (0) | 3,975/1,077 (27) | 4,898/1,418 (29) | 1,147 |
| All | 1,827/751 (41) | 761/196 (26) | 5,094/1,465 (29) | 7,682/2,412 (31) | 2,587 |

*RSIL, Health Protection Agency (which became Public Health England on April 1, 2013) Respiratory and Systemic Infection Laboratory (which became the Respiratory and Vaccine Preventable Reference Unit on April 1, 2013). Data are based on samples submitted, and >1 sample/patient might have been submitted.

†Culture testing is excluded because RSIL does not undertake initial culture testing; therefore, the total number of samples submitted for testing nationally is not known.

‡PCR testing is not routinely available for patients >1 y of age with suspected pertussis.

§Culture testing accounted for >50% of all pertussis confirmations for infants <1 y of age, but the total number of samples submitted for testing is not known.

protects infants from birth, through intrauterine transfer of maternal antibodies, until they could be actively protected by the routine infant vaccination program.

Over recent years, several other countries, including the United States (26), Australia (27), and Canada (28,29), have experienced increased pertussis activity, and these 3 countries have made PCR testing widely available. However, the availability of a noninvasive test method for children might be more acceptable to parents/guardians and health professionals, especially among children who are not severely ill. The lower proportion of samples submitted for serologic pertussis testing for children 1–14 years of age (compared with the percentage submitted for adults) suggests that providing blood samples is unpopular and that oral fluid is a useful alternative. Milder illness is also more likely to result in persons seeking care later in the course of illness, when culture and PCR are less sensitive. Similarly, the data collected through national pilot testing in England and Wales suggest that oral fluid surveillance improved ascertainment of milder cases beyond those confirmed through the testing that was already in place (culture, PCR [infants only], and serology). This improved ascertainment is useful because mild cases are problematic for surveillance because they are underdiagnosed and contribute to sustained transmission of pertussis within the community. Furthermore, underascertainment of milder infections causes bias, leading to overestimation of vaccine effectiveness (30). Unlike other available methods, oral fluid testing

was acceptable for self-sampling and did not require health care provider time or expertise, which made it more cost-effective for surveillance. Oral fluid testing has been considered ideal for the primary care setting (31).

Successful programs for vaccination of pregnant women would directly reduce the number of cases among infants <3 months of age. If high levels of activity persist in other age groups, however, increased risk of acquiring infection during infancy would also persist. The finding that the level of positivity for samples submitted for diagnostic testing is low underlines how problematic diagnosis of pertussis can be for patients in age groups that tend to not show classic symptoms. Higher rates of positivity among adolescent/teenage children may be consistent with a real increased risk for pertussis for persons in this age group or could indicate continued underascertainment of cases in this age group despite the availability of a noninvasive test method.

In view of the increased disease incidence among children 10–14 years of age that started in October 2011 and the concerns that serologic testing might be suboptimal for patients in this age group, in 2013, oral fluid testing was made routinely available in England for patients 5–16 years of age with cases of pertussis that had not been confirmed by other laboratory methods (32). Making noninvasive testing available for patients in this age group is considered prudent, given the increased number of cases observed among children 7–10 years of age and adolescents in other countries

Table 2. Total and proportion of confirmed pertussis cases, England and Wales, June 2007–August 2009

| Patient age | Test method, no. (%)* | | | | Total |
|-------------|-----------------------|----------|------------|------------|-------|
| | Culture | PCR | Serology | Oral fluid | |
| <3 mo | 211 (60) | 118 (34) | 11 (3) | 11 (3) | 351 |
| 3–5 mo | 46 (53) | 27 (31) | 4 (5) | 10 (11) | 87 |
| 6–11 mo | 9 (53) | 2 (12) | 3 (18) | 3 (18) | 17 |
| 1–4 y | 17 (14) | 5 (4) | 36 (30) | 63 (52) | 121 |
| 5–9 y | 5 (4) | 0 | 57 (41) | 77 (55) | 139 |
| 10–14 y | 12 (3) | 3 (1) | 253 (60) | 153 (36) | 421 |
| ≥15 y | 21 (2) | 0 | 1,012 (77) | 274 (21) | 1,307 |
| All | 321 | 155 | 1,376 | 591 | 2,443 |

*When >1 test method was used, culture takes precedence over PCR, which takes precedence over serology, which takes precedence over oral fluid testing (e.g., a case confirmed by culture and serologic testing is listed under culture).

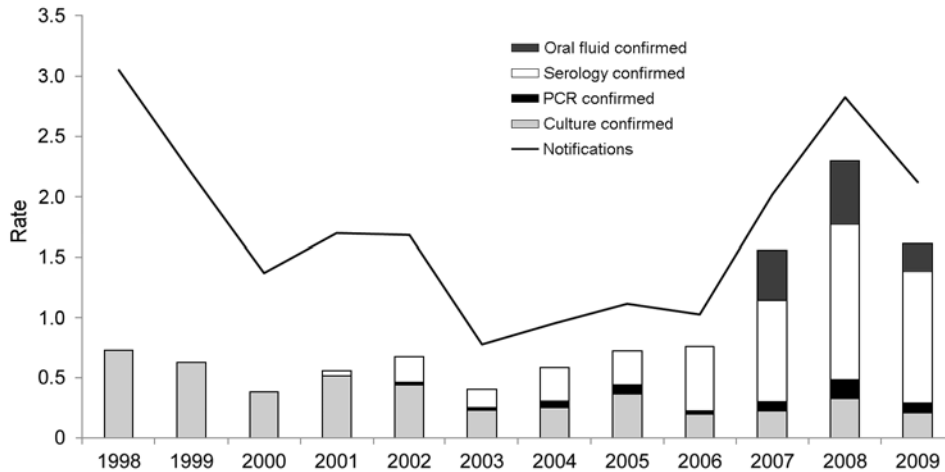


Figure 2. Rates of pertussis notification and laboratory confirmation (no. cases/100,000 population), by test method, England and Wales, 1998–2009. When >1 test method was used, culture takes precedence over PCR, which takes precedence over serology, which takes precedence over oral fluid (e.g., a case confirmed by culture and serologic testing is listed under culture).

(26,33–35). There is ongoing discussion about the need for boosters for adolescents and the optimal age at which they should be administered. If immunity does wane more rapidly after vaccination with acellular pertussis vaccines than with whole-cell vaccines, then countries such as the United States and Canada might benefit from improved surveillance to further inform the timing of booster vaccinations.

Although residual antibodies from vaccinations received while in preschool could potentially affect some positive oral fluid results for children <8 years of age in this study (17), we excluded those known to be vaccinated <1 year before oral fluid (and/or serologic) testing only through routine follow-up. The individual titers of 14 patients with cases confirmed by oral fluid testing only at >1 and <3 years after booster pertussis vaccination were well above the cut-off with a high positive predictive value for each case (mean 96.8%, range 85.1%–100%), and on this basis it was highly likely that only true cases of pertussis were included.

In conclusion, the introduction of serologic testing followed by oral fluid testing has successively narrowed the gap in surveillance for pertussis in England and Wales.

Broader use of PCR testing is currently being pilot tested in participating regions in England as a way to further improve pertussis surveillance for patients seeking care earlier in the course of illness. In countries that already widely use PCR and/or serologic testing, oral fluid testing could improve diagnosis for patients who seek care later in the course of illness, thereby ruling out other potential causes and preventing unnecessary intervention. Although the oral fluid assay is only performed at the Respiratory and Systemic Infection Laboratory, this technology has the potential for broader application and wider availability. Oral fluid testing is an additional surveillance tool that offers higher acceptability and lower cost than other available methods.

Pertussis is a rapidly reemerging disease; in several countries, reported incidence rates are high already, and rates could yet increase in other countries where disease is currently well controlled. More complete ascertainment is needed globally to better understand pertussis epidemiology and transmission, thereby facilitating the development of improved vaccines and vaccination strategies to improve future disease control.

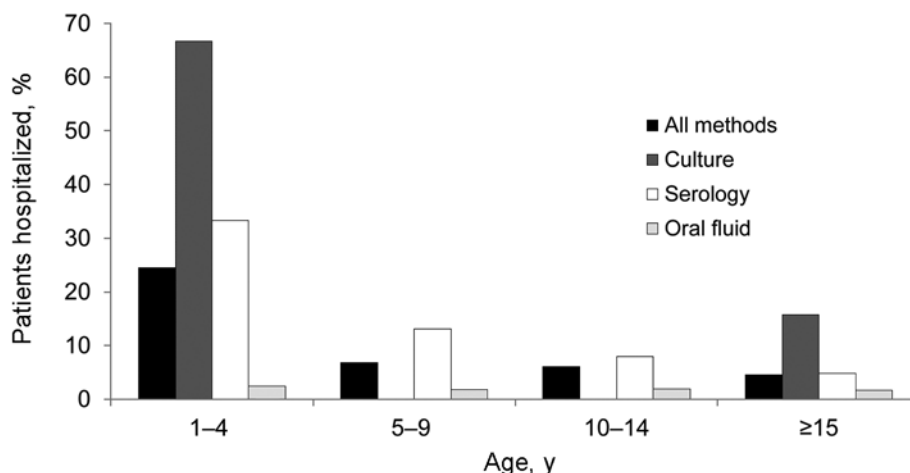


Figure 3. Proportion of cases hospitalized by age group and test method, England and Wales, June 2007–August 2009. When >1 test method was used, culture takes precedence over PCR, which takes precedence over serology, which takes precedence over oral fluid (e.g., a case confirmed by culture and serologic testing is listed under culture).

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Ms Campbell is a senior clinical scientist and lead epidemiologist for pertussis and meningococcal disease at Public Health England, London, where she also is responsible for the national surveillance of subacute sclerosing panencephalitis, congenital rubella, and vaccines given during pregnancy.

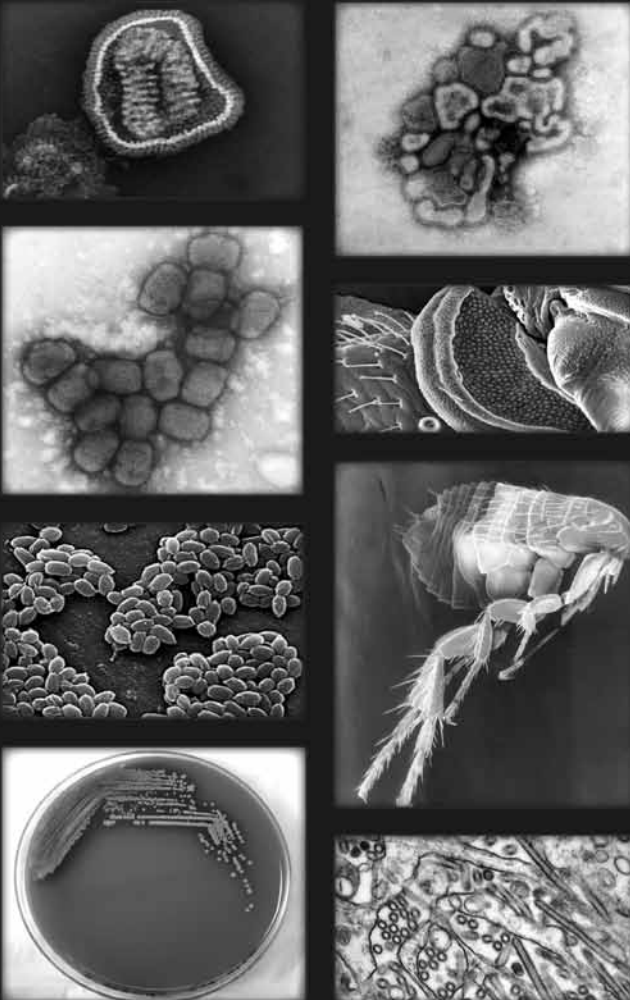
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Address for correspondence: Helen Campbell, Immunisation Hepatitis and Blood Safety Department, Public Health England, 61 Colindale Ave, Colindale, London NW9 5EQ, UK; email: helen.campbell@phe.gov.uk

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High Prevalence of *Ancylostoma ceylanicum* Hookworm Infections in Humans, Cambodia, 2012

Tawin Inpankaew, Fabian Schär, Anders Dalsgaard, Virak Khieu, Wissanuwat Chimnoi, Chamnan Chhoun, Daream Sok, Hanspeter Marti, Sinuon Muth, Peter Odermatt, and Rebecca J. Traub

Ancylostoma ceylanicum, a hookworm of canids and felids in Asia, is becoming the second most common hookworm infecting humans. In 2012, we investigated the prevalence and infection dynamics of and risk factors for hookworm infections in humans and dogs in a rural Cambodian village. Over 57% of the population was infected with hookworms; of those, 52% harbored *A. ceylanicum* hookworms. The greatest intensities of *A. ceylanicum* eggs were in persons 21–30 years of age. Over 90% of dogs also harbored *A. ceylanicum* hookworms. Characterization of the cytochrome oxidase-1 gene divided isolates of *A. ceylanicum* hookworms into 2 groups, 1 containing isolates from humans only and the other a mix of isolates from humans and animals. We hypothesize that preventative chemotherapy in the absence of concurrent hygiene and animal health programs may be a factor leading to emergence of *A. ceylanicum* infections; thus, we advocate for a One Health approach to control this zoonosis.

Human infections with *Necator americanus* and *Ancylostoma duodenale* hookworms continue to be recognized as a leading cause of iron deficiency anemia and protein malnutrition in developing countries (1). On

Author affiliations: University of Copenhagen, Copenhagen, Denmark (T. Inpankaew, A. Dalsgaard); Kasetsart University, Bangkok, Thailand (T. Inpankaew, W. Chimnoi); Swiss Tropical and Public Health Institute, Basel, Switzerland (F. Schär, V. Khieu, H. Marti, P. Odermatt); University of Basel, Basel (F. Schär, V. Khieu, H. Marti, P. Odermatt); National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia (V. Khieu, S. Muth); Fisheries Administration, Phnom Penh (C. Chhoun, D. Sok); The University of Queensland, Gatton, Queensland, Australia (R.J. Traub); and University of Melbourne, Parkville, Victoria, Australia (R.J. Traub)

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the basis of parasitologic surveys of fecal samples, hookworms are estimated to infect 576–740 million persons globally, and over half of the infections occur in Asia and the Pacific regions (2). Recent molecular-based epidemiologic surveys have shown *Ancylostoma ceylanicum* to be the second most common hookworm species infecting humans in Asia. In Thailand, Laos, and Malaysia, 6%–23% of persons positive for hookworm eggs were infected with *A. ceylanicum* helminths (3–6). There are an estimated 19–73 million *A. ceylanicum* hookworm-infected persons in regions where this zoonotic helminth is known to be endemic (7). Dogs and cats act as natural reservoirs for hookworm transmission to humans, and the prevalence of *A. ceylanicum* hookworms in these animals ranges from 24% to 92% in the Asia-Pacific region (6,8–10). Much like anthroponotic helminths, *A. ceylanicum* hookworms have the potential to produce clinical symptoms of ground itch (a pruritic papular hypersensitivity response caused by the entry of helminths into the skin), epigastric pain, diarrhea, and anemia in humans (11–15). However, despite these reports, relatively little is known about the clinical significance and infection dynamics of this zoonotic hookworm in humans, dogs, and cats. Differentiation of the genus of hookworms infecting humans is imperative because each species varies in its biology, life cycle, pathophysiology, and epidemiology, and these differences have key implications when assessing hookworm-associated illnesses and establishing control measures.

The internal transcribed spacer (ITS) –1 and –2 regions and the 5.8S region have been used to detect and characterize hookworm infections directly from eggs in human and animal feces (6,10,16,17). In addition, sequencing of the cytochrome c oxidase subunit 1 (*cox1*) gene has been successfully used to establish intraspecies genetic differences of many strongylid nematodes, including hookworms (18–21).

The aim of our study was to determine the prevalence, associated risk factors, and infection dynamics of hookworm species infection in humans and dogs living in a rural Cambodian village. To carry out this investigation, we used a combination of conventional parasitologic and molecular epidemiologic approaches.

Materials and Methods

Study Site and Sample Collection

The study was conducted in May 2012 in Dong, a rural village in Rovieng District, Preah Vihear Province, Cambodia. Preah Vihear Province is located in northern Cambodia, bordering Thailand and Laos (13°47'N 104°58'E). The climate is tropical; temperatures are warm and hot all year round, and seasons alternate between dry and wet. Subsistence farming (rice, vegetables, and fish) constitutes the primary source of income for the community. Drinking water is sourced from wells, well pumps, and rain water tanks, and just over half of the households own a latrine. All household electricity is battery or generator powered. Approximately half the households feed semidomesticated, free-roaming community dogs. These dogs are allowed to defecate indiscriminately within the village or outside the homes of their owners.

The study protocol was approved by the Ethics Committee of the Canton of Basel and Baselland, Switzerland, and the National Ethics Committee Health Research, Ministry of Health, Cambodia. Dong, the village selected for study, had previously been categorized as having endemic soil-transmitted helminths (22). According to the treatment guidelines of the Cambodian helminths control program, all children attending primary school in the village were administered albendazole (400 mg) and mebendazole (500 mg) twice a year. At completion of the study, all participants who were found positive for *Strongyloides* spp. were treated with ivermectin (200 µg/kg body weight), and participants infected with other soil-transmitted helminths were treated with albendazole (400 mg).

A cross-section of 67 households was randomly selected from a list provided by the Dong village authority. A total of 218 persons from those households were enrolled in the study. Of the 218 persons, 99 (45.4%) were male. The average age of participants was 30.0 years (range 2–84); female participants were marginally older, on average, than male participants (30.3 vs. 29.8 years of age). On the first day of the study, informed consent was obtained from the enrolled participants, and questionnaires were administered during interviews. Interviews with children (i.e., participants 2–17 years of age) were conducted with the assistance of a parent or legal guardian. All study participants responded to a questionnaire covering demographics, dietary habits, personal hygiene, and level of household income and assets.

Prelabeled stool containers were distributed to the 218 study participants for collection of feces on the second morning of the study. Fecal samples were collected from participants' dogs (N = 94), when applicable. Samples (≈3–5 g) from dogs were collected directly from the rectum at time of the participant interview and placed into a sterile plastic container. If insufficient stool was obtained from a dog, the animal was confined within the owners property and resampled on the second morning of the study. All fecal samples were chilled immediately in a cool box and transported to a laboratory in Rovieng Health Center (Rovieng District, Preah Vihear Province) within 2 h after collection. After fecal samples arrived at the laboratory, a minimum of 2 g of each sample was placed into a 15-mL centrifuge tube containing 10% formaldehyde for parasitologic analysis, and 1–2 g of each sample was placed into a 15-mL centrifuge tube containing 2.5% potassium dichromate for molecular analysis. These samples were then shipped at room temperature to the School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia, for further analysis.

Parasitologic Procedures

All fecal samples were examined by microscope. The relative intensity of hookworm infection, in eggs per gram, was determined by floatation, using a sodium nitrate solution (specific gravity 1.20) (23).

DNA Extraction

Genomic DNA was extracted directly from fecal samples by using the PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's instructions, with the exception that fecal samples were subjected to a 5-min disruption by using 0.5-mm Zirconia/Silica beads (BioSpec Products, Inc., Bartlesville, OK, USA) instead of the beads provided by the manufacturer. Final elution of DNA was made in 100 µL of elution buffer. The extracted DNA was stored at -20°C until required for PCR amplification.

Molecular Characterization of Hookworm Species in Humans

PCR was conducted by using primers RTHW1F and RTHW1R (10) in 25-µL volumes; each final reaction contained 1× CoralLoad PCR Buffer (QIAGEN Pty Ltd, Hilden, Germany), 12.5 pmol of each primer, 0.5 U of Hot-Star Taq DNA Polymerase (QIAGEN), and 2 µL of DNA. The cycling conditions were the same as the published protocol (10) except for an initial denaturation of 5 min at 95°C. A positive control of *N. americanus* and *A. ceylanicum* hookworms and negative controls of distilled water were included in each run. PCR amplicons that were ≈380 bp in size, corresponding to *Ancylostoma* spp. hookworms,

were purified by using the PureLink Quick PCR Purification Kit (Life Technologies, Carlsbad, CA, USA) and submitted to the University of Queensland Animal Genetics Laboratory, Gatton, for bidirectional DNA sequencing.

Molecular Characterization of Hookworm Species in Dogs

PCR–restriction fragment length polymorphism (RFLP) characterization of hookworms from dogs was carried out as described (17,24). In brief, RTGHF1 and RTABCR1 primers were used to amplify a 545-bp region of ITS-1, 5.8S, and ITS-2 of *A. caninum*, *A. ceylanicum*, and *Uncinaria stenocephala* hookworms. In a separate PCR, a 673-bp region of an *A. braziliense* hookworm was amplified by using RTGHF1 and a specific reverse primer, RTAYR1. Both PCR reactions consisted of 1× CoralLoad PCR Buffer (QIAGEN), 12.5 pmol of each primer, 0.2 mL of 20 mg/mL bovine serum albumin, 2 mL DNA, and 1 U of HotStar Taq Polymerase (QIAGEN) in a 25-mL reaction. The cycling conditions were as published (17,24), except for an initial denaturation time of 5 min at 95°C. Amplified PCR product (10 mL; RTGHF1/RT-ABCR1) was digested with *HinFI* and *RsaI* endonucleases in separate reactions at 37°C for 3 h. The RFLP patterns generated by each sample were then compared to the expected RFLP profiles for each hookworm species.

PCR and DNA Sequencing of *cox1* of *A. ceylanicum* Hookworm

Samples from dogs and humans that were positive for *A. ceylanicum* hookworms were further characterized to a haplotype level by analysis of the mitochondrial gene (*cox1*). AceyCOX1F (5'-GCTTTTGGTATTGTA-AGACAG-3') and AceyCOX1R (5'-CTAACAAACATAATAAG-TAT-CATG-3') were specifically designed to amplify a 377-bp region of the *cox1* gene of *A. ceylanicum* hookworm. The PCR was carried out in 25-mL volumes, with each reaction containing 1× CoralLoad PCR Buffer, 12.5 pmol of each primer, 0.5 U of HotStar Taq DNA polymerase, and 2 mL of DNA. The cycling conditions were 95°C for 5 min, followed by 50 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. A positive control of *A. ceylanicum* hookworm and a negative control of distilled water were included in the run. PCR-positive samples were purified by using the PureLink Quick PCR Purification Kit according to the manufacturer's protocol. Bidirectional DNA sequencing was performed by the University of Queensland Animal Genetics Laboratory.

Phylogenetic Analyses

DNA sequences were analyzed by using the Finch TV version 1.4.0 trace viewer (Geospiza, Inc., Seattle, WA, USA) and aligned by using BioEdit version 7.2.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html) together with the

cox1 gene sequences from the following hookworm species: *A. ceylanicum* Malaysia isolates (GenBank accession nos. KC247727–KC247745, Pos Iskandar [Human] and Sg Bumbun [Human]); *A. caninum* and *A. duodenale* (GenBank accession nos. NC012309 and NC003415, respectively); and *A. ceylanicum* Thailand genotype (GenBank accession no. KF896595). Neighbor-joining analyses were conducted by using Tamura-Nei parameter distance estimates, and the tree was constructed by using Mega4.1 (www.megasoftware.net). Bootstrap analyses were conducted using 1,000 replicates.

Statistical Analyses

We used STATA version 12 (StataCorp LP, College Station, TX, USA) for data entry and statistical analyses. The prevalence of hookworm infection was calculated by using descriptive statistics for microscopy and molecular results. A univariate model was used to assess potential risk factors associated with hookworm infection; odds ratios and 95% CI were reported. The level of statistical significance was set at $p < 0.05$. Factors that were significant in univariate analysis were evaluated by multivariate analysis, when applicable.

Results

Prevalence of Hookworm Infection

The prevalence of hookworm infection among the 218 persons tested in Dong village was 26.6% (58/218) as determined by microscopic examination and 57.4% (124/218) as determined by PCR based on amplification of the partial ITS gene. Among dogs, 80.9% (76/94) were positive for hookworms by microscopic examination, and 95.7% (90/94) were positive by PCR based on amplification of the partial ITS gene.

Molecular Characterization of Hookworm Species

Of the 124 persons with positive samples, 64 (51.6%) harbored *A. ceylanicum* hookworms; 57 (89.0%) of these infections were single infections. An equal percentage of persons, 64 (51.6%), were infected with *N. americanus* hookworms, mostly as single infections (59/64 [92.2%]), and 4 (3.2%) persons were infected with *A. duodenale* hookworms (Table).

Of the 90 dogs with positive samples, 85 (94.4%) were infected with *A. ceylanicum* hookworms, mostly (81/85 [95.3%]) as single infections, and 8 (8.9%) were infected with *A. caninum* hookworms. One dog was found to be shedding *N. americanus* eggs (Table).

Phylogenetic Analysis of *cox1* Gene of *A. ceylanicum*

Of 68 human and 82 dog samples positive for hookworms, 28 (41.2%) and 65 (79.3%), respectively, were

Table. Hookworm species found in humans and dogs, Dong village, Rovieng District, Preah Vihear Province, Cambodia, 2012*

| Infected host, hookworm species | No. (%) positive |
|---|------------------|
| Humans | |
| <i>Necator americanus</i> | 59 (47.6) |
| <i>Ancylostoma ceylanicum</i> | 57 (46.0) |
| <i>A. duodenale</i> | 1 (0.8) |
| <i>N. americanus</i> and <i>A. ceylanicum</i> | 4 (3.2) |
| <i>A. ceylanicum</i> and <i>A. duodenale</i> | 2 (1.6) |
| <i>N. americanus</i> and <i>A. ceylanicum</i> and <i>A. duodenale</i> | 1 (0.8) |
| <i>N. americanus</i> and <i>A. duodenale</i> | 0 |
| Total | 124 (100.0) |
| Dogs | |
| <i>A. ceylanicum</i> | 81 (90.0) |
| <i>A. caninum</i> | 5 (5.6) |
| <i>A. ceylanicum</i> and <i>A. caninum</i> | 3 (3.3) |
| <i>A. ceylanicum</i> and <i>N. americanus</i> | 1 (1.1) |
| Total | 90 (100.0) |

*The presence of hookworms was determined by PCR amplification of the internal transcribed spacer-1 and -2 regions and the 5.8S region and by DNA sequencing.

successfully amplified at the *cox1* gene. Of these, 21 PCR-positive amplicons from human samples and 27 PCR-positive amplicons from dog samples were randomly selected for DNA sequencing and subsequent phylogenetic analysis.

The phylogenetic tree distinctly separated into 3 clusters; the *A. ceylanicum* hookworm isolates grouped together and were genetically distinct from *A. caninum* hookworm isolates (GenBank accession nos. NC012309 and FJ483518) and *A. duodenale* hookworm isolates (GenBank accession nos. NC003415 and AJ417718). Within *A. ceylanicum* hookworm isolates, there was strong bootstrap support (100%) for the division of isolates from various geographic locations into 2 clades. The first clade comprised 4 human isolates, 1 from the current study in Cambodia (Human 19) and 3 previously reported human isolates from Malaysia (GenBank accession no. KC772445; Human [Sg Bumbun]; Human [Gurney] and Human [Pos Iskandar]). The second clade comprised a mix of isolates from humans ($n = 20$) and dogs ($n = 27$) from villages in Cambodia; humans ($n = 5$), dogs ($n = 11$), and cats ($n = 2$) from Malaysia (21); and 1 dog in Thailand (GenBank accession no. KF896595). For human- and dog-derived *A. ceylanicum* hookworms, representative DNA sequences at each *cox1* haplotype were submitted to GenBank under accession nos. KF896596–KF896605 (see sequences marked with asterisks in the online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/6/13-1770-Techapp1.pdf).

Age-related Prevalence and Intensity of *N. americanus* and *A. ceylanicum* Hookworm Infections

The prevalence of *N. americanus* hookworms peaked in persons 31–50 years of age, whereas the prevalence of *A. ceylanicum* hookworms peaked in persons 15–20 years of age and again in persons 31–50 years of age (Figure). The highest egg intensities for single infections attributed to *N. americanus* and *A. ceylanicum* hookworms occurred in persons 21–30 years of age (Figure).

Risk Factors Associated with Hookworm Infection of Humans and Dogs

The results of regression analysis showed an increased risk for hookworm infection in persons who did not wear shoes while defecating (odds ratio 6.0, 95% CI 1.1–28.6; $p = 0.038$). No significant associations were found between the prevalence and intensity of hookworms by age group, sex, household income, or dietary practices. No risk factors of significance were associated with hookworm infection in dogs.

Discussion

In this study, zoonotic ancylostomiasis caused by *A. ceylanicum* hookworms was found to be highly endemic among humans in Dong village, Preah Vihear Province, Cambodia; community dogs were the likely zoonotic reservoir. This finding is in stark contrast to the consistent finding by other molecular-based prevalence studies in the region that *N. americanus* is the predominant hookworm species in humans, followed by *A. ceylanicum* and *A. duodenale* hookworms (3,5,6,10). PCR proved a superior alternative to microscopy-based techniques for the detection of hookworms in fecal samples (25,26). In Dong village, the prevalence of *A. ceylanicum* hookworms matched that of their anthroponotic counterpart, *N. americanus* hookworms, and infections with *A. ceylanicum* hookworms substantially out-numbered those with *A. duodenale* hookworms. In addition, most infected persons harbored single-species hookworm infections; just over 10% of hookworm-positive persons had mixed-species infections. These results raise questions about the potential infection dynamics between hookworm species within individual hosts. Our study supports an earlier hypothesis (7) that anthroponotic hookworms may have a cross-protective role in expelling and preventing the subsequent establishment of *A. ceylanicum* hookworms via a T helper 2 cell response (27). The major immunologic

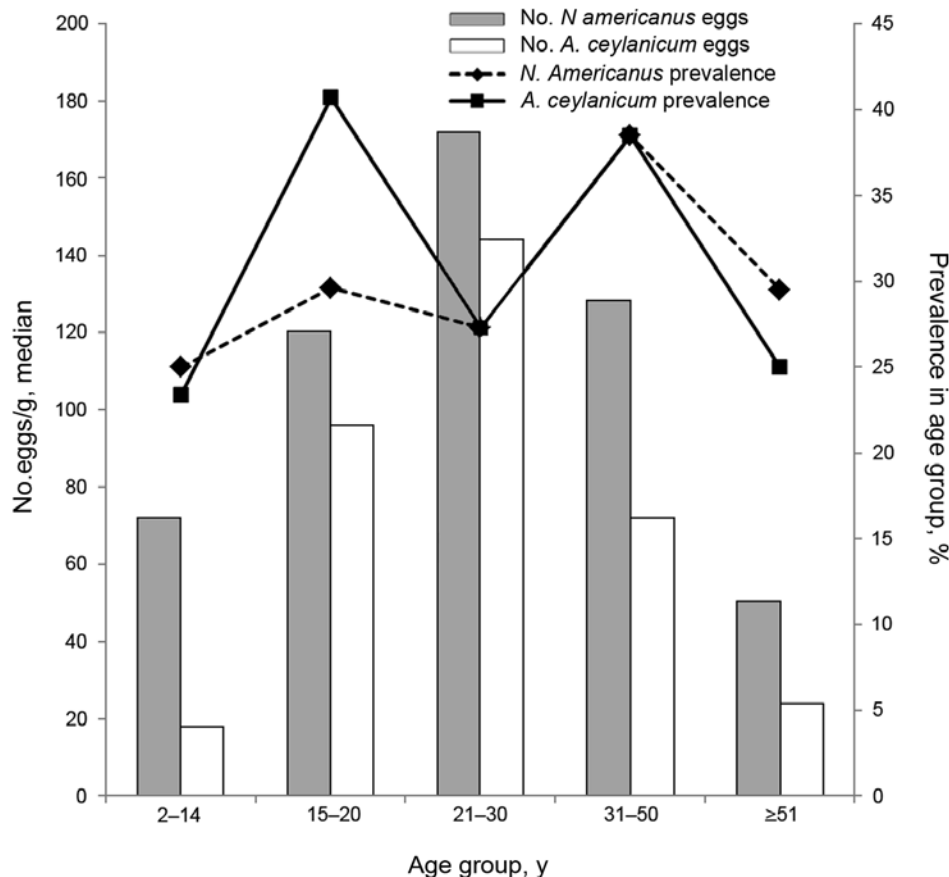


Figure. Prevalence and intensity (eggs per gram) of *Necator americanus* and *Ancylostoma ceylanicum* hookworm infections in humans of different ages in rural Dong village, Rovieng District, Preah Vihear Province, Cambodia, 2012.

action against incoming L3 larvae (infective filariform larvae) and L4 larvae (final larval stage within the intestine) is regulated by the infection itself (28). Thus, the presence of a stable and long-lived (3–6 years) infection with anthroponotic species (29) may play a role in providing an unsuitable environment for the establishment of incoming larvae of another closely related (albeit potentially shorter-lived and suboptimally host adapted) species—in this case, *A. ceylanicum* hookworms. Reduced burdens of anthroponotic hookworm species may also have the added advantage of easing density-dependent intraspecific competition for limited resources within the intestinal niche (30), leading to an opportunistic establishment of *A. ceylanicum* hookworms. Although data on the natural life span of *A. ceylanicum* hookworms in humans do not exist, infections in Dutch servicemen 5 months after their return from New Guinea (31) suggest that chronic infections with this hookworm may occur.

The initiating or causal factor for the emergence of highly endemic levels of monospecific infections with *A. ceylanicum* hookworms in Dong village remains unclear. In this study, potential causal factors for human infection are likely related to the high levels of *A. ceylanicum* hookworm infections in community dogs. In rural

Malaysia, close contact with community dogs and cats was shown often to be associated with human infection with *A. ceylanicum* hookworms (6). In Dong village, dogs were reported to defecate indiscriminately in environments shared with humans, leading to widespread environmental contamination with infective *A. ceylanicum* hookworm larvae. For humans, defecating while bare foot was shown to be the most significant risk factor for infection with both species of hookworms. Whether these factors, coupled with the administration of preventative chemotherapy, led to an increased opportunity for the *A. ceylanicum* hookworm to replace the niche of its anthroponotic competitors remains unanswered. Either way, integrated control programs aimed at combining chemotherapeutic interventions with improvements in community hygiene and animal health programs will aid in curbing this potentially opportunistic zoonosis.

Molecular epidemiologic data gathered from characterization of the *cox1* gene of *A. ceylanicum* hookworms strongly support previous findings (21) that *A. ceylanicum* hookworm isolates from humans and animals formed 2 genetically distinct groups, 1 comprising isolates specific to humans and the other comprising isolates from humans, dogs, and cats. Most *A. ceylanicum* hookworm isolates

from humans in Dong village clustered within the zoonotic haplotype, confirming that transmission from dogs to humans has occurred. Genetic groups inferred by the *cox1* gene of *A. ceylanicum* hookworms were found to be independent of geographic source. Whether the 2 primary haplotypes differ in biologic, epidemiologic, and pathophysiologic characteristic warrants further investigation.

The transmission dynamics of *A. ceylanicum* hookworms in humans of different ages largely paralleled that of *N. americanus* hookworms: persons 21–30 years of age excreted the highest number of eggs. This highly unexpected finding has key implications. First, this finding suggests that the previous classification of *A. ceylanicum* as an abnormal and minor hookworm of humans (32) no longer stands. Second, monospecific infections of humans with ≤ 100 *A. ceylanicum* worms have been reported to cause anemia, even in well-nourished persons (14,33). Thus, attention must be directed to *A. ceylanicum* infection as a major cause of human illness in areas where this zoonosis is endemic.

The zoonotic helminth *A. ceylanicum* can no longer be classified as an abnormal hookworm of humans. Although previous studies have reported this hookworm's emergence as the second most common human hookworm species in Southeast Asia, our study demonstrates its ability to infect humans at prevalence and intensity levels at par with that of its anthroponotic competitor, the *N. americanus* hookworm. We hypothesize that expansion of preventative chemotherapy in the absence of concurrent hygiene and animal health programs is a potential causal factor for the emergence of this zoonosis. Attention must be directed to the effects of *A. ceylanicum* hookworm infections on human health, and a One Health approach should be adopted for the control of this zoonosis.

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Dr Inpankaew, a veterinarian, is a PhD student at the University of Copenhagen, Denmark, and a lecturer in the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Thailand. His research interests include molecular diagnostics and epidemiology.

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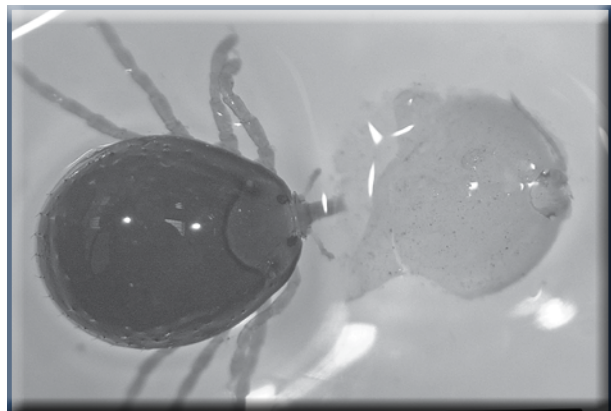
Address for correspondence: Tawin Inpankaew, Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, 50 Phahonyothin Rd, Chatuchak, Bangkok, Thailand 10900; email: fvettwi@gmail.com

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Characteristics of Patients with Mild to Moderate Primary Pulmonary Coccidioidomycosis

Janis E. Blair, Yu-Hui H. Chang, Meng-Ru Cheng, Laszlo T. Vaszar, Holenarasipur R. Vikram, Robert Orenstein, Shimon Kusne, Stanford Ho, Maria T. Seville, and James M. Parish

In Arizona, USA, primary pulmonary coccidioidomycosis accounts for 15%–29% of community-acquired pneumonia. To determine the evolution of symptoms and changes in laboratory values for patients with mild to moderate coccidioidomycosis during 2010–2012, we conducted a prospective 24-week study of patients with primary pulmonary coccidioidomycosis. Of the 36 patients, 16 (44%) were men and 33 (92%) were White. Median age was 53 years, and 20 (56%) had received antifungal treatment at baseline. Symptom scores were higher for patients who received treatment than for those who did not. Median times from symptom onset to 50% reduction and to complete resolution for patients in treatment and nontreatment groups were 9.9 and 9.1 weeks, and 18.7 and 17.8 weeks, respectively. Median times to full return to work were 8.4 and 5.7 weeks, respectively. One patient who received treatment experienced disseminated infection. For otherwise healthy adults with acute coccidioidomycosis, convalescence was prolonged, regardless of whether they received antifungal treatment.

Coccidioidomycosis is a fungal infection caused by fungi of the genus *Coccidioides*. This illness is endemic to the southwestern United States. An estimated 150,000 infections occur annually, ≈60% in Arizona (1). The incidence of infection in this coccidioidomycosis-endemic area has considerably increased from 5.3 cases per 100,000 population in 1998 to 42.6 cases per 100,000 population in 2011 (2). Every year, ≈3% of area inhabitants become infected (3) through inhalation of airborne arthroconidia

Author affiliations: Mayo Clinic Hospital, Phoenix, Arizona, USA (J.E. Blair, H.R. Vikram, R. Orenstein, S. Kusne, M.T. Seville); Mayo Clinic, Scottsdale, Arizona, USA (Y.-H. H. Chang, M.-R. Cheng, L.T. Vaszar, J.M. Parish); and Arizona State University, Tempe, Arizona, USA (S. Ho)

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(spores), which results in mild to severe febrile respiratory illness (4,5). Extrapulmonary infection occurs in 1%–5% of patients with symptomatic infections (5,6).

Among Arizona patients with community-acquired pneumonia, 15%–29% have primary pulmonary coccidioidomycosis (7–9). Differentiating coccidioidal infection from pneumonia caused by viruses or bacteria is difficult. However, unlike other causes of community-acquired pneumonia, coccidioidomycosis is characterized by slow resolution of symptoms and extreme fatigue (10,11).

Symptomatic primary pulmonary coccidioidomycosis can range from mild to severe. Severe coccidioidomycosis has been defined as infection requiring hospitalization (12–14). Little research has focused on milder symptomatic forms. Although mild to moderate infection has not been clearly defined, it is characterized by symptomatic illness that does not require patient hospitalization. In the study reported here, we sought to describe the clinical course of mild to moderate pulmonary coccidioidomycosis in patients who did or did not receive antifungal therapy.

Methods

From March 1, 2010, through October 31, 2012, at Mayo Clinic in Scottsdale, Arizona, USA, we conducted a 24-week, prospective, observational study of patients with mild to moderate symptomatic primary coccidioidomycosis. Our goal was to describe the course of illness-related signs and symptoms, laboratory values, and radiographic findings. This study was approved by the Mayo Clinic Institutional Review Board and included only those patients who had previously consented to the use of their medical records for research purposes. To be eligible, patients must have been ≥18 years of age, had primary pulmonary coccidioidomycosis, been symptomatic for ≤2 months, and had ≥2 signs or symptoms at enrollment. Signs and symptoms

included (but were not limited to) fever, chills, night sweats, headache, joint aches, muscle pains, cough, rash, fatigue, inspiratory chest pain, and shortness of breath. The diagnosis was either confirmed (according to positive culture results or histologic findings) or probable (according to typical symptoms and radiographic abnormalities, with positive serologic test results). Also for patient eligibility, serologic test results were required to be positive for IgG against *Coccidioides* spp. by enzyme immunoassay (Meridian Bioscience, Inc., Cincinnati, OH, USA), immunodiffusion, or complement fixation, or for IgM by immunodiffusion. No remuneration or other incentive was provided for study participation. Exclusion criteria were as follows: hospitalization, clinical evidence of overtly extrathoracic coccidioidomycosis, laboratory or radiographic findings of severe or disseminated infection (e.g., an initial complement fixation titer $\geq 1:32$, chest radiographic abnormalities with miliary distribution, lung involvement $>50\%$, or large pleural effusion), concurrent conditions associated with increased risk for severe or disseminated coccidioidomycosis (e.g., any viral load of HIV, chemotherapy within 6 months for cancer, solid organ or hematologic transplantation, hematologic malignancy [active or remote], diabetes mellitus, or pregnancy), receipt of immunosuppressive medications (e.g., tumor necrosis factor inhibitors, calcineurin inhibitors, mycophenolate mofetil, sirolimus, or chronic oral corticosteroids [equivalent dose of >5 mg/day, excluding inhaled, topical, or limited and transient oral corticosteroids for <5 days]), concurrent cardiopulmonary conditions (e.g., pulmonary coinfection, asthma or chronic obstructive pulmonary disease, cardiomyopathy), or underlying liver disease or stage 4 or 5 kidney disease (glomerular filtration rate ≤ 29 mL/min/1.73 m²).

Treatment decisions were determined by the treating physicians, and whether a patient received antifungal therapy before enrollment was recorded (medication name, dose, frequency, duration). After patients were enrolled, physician investigators determined the need to initiate or continue antifungal medication on a case-by-case basis. Patients were assigned to the treatment group if at any time before enrollment through study completion they received any antifungal treatment.

The medical care for coccidioidomycosis was provided by physician investigators and conducted in a standardized fashion. The initial evaluation included a complete blood cell count, comprehensive metabolic panel, serologic testing for HIV, pregnancy testing, and serologic testing for coccidioidomycosis (by enzyme immunoassay, immunodiffusion, and complement fixation); collection of microbiological specimens, if applicable (mostly sputum for fungal culture); and analysis of chest radiographs. Patients were evaluated clinically, serologically, and radiographically (chest) at enrollment and at 4, 12, 16, and 24 weeks.

Signs and symptoms were assessed by using modified standardized Mycosis Study Group symptom scores previously used in coccidioidomycosis clinical trials (15–17). Symptom scores were based on answers to a questionnaire listing common symptoms of coccidioidomycosis (fever [subjective or measured], chills, night sweats, headache, joint aches, muscle pain, rash, fatigue, anorexia or weight loss, swelling, cough, shortness of breath, pain during inspiration, hemoptysis). Additional symptoms noted by patients at enrollment were added to the list. Each symptom was scored as 1 point, and points were tallied for an enrollment symptom score. Each time signs and symptoms were assessed, patients were directly asked about symptom presence or absence within the preceding week. Symptoms could be added to the score as the course of illness progressed. After patient enrollment, symptoms were tallied weekly. When this score declined to 50% of the enrollment score for 2 consecutive weeks, symptoms were assessed every 2 weeks for 2 episodes, then monthly for 6-month follow-up visits. Baseline and monthly fatigue levels were assessed by using the fatigue severity scale (10,18), and health-related quality of life was assessed by using the 36-Item Short Form Health Survey (19). Full-time or part-time attendance at work or school was recorded during assessments. The coccidioid radiology score was based on Mycosis Study Group scoring (15,16) as follows: size (1 point for lesions <5.0 cm; 2 for ≥ 5.0 cm), spread (1 point if unilateral; 2 if bilateral), and other characteristics (1 point each for pulmonary cavity, hilar lymphadenopathy, or pleural effusion).

All end points were established a priori. The primary end point was time required to achieve a 50% decrease in symptom score. Secondary end points included time to 100% symptom resolution (excluding fatigue), time to resumption of all activities of daily living, time to achieve 50% and 100% reductions in fatigue score, time to 50% reduction in Mycosis Study Group score, time to full attendance at work or school, and time to 50% improvement in quality-of-life score. Secondary end points included comparison of end points between patients in the treatment and nontreatment groups. All recorded times were normalized to time of symptom onset rather than time from enrollment.

Patient characteristics and the occurrence of symptoms were summarized as counts and percentages and were compared between treatment groups by using the χ^2 test or the Fisher exact test. The total symptom score, time to resolution, and quality-of-life summary scores were summarized as medians and interquartile ranges and were compared by using the Wilcoxon rank-sum test. The prevalence of each symptom over time was plotted. The prevalence of each symptom was modeled by the generalized estimating equation, and the difference of the prevalence between time points was evaluated statistically. All analyses were

performed by using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). All hypothesis tests were 2 sided, and statistical significance was defined as $p < 0.05$.

Results

During the study period, 45 patients with primary pulmonary coccidioidomycosis were enrolled; 9 withdrew consent or did not fully seroconvert, leaving 36 with probable infection for inclusion in the study (Figure 1). Of these, 27 (75%) patients remained in the study through week 24 of their illness, and 20 (56%) completed the entire 24 weeks of observation after enrollment. Median time from symptom onset to enrollment was 33 days.

At the time of enrollment, 16 (44%) patients had not received antifungal therapy (Table 1). Among the 20 (56%) patients who had received antifungal therapy, treatment was initiated by nonstudy medical practitioners before enrollment for 17 and by study physicians on the day of enrollment for 3. Antifungal treatment was initiated at a median of 21 days of symptoms (range 4–46 days after onset [interquartile range 11–32 days]). Of the 20 who received treatment, 18 received fluconazole at 400 mg per day for a median of 8.5 weeks (range 1.5–28.0 weeks). The median weight of patients in the treatment group was 84.8 kg (interquartile range 73–91 kg). Twenty-six patients (16 treatment, 10 nontreatment) had received empiric treatment with 1 or 2 courses of antibacterial drugs before their coccidioidomycosis diagnosis.

At enrollment, ongoing fever was more common among patients who had received treatment than among those who had not (8/20 [40%] vs. 1/16 [6.2%], respectively; $p = 0.02$), although other symptoms did not differ by group (Table 1). At enrollment, symptom scores

were higher among patients in the treatment than in the nontreatment group (median 5.5 vs. 4.0, respectively; $p = 0.02$).

In terms of occupation, 22 patients were employed and 3 were full-time students (Table 1). The median number of whole workdays missed was 10 (range 1–28 days). One student missed 10 days of school.

At enrollment, no significant differences in serologic or radiographic findings were noted among patients in the treatment and nontreatment groups (Table 1). The percentages of patients with detectable complement fixation antibody at enrollment and at 4, 12, and 24 weeks were 38% (12/32), 61% (17/28), 59% (16/27), and 23% (5/22), respectively. Peak complement fixation titers (range 1:2–1:32) occurred 4 weeks after enrollment. Radiographic scores did not differ by group; and for most patients, a unilateral radiographic abnormality < 5 cm was seen. Although radiographic abnormalities improved over time, abnormalities on chest radiographs, as reflected in median scores, did not decline from 2.0 at enrollment. At 24 weeks, one-half of the patients had residual granuloma.

Table 2 summarizes primary and secondary end points of the study and shows that times to most end points were similar for both groups; for the nontreatment and the treatment groups, the median times to 50% and 100% resolution of symptoms were 9.1 and 9.9 weeks and 17.8 and 18.7 weeks, respectively. The median times to 50% resolution of fatigue were 9.8 and 12.9 weeks, respectively. Of 27 patients, 13 (48%) indicated continued fatigue by week 24. Patients in the nontreatment group returned to full-time work sooner than did those in the treatment group (5.7 vs. 8.4 weeks, respectively) ($p = 0.02$).

Figure 2 and Table 3 summarize symptom resolution for the 36 patients over time. Although symptom curves seemed to separate, especially from week 16 on, there was no statistical significance between these curves.

The course of convalescence was typical for 35 patients and atypical for 1 patient. This previously healthy 34-year-old White man was initially seen at an external institution for a 3-week history of fever, night sweats, dry cough, headache, and rash. Serologic test results were positive for *Coccidioides* spp. by enzyme immunoassay and immunodiffusion, and chest radiographs demonstrated a 3-cm nodular infiltrate; the physician prescribed a nonstandard antifungal regimen of ketoconazole at 400 mg/day. When the patient was referred to our institution (Mayo Clinic Hospital, Phoenix, AZ, USA) for possible study participation 10 days later, his symptoms were nearly resolved and ketoconazole was discontinued by the study physician. One week after enrollment, the patient experienced a severe headache; subsequent cerebrospinal fluid analysis was consistent with aseptic meningitis, presumed to be caused by *coccidioides*. He was given

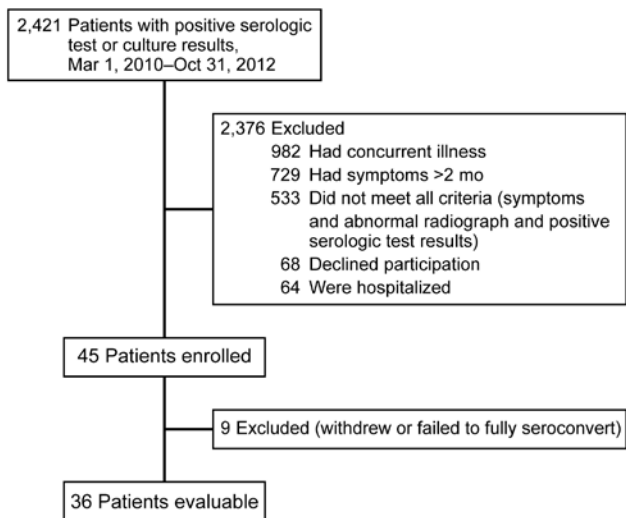


Figure 1. Coccidioidomycosis patient enrollment and exclusions, Arizona, USA, March 1, 2010–October 31, 2012.

fluconazole at 800 mg/day and promptly improved clinically. Lifelong treatment is anticipated.

Discussion

Over the past 2 decades, the incidence of coccidioidomycosis has markedly risen in the disease-endemic area (1,2). Until recently, little research has characterized the course of uncomplicated symptomatic illness, although experienced clinicians have observed that affected patients eventually recover (11,20). Each year in the disease-endemic area, an estimated 3% of the population becomes infected (3); therefore, even if 60% of the infected population is asymptomatic, the potential number of patients who may become ill enough to be unable to perform daily activities or work is substantial. This study prospectively characterized the prolonged clinical

course of patients with mild to moderate primary pulmonary coccidioidomycosis.

A 2007 survey found that patients with coccidioidomycosis undifferentiated by severity, status of dissemination, or duration (acute or chronic) recalled experiencing symptoms for a median of 120 days and missing 14 days of work or 9 days of school (21). The study was limited by its retrospective nature, relying only on the ability of patients to recall details of their illness from the previous year, and these self-reports were not correlated with severity of illness (mild vs. severe or pulmonary vs. disseminated). Other investigators have demonstrated a median time of 95–98 days (roughly 14 weeks) to 50% resolution of symptoms (11). Still others have found that fatigue is severe at baseline and 4 months later (10) and that >25% of otherwise healthy college students with primary coccidioidomycosis

Table 1. Characteristics of 36 patients with primary pulmonary coccidioidomycosis, Arizona, USA, March 1, 2010–October 31, 2012*

| Characteristic | Total, N = 36 | Antifungal treatment | | p value |
|---|------------------|----------------------|-----------------|---------|
| | | Yes, n = 20 | No, n = 16 | |
| Sex, no. (%) | | | | |
| M | 16 (44) | 11 (55) | 5 (31) | 0.15† |
| F | 20 (56) | 9 (45) | 11 (69) | 0.15† |
| Age, y, median (range) | 53 (21–79) | 52 (28–79) | 53 (21–68) | 0.48‡ |
| Race/ethnicity, no. (%) | | | | 0.65† |
| White | 33 (92)§ | 18 (90) | 15 (94) | |
| Hispanic | 1 (3) | 1 (5) | 0 | |
| Asian | 2 (6) | 1 (5) | 1 (6) | |
| Follow-up time, median (IQR), wk | 24 (24.0–24.0) | 24 (21.5–24.0) | 24 (24.0–24.0) | |
| Concurrent illness¶ | | | | |
| Rheumatologic, no. (%)# | 1 (3) | 1 (5) | 0 | 0.35† |
| Prior remote cancer, no recurrence, no./total (%)# | 3/35 (9) | 2/19 (11) | 1 (6) | 0.65† |
| Employment | | | | |
| Employed at time of coccidioidomycosis, no. (%) | 22 (61) | 14 (70) | 8 (50) | 0.22† |
| Illness resulted in work absences, no. (%) | 18/22 (82) | 12/14 (86) | 6/8 (75) | 0.53† |
| Days absent, median no. (IQR), [range] | 10 (5–14) [1–28] | 10 (5–15) [2–28] | 7 (4–12) [1–13] | 0.32‡ |
| School attendance | | | | |
| Attending at time of coccidioidomycosis, no. (%) | 3 (8) | 2 (10) | 1 (6) | 0.68 |
| Illness resulted in absences, no./total (%) | 1/3 (33) | 0 | 1/1 (100) | 0.08 |
| Coccidioidal symptoms, ever present, no. (%) | | | | |
| Fatigue | 36 (100) | 20 (100) | 16 (100) | >0.99† |
| Fever | 31 (86) | 19 (95) | 12 (75) | 0.08† |
| Chills | 32 (89) | 19 (95) | 13 (81) | 0.19† |
| Cough | 34 (94) | 18 (90) | 16 (100) | 0.19† |
| Night sweats | 29 (81) | 17 (85) | 12 (75) | 0.45† |
| Headache | 29 (81) | 16 (80) | 13 (81) | 0.93† |
| Chest pain | 25 (69) | 15 (75) | 10 (62) | 0.52† |
| Arthralgia | 25 (69) | 13 (65) | 12 (75) | 0.59† |
| Rash | 23 (64) | 12 (60) | 11 (69) | 0.58† |
| Coccidioidal symptoms score at enrollment, median (IQR) | 5.0 (3.0–7.0) | 5.5 (5.0–7.5) | 4.0 (3.0–5.0) | 0.02‡ |
| Diagnostic test results | | | | |
| Median chest radiograph score at enrollment | 2.0 | 2.0 | 2.0 | 0.72‡ |
| Positive serologic results at enrollment, no./total (%) | | | | |
| EIA IgM | 34/35 (97) | 18/19 (95) | 16/16 (100) | 0.35† |
| EIA IgG | 26/35 (74) | 12/19 (63) | 14/16 (88) | 0.1† |
| ID IgM | 14/36 (39) | 7/20 (35) | 7/16 (44) | 0.59† |
| ID IgG | 19/35 (53) | 10/20 (50) | 9/16 (56) | 0.71† |
| Positive IgG by CF | 12/32 (38) | 6/16 (38) | 6/16 (38) | >0.99† |

*IQR, interquartile range; EIA, enzyme immunoassay; ID, immunodiffusion; CF, complement fixation.

†By χ^2 test.

‡By Wilcoxon rank-sum test.

§95% CI for White race was 77.5%–98.2%.

¶No patients had pulmonary, cardiovascular, kidney, or liver disease.

#Patient was not receiving any immunosuppressive treatment or chemotherapy.

Table 2. Comparison of onset of symptoms to time to study end points among 36 patients with primary pulmonary coccidioidomycosis, Arizona, USA, March 1, 2010– October 31, 2012*

| Study end point | Antifungal treatment | | p value |
|--|----------------------|------------|---------|
| | Yes, n = 20 | No, n = 16 | |
| Time to 50% reduction in symptom score, wk | | | 0.84† |
| Median | 9.9 | 9.1 | |
| Q1, Q3 | 7.0, 13.4 | 7.4, 17.1 | |
| Range | 4.0–24.0 | 3.7–24.4 | |
| Time to complete symptom resolution, wk | | | 0.65‡ |
| Median | 18.7 | 17.8 | |
| Q1, Q3 | 13.6, 25.0 | 12.1, 24.0 | |
| Range | 8.9–29.6 | 8.7–27.1 | |
| Time to 50% reduction in fatigue, wk§ | | | 0.59‡ |
| Median | 12.9 | 9.8 | |
| Q1, Q3 | 8.0, 16.0 | 8.4, 15.9 | |
| Range | 5.0–29.6 | 4.4–25.6 | |
| Time to full attendance at work, wk | | | 0.02† |
| Median | 8.4 | 5.7 | |
| Q1, Q3 | 6.7, 14.8 | 5.1, 6.0 | |
| Range | 9.0–29.6 | 2.4–7.6 | |
| Time to full attendance at school, wk | | | |
| Median | | 13.7 | |
| Q1, Q3 | | 13.7, 13.7 | |
| Time to 50% improvement in PCS, wk | | | 0.08‡ |
| Median | 20.4 | 13.8 | |
| Q1, Q3 | 14.7, 26.0 | 10.4, 18.0 | |
| Time to 50% improvement in MCS, wk | | | 0.21‡ |
| Median | 12.0 | 8.1 | |
| Q1, Q3 | 9.3, 14.7 | 6.6, 9.7 | |

*Q1, first quartile; Q3, third quartile; PCS, physical activity score as measured on the SF-36 General Health Survey; MCS, mental activity score as measured on the SF-36 General Health Survey.

†By the Wilcoxon rank-sum test.

‡By the equal variance *t* test.

§As reported on the Fatigue Severity Scale.

required medical care for at least 4 months (22). Although our study focused only on persons with mild to moderate infection and no substantial concurrent conditions, results are similar to those of previous studies (long duration of symptoms, fatigue, and illness caused by primary coccidioidal infection).

In our study, we noted a typical pattern of clinical resolution and resumption of normal activities. Fever and chills were relatively short-lived (days to weeks), but other symptoms, such as cough and fatigue, lasted weeks to months.

Figure 2 delineates resolution of symptoms over time among patients in the treatment and nontreatment groups. Statistical comparison of these curves did not identify any differences. For many symptoms, a bimodal curve appeared for the treatment group but not for the nontreatment group, suggesting that patients who received treatment experienced more symptoms in the second half of the observation period. However, when we fitted a longitudinal model to examine whether a difference existed between weeks 16 and 20 for each of the symptoms, no statistically significant differences were identified.

Most (82%) patients missed work for a median of 10 workdays. Those in the treatment group did not miss more workdays than those in the nontreatment group, but they did return to full-time employment more slowly (median 8.4 vs. 5.7 weeks). Whether this finding was the result of

more severe illness in patients in the treatment group or other factors is not certain. However, this lost work productivity highlights the potentially profound economic cost of this illness in the coccidioidomycosis-endemic area.

Although the current study was strictly observational, we enrolled similar numbers of patients with similar demographic characteristics, regardless of treatment received. We classified the groups by any antifungal treatment and classified patients as having received treatment even if treatment was given for a short time or at a sub-optimal dosage. Two patients received treatment for <1 month (1.5 weeks and 2 weeks), and another received nonstandard treatment at an external institution (ketoconazole at 400 mg/day). However, because our results showed no difference with and without inclusion of such patients (data not shown), these data did not influence our overall findings. Of 20 patients in the treatment group, 18 received fluconazole at 400 mg/day for a median duration of 8.5 weeks. For some patients, antifungal medications were discontinued because of medication intolerance; others initially received antifungal treatment from non-study medical providers and were subsequently determined by study physicians to not require treatment, which was then discontinued. The optimal duration of treatment for mild to moderate coccidioidomycosis has never been defined, and the fact that discontinuation of some

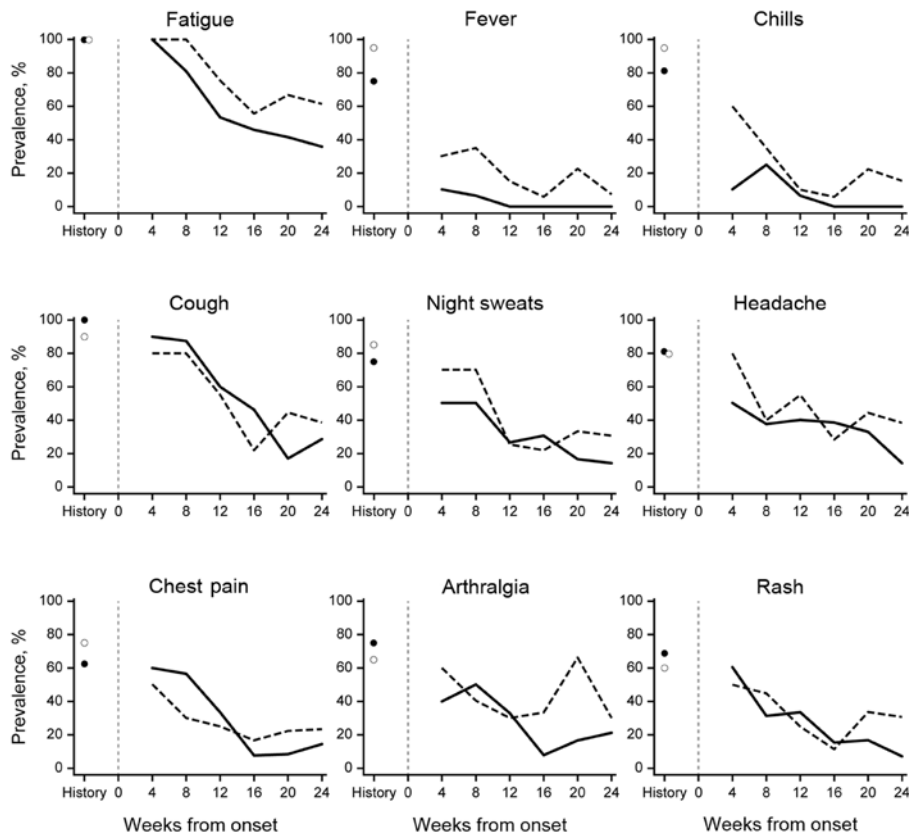


Figure 2. Presence of coccidioidomycosis symptoms from time of symptom onset, by treatment group, Arizona, USA, March 1, 2010–October 31, 2012. The graphs represent the percentages of patients who reported each symptom, from the time since onset of symptomatic illness. Solid lines and filled circles indicate the nontreatment group, and dashed lines and open circles indicate the treatment group. The vertical line indicates time of symptom onset. The circles to the left of the vertical line indicate the presence of symptoms at any time, including before study enrollment.

antifungal medications occasionally preceded complete resolution of prolonged symptoms (e.g., occasional cough or prolonged fatigue) reflected the practices of our clinical investigators. Neither study design nor study power enabled identification of small differences in the clinical courses of disease among patients in the treatment versus nontreatment groups. However, we did not identify any clinical end points that showed a benefit to patients in the treatment group, who were statistically more symptomatic according to symptom score at study enrollment. Our results are similar to those of Ampel et al. (11), who found that patients in treatment and nontreatment groups reached 50% symptom reduction at the same time.

Patients in this study began receiving antifungal treatment at a median of 21 days from the onset of coccidioidomycosis symptoms. Most patients sought care from their medical providers within days of symptom onset, but when they had no clinical response to empirically prescribed antibacterial agents, further testing identified the coccidioidal etiologic agent of disease. Therefore, the delay in early treatment probably reflects the lack of recognition of coccidioidal illness (which causes nonspecific symptoms), the lack of an early and reliable diagnostic test, or both.

Although the Infectious Diseases Society of America treatment guidelines acknowledge differences of expert

opinion regarding the need to treat primary coccidioidomycosis (23), the guidelines suggest identifying characteristics to facilitate diagnosis of moderate to severe infection in patients who might benefit from treatment (23). These guidelines recommend possible antifungal treatment for patients with symptoms lasting >2 months, night sweats >3 weeks, weight loss of >10%, inability to work, serologic complement fixation titer >1:16, bilateral infiltrates or involvement of at least one half of 1 lung, or prominent or persistent hilar adenopathy (23). In retrospect, many of the patients in our nontreatment group met ≥ 1 of these criteria, yet their illness resolved no more slowly than that of patients in the treatment group; this finding mandates further study to determine which patients with mild to moderate pulmonary coccidioidomycosis will benefit from antifungal treatment.

One challenge posed by previous and current studies is the lack of tests sensitive enough to identify coccidioidal infection early in its course. All current serologic tests take a few weeks to several weeks to show positivity (24). Because of potential diagnostic delays resulting from delayed seroconversion or the lack of clinical recognition of primary pulmonary coccidioidomycosis, patients received a diagnosis, received treatment, and were enrolled in the study at variable points relative to illness onset, which was

Table 3. Symptoms reported after onset of coccidioidal illness among 36 patients with primary pulmonary coccidioidomycosis, Arizona, USA, March 1, 2010–October 31, 2012

| Symptom* | No. (%) patients | | | | |
|--------------|------------------|----------------|-----------------|-----------------|-----------------|
| | Week 4, n = 20† | Week 8, n = 36 | Week 12, n = 35 | Week 16, n = 31 | Week 24, n = 27 |
| Fatigue‡ | 20 (100) | 33 (92) | 22 (66) | 16 (52) | 13 (48) |
| Fever | 4 (25) | 8 (22) | 3 (9) | 1 (3) | 1 (4) |
| Chills | 7 (35) | 11 (31) | 3 (9) | 1 (3) | 2 (7) |
| Cough | 17 (85) | 30 (83) | 20 (57) | 10 (32) | 9 (33) |
| Night sweats | 12 (60) | 22 (61) | 9 (26) | 8 (26) | 6 (22) |
| Headache | 13 (65) | 14 (39) | 17 (49) | 10 (32) | 7 (26) |
| Chest pain | 11 (55) | 15 (42) | 10 (29) | 4 (13) | 5 (19) |
| Arthralgia | 10 (50) | 16 (44) | 11 (31) | 7 (23) | 7 (26) |
| Rash | 11 (55) | 14 (39) | 10 (29) | 4 (13) | 5 (19) |

*As reported by patients responding directly to a question about presence of the symptom.

†Sixteen patients had not yet enrolled in the study by the fourth week of symptom onset.

‡By direct question for presence of fatigue, not by Fatigue Severity Score.

typically 4–7 weeks. Therefore, the end points of the study were normalized to symptom onset.

Some potential participants were excluded from the study for lack of any positive serologic results other than detection of IgM by enzyme immunoassay. For some, an IgG response might have been inhibited by preexisting treatment with fluconazole, which has been reported (25). For others, detection of IgM by enzyme immunoassay might have been associated with false positivity, although published reports are divided on this point (26,27). Thus, in our effort to ensure that all study participants truly had coccidioidomycosis, we might have excluded patients with an incomplete serologic response.

Other limitations of this study are noteworthy. The study is small because we were able to identify and study only 36 patients within the given time frame. During enrollment, we recognized that some patients with typical signs and symptoms and coccidioidal seroconversion lacked identifiable abnormalities on chest radiographs early in their illness or had typical radiographic abnormalities but no definitive serologic test results. Thus, we probably excluded patients who had even milder forms of infection. Other enrollment difficulties included a delay to recognition and diagnosis beyond 2 months of illness, restrictive exclusion criteria, and the requirement for frequent follow-up visits at specific times over an extended period. Study dropout was a problem because the study was long, repetitive, and time intensive. Although most (27/36 [75%]) patients continued in the study through week 24 of their illness, 9 (25%) did not, which resulted in low numbers at the end of the study and reduced percentages of patients with various symptoms. More dropouts came from the treatment than the nontreatment group (7 vs. 2), and some patients dropped out before becoming asymptomatic. Last, if a patient's symptoms had a different cause (e.g., viral bronchitis or fluconazole-related rash) during follow-up, their symptoms might have been misinterpreted by the patient and reported as coccidioidal symptoms; therefore, our results must be interpreted with caution.

Our study cohorts were selected for the absence of concurrent illnesses that might otherwise have affected manifestations or outcomes of coccidioidal illness, which might have given them the best possible course of illness resolution. We also enrolled primarily White patients (reflecting the 85% White [non-Hispanic] population of Maricopa County [28] and the proportion of White patients with coccidioidal infection reported to the Arizona Department of Health Services [82%] [29]); disseminated infection is generally less likely to develop in members of this group than in members of other racial or ethnic groups (e.g., Africans or Filipinos) (30). This narrow cohort limits the generalizability of our findings to other patient groups.

In conclusion, we believe that mild to moderate primary pulmonary coccidioidomycosis is a consequential illness that affects numerous persons residing in or traveling to the disease-endemic area. Our detailed description of patients with mild to moderate signs and symptoms of infection and the slow resolution of those signs and symptoms over time can better inform diagnosis, treatment, and prognosis for patients with coccidioidomycosis. Although we found no benefit from antifungal treatment, the study was neither designed nor powered to optimally address that issue. Given that the coccidioidal illness is substantial and prolonged, further study is warranted to optimally identify and treat this condition in such patients.

Dr Blair is an infectious diseases consultant at Mayo Clinic, Scottsdale, Arizona, and a professor of medicine at Mayo Clinic College of Medicine. Her research interests include the study of coccidioidomycosis in immunosuppressed and healthy hosts.

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Address for correspondence: Janis E. Blair, Division of Infectious Diseases, Mayo Clinic Hospital, 5777 E Mayo Blvd, Phoenix, AZ 85054, USA; email: blair.janis@mayo.edu

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Human Polyomavirus 9 Infection in Kidney Transplant Patients

Els van der Meijden, Herman F. Wunderink, Caroline S. van der Blij-de Brouwer, Hans L. Zaaijer, Joris I. Rotmans, Jan Nico Bouwes Bavinck, and Mariet C.W. Feltkamp

Several human polyomaviruses of unknown prevalence and pathogenicity have been identified, including human polyomavirus 9 (HPyV9). To determine rates of HPyV9 infection among immunosuppressed patients, we screened serum samples from 101 kidney transplant patients in the Netherlands for HPyV9 DNA and seroreactivity. A total of 21 patients had positive results for HPyV9 DNA; positivity rates peaked at 3 months after transplantation, but the highest viral loads were measured just after transplantation. During 18 months of follow-up, HPyV9 seroprevalence increased from 33% to 46% among transplant patients; seroprevalence remained stable at \approx 30% in a control group of healthy blood donors in whom no HPyV9 DNA was detected. Further analysis revealed an association between detection of HPyV9 and detection of BK polyomavirus but not of cytomegalovirus. Our data indicate that HPyV9 infection is frequent in kidney transplant patients, but the nature of infection—endogenous or donor-derived—and pathogenic potential of this virus remain unknown.

The *Polyomaviridae* constitute a family of small DNA viruses that infect a variety of hosts. BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV), discovered in 1971 (1,2), are well-known examples of human polyomaviruses (HPyVs) that cause severe disease in immunocompromised patients. Serologic data have revealed that most polyomaviruses are ubiquitous (3–6). In case of JCPyV and BKPyV, primary infection occurs early in life, without apparent symptoms, and persists throughout life as latent infection in the kidneys, accompanied by occasional virus shedding in urine (7). When immunity is decreased, these viruses can reactivate with detectable viremia and manifestation of disease, which poses a threat to, among others, patients who receive solid-organ transplants.

Author affiliations: Leiden University Medical Center, Leiden, the Netherlands (E. van der Meijden, H.F. Wunderink, C.S. van der Blij-de Brouwer, J.I. Rotmans, J.N. Bouwes Bavinck, M.C.W. Feltkamp); and Sanquin Blood Supply Foundation, Amsterdam, the Netherlands (H.L. Zaaijer)

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For kidney transplant patients, BKPyV infection is considered the most common viral complication and causes nephropathy and graft loss in 1%–10% of cases if left untreated (8). It is not known what determines the severity of BKPyV infection and whether co-infection is involved in the pathogenesis.

Since 2007, at least 10 novel HPyVs have been discovered (9–20); of these, Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (TSPyV) have been shown to be associated with disease (11,19,21,22). Human polyomavirus 9 (HPyV9), so far without a disease association, was identified in 2011 from a serum sample from a kidney transplant patient (17). Overall seroprevalence of HPyV9 has been determined to be 25% to 50% (23–26).

Because HPyV9 was originally isolated from a kidney transplant recipient (17), we aimed to systematically study the presence of HPyV9 infection in kidney transplant patients and investigate a possible association with the known nephropathogenic BKPyV. We analyzed a cohort of 101 transplant patients who received either a kidney transplant or a simultaneous kidney–pancreas transplant for the appearance of markers for HPyV9 infection during the 18 months after transplantation. We assessed the presence of HPyV9 DNA and IgG seroresponses in serum samples. The HPyV9 findings in the transplant cohort were compared with those obtained for an age- and sex-matched cohort of healthy blood donors. Co-infection with BKPyV was investigated by comparing observed HPyV9 and BKPyV viremia levels in the transplant cohort. For comparative purposes, we also tested for cytomegalovirus (CMV), which, like polyomaviruses, frequently reactivates during immunosuppressive drug use after transplantation.

Materials and Methods

Study Population

The cohort study consisted of 101 patients who received kidney (n = 83) or kidney–pancreas (n = 18) transplants during 2002–2004 at Leiden University Medical

Center (LUMC), Leiden, the Netherlands (Table 1). This study population is part of a larger prospective European multicenter study designed to investigate the role of human papillomavirus infection in the development of skin cancer in solid-organ transplant patients (27). The study adhered to the Declaration of Helsinki Principles, and the medical ethical committee of the LUMC approved of the study design (Medical Ethical Committee no. P02.111). Participants gave written informed consent.

All patients received induction with interleukin-2 receptor blocker daclizumab (100 mg/d) on the day of transplantation and 10 days after transplantation or basiliximab (40 mg at days 0 and 4), followed by triple therapy with prednisone, tacrolimus, or cyclosporine and mycophenolate mofetil. For kidney transplant patients, the dose of the calcineurin inhibitor (tacrolimus or cyclosporine) was tapered at 6 weeks after transplantation, whereas for kidney-pancreas transplant patients, the calcineurin inhibitor was reduced at 3 months after transplantation.

The time points of serum sample collection and the number of samples collected per time point are summarized in Table 2 and shown in relation to the date of transplantation. The baseline samples were obtained in the days immediately after transplantation (time point 0, T0). To collect the subsequent samples, patients were asked to visit the LUMC outpatient clinic for follow-up sample collection at the preferred time points of 3, 6, 9, 12, and 18 months after transplantation (T3–T18). A total of 58 patients provided a sample at all 6 time points: 31, 6, 4, and 2 patients provided 5, 4, 3, or 1 samples, respectively.

Stored pretransplantation serum samples, if available, were retrieved and tested for HPyV9 DNA ($n = 65$; Table 2) and antibodies ($n = 45$, 40 of which were also included in pretransplantation DNA testing). The average dates of obtaining samples for DNA and antibody testing were 8 and 2 days before transplantation, respectively.

To obtain a healthy control population, we analyzed anonymized samples from 87 random unpaid blood donors (Table 2). For each donor, 2 follow-up serum samples were studied, collected 1 year apart (T0 and T12). The donors were matched for age and sex with the transplant patient study population (Table 1).

Viral DNA Detection and Quantification

Total nucleic acids were extracted from 200 μ L serum by using the MagNA Pure LC Total Nucleic Acid Isolation Kit–High Performance and MagNA Pure LC Instrument (Roche Diagnostics, Indianapolis, IN, USA). To monitor the quality of DNA extraction and potential PCR inhibition, we added low concentrations of phocine herpesvirus (28) to the lysis buffer. DNA was eluted in a final volume of 100 mL elution buffer, of which 10 mL was used as input for real-time quantitative PCR (qPCR).

Primers and Taqman probes were designed by using Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA). For HPyV9, we used the following primers and probe, located in the viral protein (VP) 1 gene amplifying a product of 109 nt: sense primer 5'- CCT-GTAAGCTCTCCTTA-3', antisense primer 5'- CCT-GATAAATTCTGACTTCTTC-3', and probe FAM-5'-CTTGTTCTCTGGTCTTATGCCTCA-3'-BHQ1. For BKPyV, we used the following primers and probe, located in the VP1 gene amplifying a product of 90 nt: sense primer 5'-GAAAAGGAGAGAGTGTCAGGG-3', antisense primer 5'-GAACTTCTACTCCTCCTTTTATTAGT-3', and probe FAM-5'-CCAAAAGCCAAAGGAACCC-3'-BHQ1.

The BKPyV qPCR and phocine herpesvirus PCR were duplexed for DNA quality and potential PCR inhibition monitoring. Furthermore, the BKPyV qPCR was validated to detect BKPyV genotypes I–IV. qPCR reactions were performed in a total volume of 50 μ L, containing 25 μ L HotStart Taq mastermix (QIAGEN, Hilden, Germany), 0.5 μ mol/L of each primer, 0.35 μ mol/L BKPyV probe or 0.4 μ mol/L HPyV9 probe, and 3.5 mmol/L $MgCl_2$. Reactions were performed by using a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) with the following cycle conditions: 15 min at 95°C followed by 45 cycles of amplification (30 s at 95°C; 30 s at 60°C for HPyV9 qPCR and 55°C for BKPyV qPCR; 30 s at 72°C). For quantification, a standard of pGEX 5 \times 3 HPyV9 VP1 plasmid (Genscript, Piscataway, NJ, USA) and of a quantified BKPyV-positive urine sample were used. Analytical sensitivity of the HPyV9 and BKPyV qPCRs was \approx 10 copies/mL. CMV load was measured as described (29).

Table 1. Characteristics of patients and controls for study of human polyomavirus 9 prevalence among kidney transplant patients, the Netherlands*

| Characteristic | Transplant patient type | | | p value | Blood donors |
|-----------------------|-------------------------|------------|-----------------|---------|--------------|
| | All | Kidney | Kidney–pancreas | | |
| Patients and controls | 101 | 83 (82) | 18 (18) | | 87 |
| Mean age, y (range) | 47 (21–74) | 48 (21–74) | 43 (30–54) | 0.129† | 52 (29–68) |
| Sex | | | | | |
| F | 34 (34) | 27 (33) | 7 (39) | 0.605‡ | 31 (36) |
| M | 67 (66) | 56 (67) | 11(61) | | 56 (64) |

*Values are no. (%) except as indicated.

†Comparison of kidney and kidney–pancreas patient groups; Student t test.

‡Comparison of kidney and kidney–pancreas patient groups; χ^2 test.

Table 2. Detection of human polyomavirus 9 DNA and viral loads in kidney transplant patients and blood donors, the Netherlands*

| Population | Mean time after transplantation or first sample collection, mo (range) | No. samples | No. (%) HPyV9 DNA positive | Mean viral load, copies/mL (range) |
|----------------------------------|--|-------------|----------------------------|------------------------------------|
| Transplantation patients | | 101 | 21 (20.8)† | 157 (25–530)‡ |
| Transplant type | | | | |
| Kidney | | 83 | 17 (20.5)† | 135 (25–530)‡ |
| Kidney and pancreas | | 18 | 4 (22.2)† | 250 (89–472)‡ |
| No. serum samples | | 541 | 27 (5.0) | 137 (25–530) |
| Mo after transplant | | | | |
| Pretransplant§ | –0.3 (–1.4 to 0) | 65 | 0 | NA |
| 0 | 0.4 (0.1–1.2) | 99 | 3 (3.0) | 203 (141–265) |
| 3 | 3.5 (2.3–5.5) | 98 | 7 (7.1) | 172 (52–530) |
| 6 | 6.5 (5.5–9.6) | 97 | 6 (6.2) | 141 (25–472) |
| 9 | 9.6 (7.6–12.6) | 80 | 5 (6.3) | 125 (45–213) |
| 12 | 12.6 (9.3–16.0) | 87 | 4 (4.6) | 80 (66–92) |
| 18 | 18.2 (16.0–21.3) | 80 | 2 (2.5) | 51 (38–63) |
| Blood donors | | 87 | 0 | NA |
| No. serum samples | | 174 | 0 | NA |
| Mo after first sample collection | | | | |
| 0 | 0 | 87 | 0 | NA |
| 12 | 13.4 (9.9–18.1) | 87 | 0 | NA |

*NA, not applicable.

†Patients HPyV9 positive in the follow-up period after transplant.

‡Mean load of HPyV9 DNA–positive patients based on the first positive sample per patient.

§Pretransplant samples were retrieved from the serum sample archive at the Leiden University Medical Center Clinical Microbiology Laboratory (Leiden, the Netherlands).

with minor adjustments: 0.5 $\mu\text{mol/L}$ of each primer, 0.2 $\mu\text{mol/L}$ probe, and HotStart Taq mastermix (QIAGEN) was used with the following cycle conditions: 15 min at 95°C, followed by 45 cycles of amplification (5 s at 95°C, 15 s at 63°C, 15 s at 72°C). Analytical sensitivity of the CMV qPCR was ≈ 100 copies/mL. On each plate, 3 negative controls were included; these controls tested negative in all PCR assays. PCR results with a cycle threshold ≥ 40 were considered negative.

HPyV9 DNA Sequencing

HPyV9-positive PCR samples were confirmed by sequencing. PCR products were cloned by using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and subsequently sequenced. Sequence reactions were performed by using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

HPyV9 Serologic Testing

To detect IgG seroresponses against the major capsid protein VP1 of HPyV9, we performed an antibody-binding assay using Luminex xMAP technology (30), as described (26). Briefly, the assay is based on cross-linking of glutathione to casein, which is subsequently coupled to fluorescent polystyrene beads (Bio-Rad). Glutathione S-transferase HPyV9 VP1 fusion protein was affinity purified on the beads. Serum samples were tested in a 1:100 dilution, and VP1-bound antibodies were detected with biotinylated goat anti-human IgG (H+L; Jackson Immuno Research, West Grove, PA, USA), followed by streptavidine-R-phycoerythrin

(Invitrogen). Finally, the beads and the phycoerythrin signal were analyzed in a Bio-Plex 100 Analyzer (Bio-Rad), which gave results in median fluorescent intensity (MFI). For background correction, MFI values of glutathione S-transferase alone were subtracted to obtain HPyV9 VP1-specific signals. Quality control was performed on each plate with a serum pool consisting of 4 serum samples that had been analyzed in a 1:4 serial dilution, starting with a dilution of 1:100 up to 1:409,600. Little interplate variance was observed.

Cutoff Value Determination

The cutoff value of the antibody-binding assay was defined on the basis of a group of healthy children 0.5–2 years of age and determined as described by van der Meijden et al. (26). The transplant patients and the healthy blood donors were analyzed in 2 independent antibody-binding assays. HPyV9 cutoff values of 252 MFI and 311 MFI were determined for the transplant patient group and the blood donor group, respectively.

Statistical Analyses

Differences between groups in terms of HPyV9 DNA or seroprevalence were assessed by using the Pearson χ^2 or Fisher exact test, as appropriate for population size. Independent Student *t* tests or analyses of variance were used for comparisons of mean values between groups. Occurrence of HPyV9 viremia at transplantation was calculated by using the Kaplan-Meier method, with the time from transplantation to the next detected HPyV9 DNA as the outcome variable for HPyV9-seronegative and -positive patients at baseline (T0). HPyV9 seroconversion at

transplantation was calculated by using the Kaplan-Meier method with the time from transplantation to the next seropositive sample as the outcome variable for HPyV9 viremic and nonviremic patients during follow-up. For all tests, 2-tailed p values ≤ 0.05 were considered significant. The statistical analyses in this study were performed by using SPSS 20 (IBM, Armonk, NY, USA) and Prism 3 statistical software (GraphPad Software Inc., San Diego, CA, USA).

Results

HPyV9 Viremia in Kidney Transplant Patients

To determine HPyV9 viremia in the transplant patients, we assessed the presence of HPyV9 DNA in the complete sample set. During the 18 months after transplantation, HPyV9 DNA was detected at some point in 21 (20.8%) of the 101 patients (Table 2). No significant difference in the detection of HPyV9 DNA was observed between kidney and kidney-pancreas transplant patients (20.5% vs. 22.2%, respectively). For 3 (3.0%) patients, results were positive for consecutive serum samples; persistent HPyV9 DNA detection throughout the follow-up period was observed for 1 patient. Shortly after transplantation (T0, on average 11 days after transplantation), HPyV9 DNA was detected in 3.0% of patients. Detection of HPyV9 DNA peaked 3 months after transplantation (7.1% positivity) and gradually decreased to 2.5% 18 months after transplantation (Figure 1; Table 2).

Lack of HPyV9 Viremia before Transplantation and in Controls

To explore the possibility that the observed viremia was not related to the transplant and immunosuppression but to the underlying cause of the kidney disease (e.g., diabetes), we retrieved and analyzed pretransplantation serum samples for 65 (64%) of the 101 transplant patients. In addition, the group of 87 healthy blood donors was analyzed for the

presence of HPyV9 DNA. No DNA was detected in either of these sample sets. To confirm the HPyV9-specificity of our PCR findings in the transplant patients, we cloned and sequenced 13 of the 27 HPyV9-positive PCR products, 109 nt in length. The results revealed a complete match with the described HPyV9 DNA sequence in GenBank (accession no. NC_015150) for 12 of 13 samples. In 1 sample, a single nonsynonymous nucleotide mismatch was observed (A→G at position 2403), resulting in an I321V amino acid mutation in the VP1 capsid protein.

Peak of HPyV9 Viral Load Immediately after Transplantation

The mean HPyV9 DNA load after transplantation was 137 copies/mL (range 25–530 copies/mL). On average, the highest viral loads were observed immediately after transplantation (Figure 1). The kidney-pancreas transplant patients tended to show higher HPyV9 DNA loads than did kidney transplant patients (mean values of 250 and 135 copies/mL, respectively; Table 2), but this difference was not statistically significant ($p = 0.123$ by Student t test).

HPyV9 Seroreactivity Increase in Transplant Patients but Not in Controls

HPyV9 seroresponses were analyzed for the complete sample set. At baseline, just after transplantation (T0), 33% of transplant patients were HPyV9 seropositive. This percentage corresponds to the percentages that we measured in healthy blood donors (29%) and in 45 pretransplantation serum samples (31%). However, at 1 year after transplantation (T12), the seropositivity rate for transplant patients rose to 46%. This percentage differed significantly from the rate measured for blood donors, which remained stable at $\approx 30\%$ during 1 year of follow-up ($p = 0.029$ by χ^2 test) (Figure 2, panel A). In total, 15 (15%) of 101 transplant patients seroconverted during follow-up (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/6/14-0055-Techapp1.pdf); these patients represent 23% (15/66) of the patients who were seronegative at baseline.

The intensity of measured HPyV9 serologic responses also increased after transplantation, whereas HPyV9 seroreactivity in blood donors was lower at baseline and remained low within a comparable follow-up period of 1 year (Figure 2, panel B). Mean HPyV9 seroreactivity in the 1-year follow-up samples was significantly higher for the transplant group than for blood donors ($p = 0.008$ by Student t test). Further analysis of the transplant population revealed that kidney-pancreas transplant patients in particular were responsible for the observed increase in HPyV9 seroreactivity after transplantation (Figure 2, panels C, D). Kidney-pancreas transplant patients had lower mean seroreactivity at baseline than did kidney transplant patients (Figure 2, panel D); the relative increase of seroreactivity

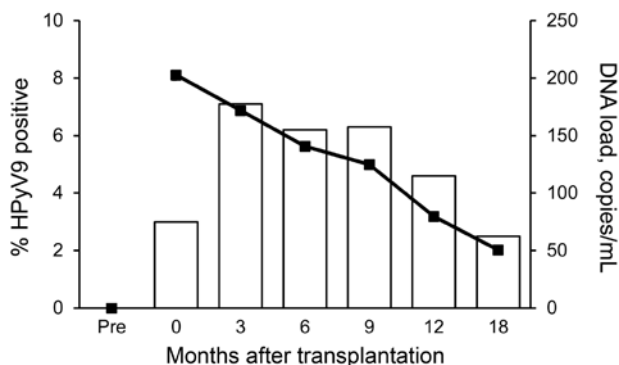


Figure 1. Human polyomavirus 9 (HPyV9) DNA positivity and mean DNA viral load in transplant patients over time, the Netherlands. Bars indicate percentage of HPyV9-positive patients; line indicates DNA load. Time points are shown as described in Table 2. Pre, pretransplant (baseline).

in kidney–pancreas transplant patients was confirmed by analyzing the complete dataset with a mixed model analysis ($p = 0.003$; data not shown).

No Correlation between HPyV9 Viremia and Seroreactivity

Because HPyV9 DNA detection and seroresponses increased after transplantation, we investigated the correlation between these parameters. Comparable proportions of patients who were HPyV9-seropositive and -seronegative at baseline became HPyV9 DNA-positive during follow-up (6/35 [17%] and 15/66 [23%], respectively; $p = 0.510$ by χ^2 test) (Figure 3, panel A), and the measured mean viral loads were comparable for the 2 groups (169 and 152 copies/mL, respectively; $p = 0.798$ by Student t test). Furthermore, we analyzed whether the presence of HPyV9 DNA influenced HPyV9 seroreactivity during follow up and found no association (Figure 3, panel B); we also found no association when we compared HPyV9 DNA positivity between high and low seroresponders (above and below median MFI) and seroconverters (data not shown). Stratified analyses for kidney–pancreas and kidney transplant patients did not alter the lack of association. HPyV9 DNA and seroreactivity profiles for patients who seroconverted and/or became viremic during follow-up are shown in the online Technical Appendix.

Correlation between HPyV9 and BKPyV Viremia

Of 541 samples tested, 225 (42%) were BKPyV DNA positive; these samples came from 86 (85%) of the 101 transplant patients. HPyV9 DNA was detected significantly more frequently in BKPyV DNA–positive samples than in BKPyV DNA–negative samples (9.8% vs. 1.6%, respectively; $p < 0.001$ by χ^2 test) (Figure 4, panel A). During follow-up, HPyV9 DNA was more often detected in BKPyV DNA–positive patients than in BKPyV–negative patients (23.3% vs. 6.7%, respectively; $p = 0.185$ by Fisher exact test) (Figure 4, panel B). Furthermore, we divided BKPyV viremic patients into 2 groups, those with high ($>10^3$ copies/mL) and low ($<10^3$ copies/mL) BKPyV DNA loads, and found HPyV9 DNA–positive patients were overrepresented among patients with high BKPyV loads ($p = 0.001$ by Fisher exact test; Figure 4, panel C). For 11 (55%) of 20 co-infected patients, BKPyV viremia coincided with HPyV9 viremia; for 8 (40%), BKPyV viremia preceded HPyV9 viremia.

We additionally assessed the presence of viremia caused by CMV, a herpes virus that is not phylogenetically related to HPyV9 and not particularly related to urinary tract infections but that frequently reactivates during immunosuppressive drug use after transplantation. CMV DNA was detected at some point after transplantation in 27 (27%)

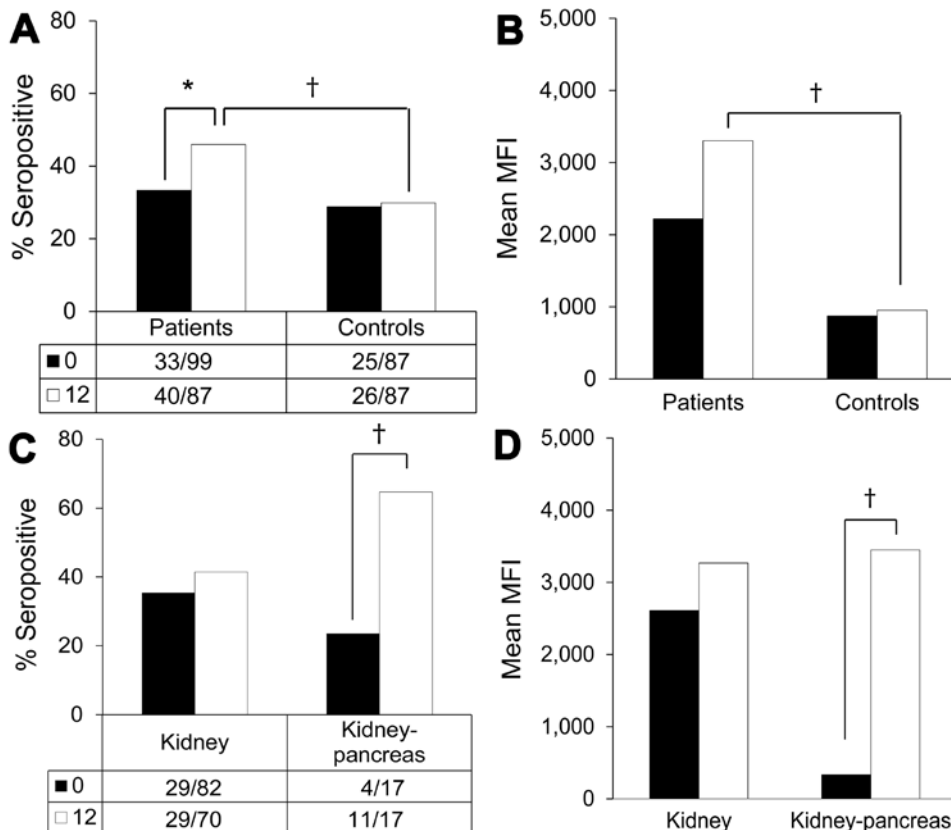


Figure 2. Human polyomavirus 9 (HPyV9) seropositivity and seroreactivity in samples from transplant patients and healthy blood donor controls collected 1 year apart, the Netherlands. Black bars, baseline samples; white bars, follow-up samples (Table 2). Values below bars indicate no. persons positive/total no. tested. A) Seropositivity percentages for transplant patients and controls; B) seroreactivity levels for transplant patients and controls; C) seropositivity percentages for kidney transplant and kidney–pancreas transplant patients; D) seroreactivity levels for kidney transplant and kidney–pancreas transplant patients. MFI, median fluorescent intensity. *Borderline significant ($0.05 < p < 0.100$); †significant ($p < 0.05$).

of the 101 patients (43 [8%] of 541 tested samples). The proportion of HPyV9 DNA–positive samples was similar among CMV DNA positive and negative samples (7% and 5%, respectively; Figure 4, panel A), and no associations were found when comparing HPyV9 DNA positivity and seropositivity among patients who were negative or positive for CMV DNA (Figure 4, panel B; data not shown).

Discussion

We systematically assessed the presence of HPyV9 DNA and IgG responses in posttransplantation serum sam-

ples from kidney transplant patients. These markers of viremia and seroreactivity were shown to increase after transplantation, indicative of active HPyV9 infection, whereas the levels remained stable in matched healthy blood donors.

HPyV9 viremia was detected in 21% of transplant patients at some point within 18 months after transplantation. Most patients were viremic at a single time point, predominantly 3 months after transplantation. The highest mean viral loads were observed immediately after transplantation and decreased gradually over time, but overall HPyV9 loads were low (25–530 copies/mL). Repeat analysis of the complete sample set of 541 serum samples reconfirmed HPyV9 viremia in the same patients (data not shown). At the same time, reanalysis of our cohort showed that the time of a positive finding sometimes differed within viremic patients, compatible with the idea that the viral loads are generally low in persons with HPyV9 viremia and sometimes fall below the PCR detection limit.

Since the identification of HPyV9 in 2011 (17), one study has reported detection of the virus in blood from 2% of immunosuppressed patients (31), whereas other studies did not find HPyV9 (32,33). These studies did not report the time of sampling in relation to transplantation and immunosuppression. Our data suggest that active HPyV9 infection is particularly found in the first year after transplantation. After 18 months, only 2.5% of our transplant patients were HPyV9 DNA–positive, with a mean viral load of 51 copies/mL. The use of different primer sets (and probes) in different studies, with different specificity and sensitivity for the detection of HPyV9 DNA, hampers an accurate comparison among studies.

We observed a peak in HPyV9 DNA detection and load in the first 3 months after transplantation, which coincides with the highest dose of immunosuppressive medication administered to these patients. HPyV9 DNA was not detected in serum samples from patients before transplantation or in serum samples from healthy blood donors. Taken together, these observations indicate a close relationship between active HPyV9 infection and transplantation and/or immunosuppression. The higher mean viral load detected in patients who received a combined kidney–pancreas transplant might be the result of the more intensified immunosuppressive regime applied to these patients. Alternatively, the underlying cause of kidney failure might predispose patients for more frequent HPyV9 infection: 94% of kidney–pancreas transplant patients had diabetes, compared with only 6% of kidney transplant patients.

During follow-up, HPyV9 seroprevalence significantly increased among transplant patients, from 33% to 46%, but remained stable at ≈30% in a control group of healthy blood donors among whom no HPyV9 DNA was detected. A previous cross-sectional study observed a comparable difference in HPyV9 seroprevalence between

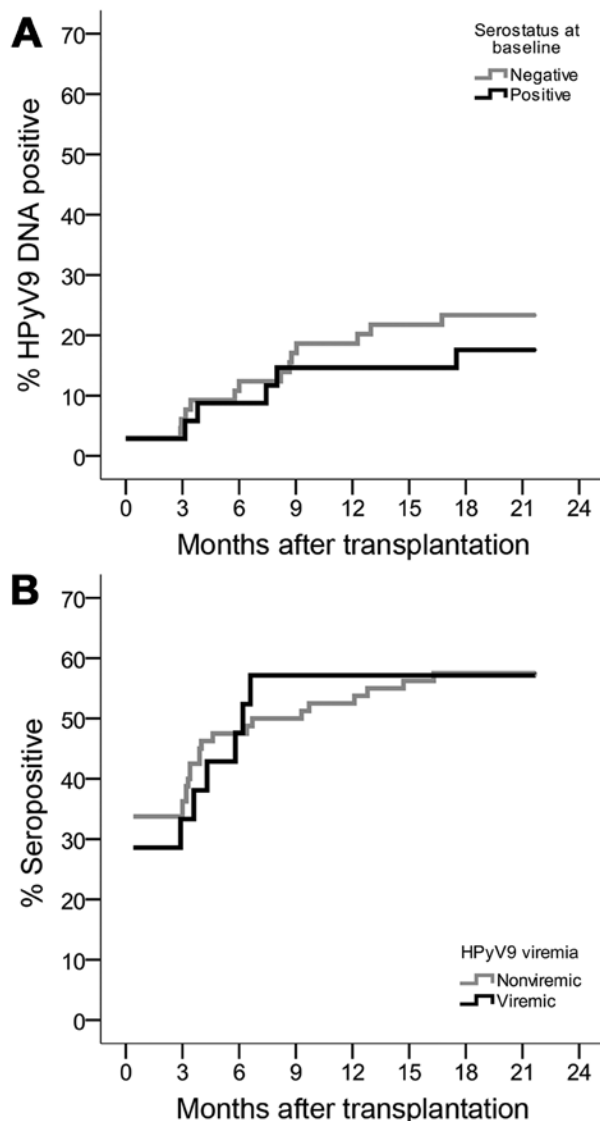


Figure 3. Kaplan-Meier curves showing proportional increase of human polyomavirus 9 (HPyV9) DNA–positive and seropositive transplant patients during 12-month follow-up, the Netherlands. A) Cumulative HPyV9 DNA positivity (viremia) for transplant patients who were seronegative (gray) or seropositive (black) at baseline. B) Cumulative HPyV9 seropositivity for transplant patients who were nonviremic (gray) or viremic (black) at baseline.

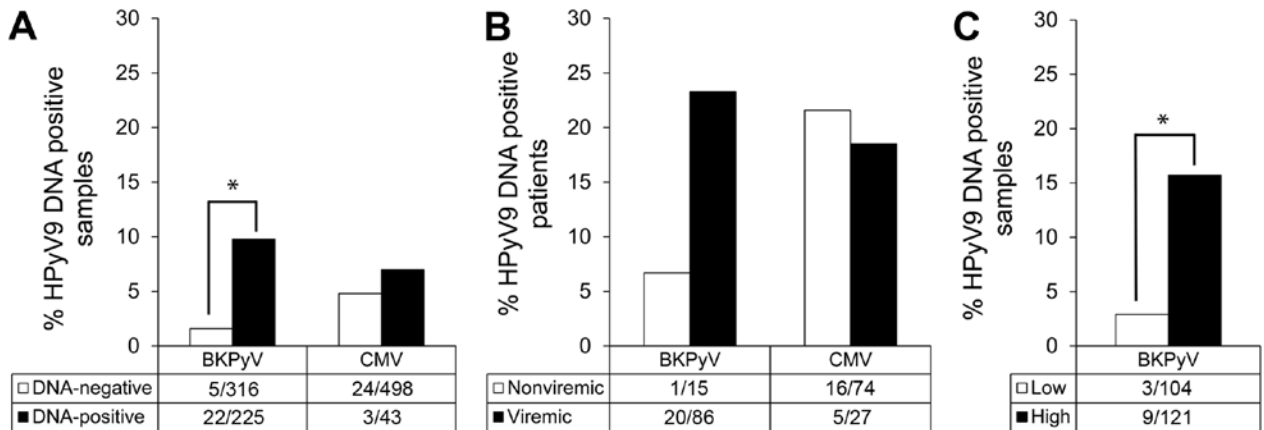


Figure 4. Association between human polyomavirus 9 (HPyV9), BK polyomavirus (BKPyV), and cytomegalovirus (CMV) infection among kidney transplant patients, the Netherlands. A) Percentage of HPyV9 DNA–positive samples among samples that tested negative (white bars) or positive (black bars) for BKPyV and CMV DNA; B) percentage of HPyV9 viremic patients among BKPyV- and CMV-nonviremic (white bars) and viremic (gray bars) patients; C) percentage of HPyV9 DNA–positive samples by measured BKPyV load within the same sample: low, $<10^3$ copies/mL (white bars) or high, $>10^3$ copies/mL (black bars). Values below bars indicate no. persons positive/total no. tested. *Significant ($p < 0.05$ by χ^2 test).

kidney transplant patients (65%) and healthy persons (45%) (25).

The detection of HPyV9 DNA and the increase in HPyV9 seroreactivity observed after transplantation could reflect primary infection and reactivation. Polyomavirus infections after transplantation and immunosuppression could result from endogenous reactivation, but proof of this concept is lacking. Infection/reactivation originating from the transplanted organ, as suggested for BKPyV (34), should be considered in the case of HPyV9, especially because HPyV9 viremia and baseline HPyV9 seroreactivity were not correlated in this study. HPyV9 viremia was frequently detected in baseline samples from seronegative transplant patients, which suggests donor-derived infection rather than endogenous reactivation.

Additional analysis of our findings showed that HPyV9 viremia was more prevalent in BKPyV DNA–positive samples and in BKPyV-viremic patients than in their BKPyV DNA–negative equivalents; this association reached statistical significance in BKPyV DNA–positive samples. Stratified analysis revealed that HPyV9 DNA positivity was correlated with high BKPyV load. Taken together, these observations suggest that these related viruses benefit from a joint risk factor present in immunosuppressed kidney transplant patients. The observation that CMV and HPyV9 viremia were not associated, however, suggests that the joint risk factor for the polyomaviruses is not simply explained by immunosuppression, which is a well-known risk factor for CMV.

Although we provide strong evidence for emergence of HPyV9 infection in kidney transplant patients and for association between HPyV9 and BKPyV infection, this study

has its limitations. The cohort of kidney transplantation patients we tested was small ($n = 101$) and was formed >10 years ago. Confirmation of our observations in a more recent and larger cohort will strengthen our findings. Because we were not able to investigate whether donor HPyV9 serostatus correlated with HPyV9 viremia in the recipient, future research might explore the possibility of the donor organ as the source of HPyV9 infection. Furthermore, studies that include urine samples that were not available for our analyses could investigate urinary excretion of HPyV9 in the infected patients and might confirm the epidemiologic correlations we found in serum samples. Finally, the sensitivity of detecting HPyV9 viremic episodes was limited by the 3-month sampling interval. Future studies using a shorter sampling interval would increase the number of measurements.

In conclusion, we identified HPyV9 as an emerging infection in immunosuppressed kidney transplant patients. The observed prevalence of HPyV9 DNA in serum samples (21%) considerably exceeded detection rates of HPyV9 found by others in skin samples of immunocompetent (0.9%) and immunocompromised (2.0%) persons (35), which suggests that HPyV9 causes systemic rather than skin infection. Whether HPyV9 is pathogenic in immunocompromised patients, alone or in concert with the well-known pathogen BKPyV, deserves further study. In this context, it would be worthwhile to investigate the course of BKPyV viremia and development of BK virus–associated nephropathy in HPyV9-positive and -negative patients.

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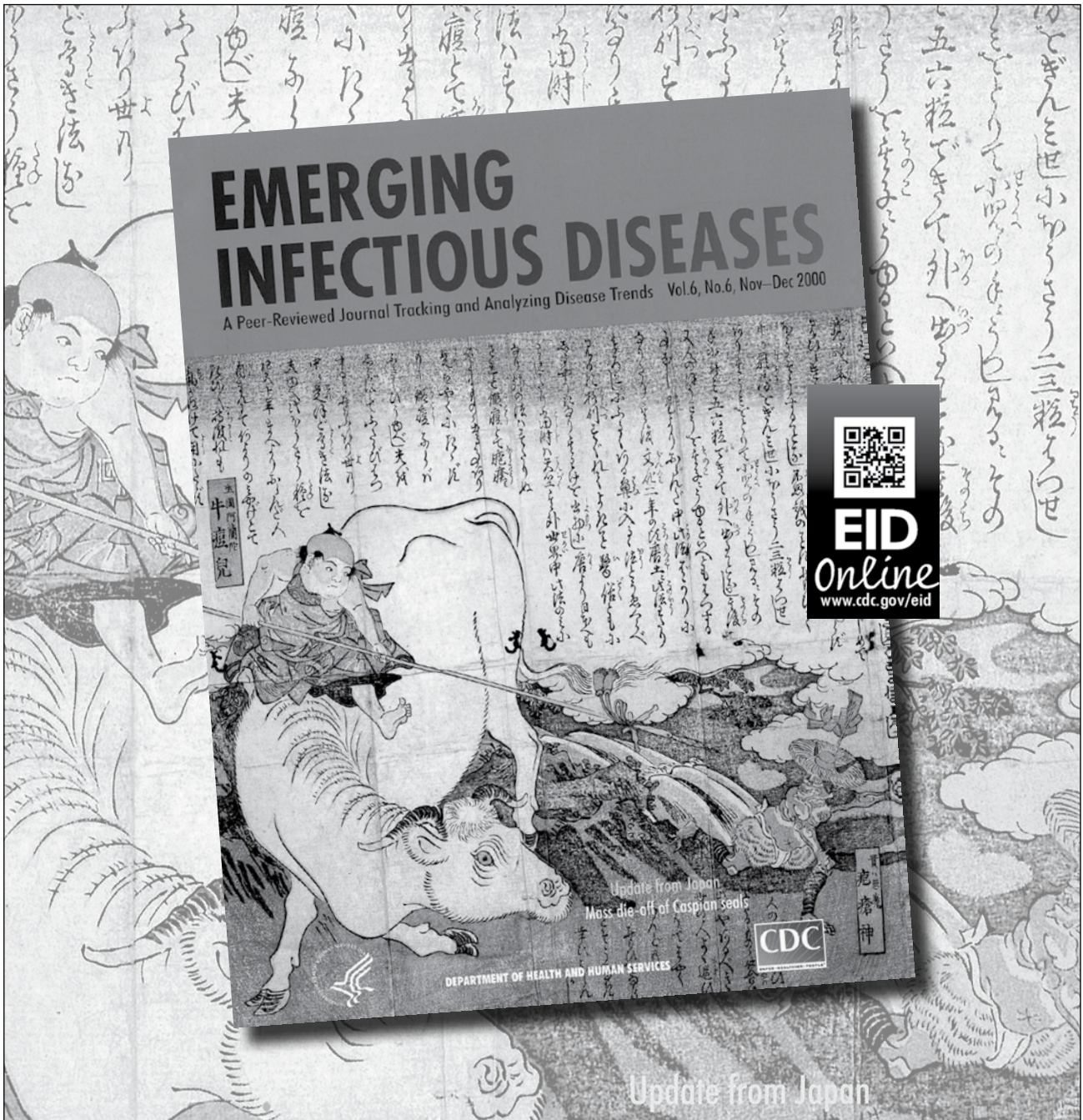
Ms van der Meijden is a PhD student at the Leiden University Medical Center. Her research interests include epidemiologic and molecular characterization of human polyomaviruses.

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Address for correspondence: Els van der Meijden, Department of Medical Microbiology E4-P, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, the Netherlands; email: p.z.van_der_meijden@lumc.nl



Infection with *Mansonella perstans* Nematodes in Buruli Ulcer Patients, Ghana

Richard O. Phillips, Michael Frimpong, Fred S. Sarfo, Birte Kretschmer, Marcus Beissner, Alexander Debrah, Yaw Ampem-Amoako, Kabiru M. Abass, William Thompson, Mabel Sarpong Duah, Justice Abotsi, Ohene Adjei, Bernhard Fleischer, Gisela Bretzel, Mark Wansbrough-Jones, and Marc Jacobsen

During August 2010–December 2012, we conducted a study of patients in Ghana who had Buruli ulcer, caused by *Mycobacterium ulcerans*, and found that 23% were co-infected with *Mansonella perstans* nematodes; 13% of controls also had *M. perstans* infection. *M. perstans* co-infection should be considered in the diagnosis and treatment of Buruli ulcer.

Buruli ulcer, caused by *Mycobacterium ulcerans*, is a neglected tropical disease common in rural parts of West Africa. Infection with *M. ulcerans* causes disfiguring skin ulcers, mainly in children. The disease is highly focal, and in Ghana, cases are reported mainly from the humid and tropical southern regions, including Ashanti and Greater Accra (1). Recent studies suggest that aquatic invertebrates serve as a reservoir for *M. ulcerans*, although complete transmission pathways remain unknown (2,3). Aquatic insects infected with *M. ulcerans* can establish

infection in mice by biting (4), but it is not clear that this is the cause of human infection (5). In southeastern Australia, evidence has been found linking infected mosquitoes with human cases (6,7), but proof of transmission is lacking.

Residents of regions in which Buruli ulcer is endemic are frequently exposed to parasitic infections such as filariasis. In Ghana, lymphatic filariasis caused by *Wuchereria bancrofti* nematodes is found in several regions to which Buruli ulcer is endemic, such as the Upper Denkyira District in the central region of Ghana, but its prevalence is unknown (8). The filarial nematode *Mansonella perstans* is endemic to countries in central and western Africa; its distribution overlaps that of other filarial nematodes *W. bancrofti*, *Loa loa*, and *Onchocerca volvulus* (9). Infective *M. perstans* larvae are transmitted through the bite of *Culicoides* midges (Diptera: Ceratopogonidae); the larvae develop over the course of months into adult worms that reside in serous cavities, particularly in the abdomen. *M. perstans* infection is not associated with a specific set of clinical signs and symptoms, but those attributed to this infection include acute swelling in the forearms, hands, and face that recedes in a few days and often recurs; itching with or without rash; arthralgia; and eosinophilia (9).

During an investigation into the immunopathogenesis of Buruli ulcer, we observed *M. perstans* nematodes in preparations of peripheral blood mononuclear cells from a patient. This finding led us to consider whether this organism was involved in the transmission or pathogenesis of *M. ulcerans* disease or if the finding was incidental. We then conducted a small case–control study to investigate the frequency of *M. perstans* co-infection in patients with *M. ulcerans* disease and the effect of this co-infection, if any, on patient response to antimicrobial drug therapy.

The Study

During August 2010–December 2012, we recruited all patients who had clinically suspected *M. ulcerans* infection and had attended a clinic in the Buruli ulcer–endemic Asante Akim North District in Ghana. Age- and sex-matched household contacts of patients were also asked to participate; all study participants were ≥ 5 years of age. The study protocol was approved by the ethics review committee of the School of Medical Sciences, Kwame Nkrumah University of Science and Technology (CHRPE/91/10).

Whole blood samples were taken at baseline, at week 6, and at week 12 from 66 patients in whom the diagnosis of Buruli ulcer disease had been confirmed by PCR for the IS2404 repeat sequence specific for *M. ulcerans* (8); samples were also obtained from 20 household contacts at the same intervals. The samples were heparinized, and peripheral blood mononuclear cells were separated from 10-mL samples. Filarial infection was confirmed on a blood film stained with Giemsa and Delafield hematoxylin and

Author affiliations: Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (R.O. Phillips, A. Debrah); Komfo Anokye Teaching Hospital, Kumasi (R.O. Phillips, F.S. Sarfo, Y. Ampem-Amoako, O. Adjei); Kumasi Collaborative Centre for Research, Kumasi (M. Frimpong, M. Sarpong Duah); Bernhard Nocht Institute of Tropical Medicine, Hamburg, Germany (B. Kretschmer, B. Fleischer); University Hospital, Ludwig-Maximilians-University of Munich, Munich, Germany (M. Beissner, G. Bretzel); Agogo Presbyterian Hospital, Agogo, Ghana (K.M. Abass, W. Thompson, J. Abotsi); St. George's University of London, London, UK (M. Wansbrough-Jones); and University Children's Hospital, Dusseldorf, Germany (M. Jacobsen)

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examined for microfilariae at $\times 10$ and $\times 40$ magnification (the Knott technique; 10). *M. perstans* nematodes were distinguished from *L. loa* and *W. bancrofti* nematodes by their small size and the absence of a sheath (Figure 1).

Patients in whom *M. ulcerans* infection was found were treated with 10 mg/kg oral rifampin and 15 mg/kg intramuscular streptomycin, administered daily at village health posts under direct observation for 8 weeks (RS8 treatment). The patients were followed up every 2 weeks in the clinic and monitored for complete healing or recurrence of skin lesions. We compared the proportion of household controls versus the proportion of Buruli ulcer patients infected with *M. perstans* nematodes and the time to complete healing of *M. ulcerans* lesions in co-infected versus monoinfected patients. Categorical variables such as sex, clinical form of *M. ulcerans* lesion, and category of *M. ulcerans* lesion were compared by using the Fisher exact test, and cumulative healing was compared by using the log-rank test.

We found all forms of *M. ulcerans* disease among the group of patients; proportions of each type and category are shown in the Table. Of 66 patients with *M. ulcerans* disease, 15 (22.7%) were co-infected with *M. perstans* nematodes, whereas 4 (13%) of 30 household controls had *M. perstans* infection ($p = 0.4$ by Fisher exact test). Three patients in the co-infected group and none in the *M. ulcerans*-monoinfected group reported pruritus. No other clinical signs of *M. perstans* infection were found.

All 66 patients completed RS8 treatment, but 9 were lost to follow-up during the 12-month follow-up period. Buruli ulcer lesions healed completely in 14 co-infected patients by 58 weeks (median 20 weeks, 95% CI 14.6–



Figure 1. *Mansonella perstans* nematode in peripheral blood mononuclear cells from Buruli ulcer patient in Ghana. Cells were stained with Giemsa (original magnification $\times 1,000$). *M. perstans* nematodes can be distinguished from *Loa loa* and *Wuchereria bancrofti* nematodes by relative small size, detection in blood samples obtained during the day, and lack of a sheath.

30.2) and in 43 monoinfected patients by 50 weeks (median 21 weeks, 95% CI 16.7–25.5). We found no difference in cumulative time to healing for co-infected versus monoinfected patients ($p > 0.05$ by log-rank test) (Figure 2). Buruli ulcer patients who had *M. perstans* nematodes co-infection were treated with doxycycline (200 mg) and ivermectin (150 $\mu\text{g}/\text{kg}$) daily for 6 weeks, starting during the second to fourth week of RS8 treatment. Viable microfilariae were still visible in peripheral blood mononuclear cell cultures from all co-infected patients after ivermectin

Table. Characteristics of patients with active *Mycobacterium ulcerans* infection, monoinfected or co-infected with *Mansonella perstans*, and of household contacts, Ghana, August 2010–December 2012*

| Characteristic | No. (%) persons with <i>M. ulcerans</i> infection | | | No. (%) household contacts, n = 30 | p value |
|---|---|----------------------|---------------|------------------------------------|---------|
| | Co-infected with <i>M. perstans</i> , n = 15 | Monoinfected, n = 51 | Total, n = 66 | | |
| Age, y | | | | | 0.514† |
| <16 | 4 (27) | 24 (47) | 28 (42) | 15 (50) | |
| 16–59 | 11 (73) | 27 (53) | 38 (58) | 15 (50) | |
| Sex | | | | | 1.000† |
| M | 9 (60) | 19 (37) | 30 (45) | 14 (47) | |
| F | 6 (40) | 32 (63) | 36 (55) | 16 (53) | |
| Clinical form of <i>M. ulcerans</i> infection | | | | | 0.049‡ |
| Nodule | 8 (53) | 11 (22) | 19 (29) | NA | |
| Plaque with edema | 2 (12) | 17 (33) | 19 (29) | NA | |
| Ulcer | 5 (35) | 23 (45) | 28 (42) | NA | |
| Category of <i>M. ulcerans</i> infection | | | | | 0.910‡ |
| I | 9 (59) | 32 (63) | 41 (62) | NA | |
| II | 4 (29) | 11 (22) | 15 (23) | NA | |
| III | 2 (12) | 8 (16) | 10 (15) | NA | |
| <i>M. perstans</i> infection | | | | | 0.408‡ |
| Yes | 15 (100) | NA | 15 (23) | 4 (13) | |
| No | 0 | NA | 51 (77) | 26 (87) | |

*NA, not applicable.

†Comparison of combined *M. ulcerans* monoinfected and *M. ulcerans* co-infected with *M. perstans* versus prevalence in household contacts, determined by 2-tailed Fisher exact test.

‡Comparison of *M. ulcerans* co-infected with *M. perstans* versus *M. ulcerans* monoinfected group, determined by 2-tailed Fisher exact test.

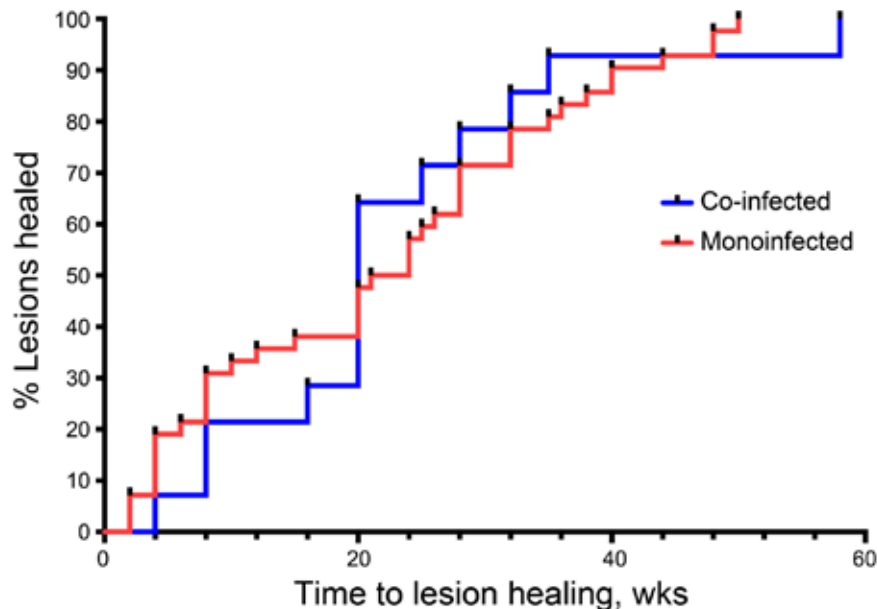


Figure 2. Survival analysis curve of cumulative healing for patients with *Mycobacterium ulcerans* infection who were co-infected with *Mansonella perstans* nematodes compared with those who had *M. ulcerans* mono-infection, Ghana, August 2010–December 2012. No difference in cumulative healing was found between the 2 groups ($p = 0.93$ by log-rank test).

and doxycycline treatment, but pruritus subsided in the 3 patients who had reported it.

Conclusions

We found co-infection with *M. perstans* in 23% of Buruli ulcer patients in a disease-endemic district in Ghana, but this prevalence was not significantly different from prevalence among household contacts who served as controls (13%). As with Buruli ulcer, *M. perstans* filariasis is predominantly found in rural populations and infection begins in childhood; the highest infection rates are found in children 10–14 years of age (11), similar to those for children at highest risk for *M. ulcerans* infection. *M. perstans* infection occurs in Ghana and was seen in the Volta region of Ghana around Hohoe during the 1990s, but its prevalence is unknown (12), and no information is available about the average number of worms per infection. In Uganda, prevalence of *M. perstans* infection has been found to range from 0.4% to 50% (13).

M. perstans nematodes are transmitted by the bites of *Culicoides* midges, but it is not known whether *M. perstans*-infected midges can be co-infected with *M. ulcerans*. In a guinea pig model, skin penetration was shown to be a requirement for establishment of *M. ulcerans* disease (14), and it has been postulated that mosquito bites cause *M. ulcerans* disease in Australia (6). These organisms might share a common route of transmission, but our findings in this small study do not support this concept.

Our findings suggest that *M. perstans* nematodes are common in rural Ghana and coincidentally infect patients with *M. ulcerans* disease, necessitating the consideration of these organisms in the management plan of Buruli ulcer patients. Although often asymptomatic, *M. perstans*

infection may cause eosinophilia, subcutaneous swellings, aches, pains, and skin rashes in a considerable proportion of patients (9). Because filarial nematodes are known to polarize the host immune responses from T-helper type 1 cells needed for protection against mycobacterial infections, toward humoral and T helper type-2 mediated immunity, we plan to undertake a study to investigate this interaction.

Acknowledgment

We are grateful to the patients and contacts from the Asante Akim North District who agreed to be part of this study.

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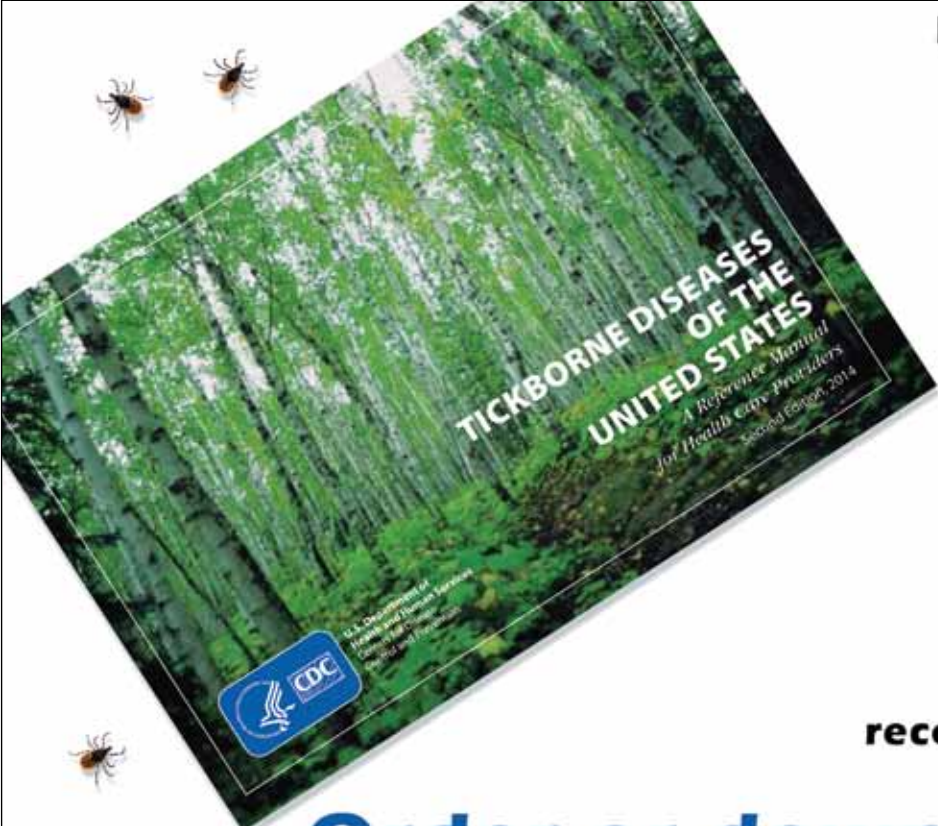
Dr Phillips is a senior lecturer at the Kwame Nkrumah University of Science and Technology. His research interest is the pathogenesis and management of *M. ulcerans* disease (Buruli ulcer).

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Address for correspondence: Richard O. Phillips, Kwame Nkrumah University of Science and Technology, School of Medical Sciences, Department of Medicine, Private Mail Bag, KNUST Kumasi, Ghana; email: rodamephillips@gmail.com



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Timeliness of Yellow Fever Surveillance, Central African Republic

Antoine Rachas,¹ Emmanuel Nakouné,
Julie Bouscaillou, Juliette Paireau,
Benjamin Selekon, Dominique Senekian,
Arnaud Fontanet, and Mirdad Kazanji

During January 2007–July 2012, a total of 3,220 suspected yellow fever cases were reported in the Central African Republic; 55 were confirmed and 11 case-patients died. Mean delay between onset of jaundice and case confirmation was 16.6 days. Delay between disease onset and blood collection could be reduced by increasing awareness of the population.

Because the number of reported cases of yellow fever has increased over the past 2 decades, it is considered a reemerging disease (1,2). The World Health Organization (WHO) requires that all affected countries report yellow fever cases. Increasing risk for resurgence, potential severity of an epidemic, and possibility of preventing its spread by vaccination make early detection of yellow fever outbreaks essential, especially in a country such as the Central African Republic, where access to healthcare is difficult because of security concerns in several areas.

There are minimal data for yellow fever surveillance in Africa. The purpose of this study was to describe the timeliness, which was defined as the delay between date of onset of jaundice reported by the patient and date of an ELISA result, of the yellow fever surveillance system in the Central African Republic and identify temporal and spatial patterns and factors associated with delays in reporting.

The Study

This study was conducted as part of epidemiologic surveillance activities of the Ministry of Public Health of the Central African Republic. Data were obtained through the yellow fever surveillance system and approved by the

Ministry of Health and WHO. All suspected cases reported during January 2007–July 2012 were included. A suspected case of yellow fever was defined as an acute onset of fever in a patient followed by jaundice within 2 weeks (3).

All blood samples from patients with suspected cases were tested at the Institut Pasteur (Bangui, Central African Republic) (IPB) for yellow fever virus–specific IgM by using the ELISA developed by the US Centers for Disease Control and Prevention (4). Positive samples were sent to the regional reference laboratory at the Institut Pasteur in Dakar, Senegal, where a plaque-reduction neutralization test (PRNT) was performed for case confirmation. A suspected yellow fever case was ruled out if ELISA or PRNT results were negative. When a suspected case was not ruled out by ELISA, health authorities were informed and an investigation was conducted so that vaccination could be implemented without delay if the case was confirmed.

The main study outcome was timeliness of the yellow fever surveillance system. Each intermediate step was studied: collection (from onset of jaundice to blood sample collection), field storage (from sample collection to shipping), transportation (from shipping to reception at IPB) and testing (from reception of sample to ELISA result).

Survival analysis was performed to determine how the following factors affected time to confirmed cases: age, sex, onset during the rainy season (May 1–October 31), province of residence, history of vaccination against yellow fever, and year of onset. Because the proportional hazard assumption was not verified for several variables, we used a parametric survival model and assumed a log-normal distribution of event times to estimate mutually adjusted time ratios. Subgroups were analyzed by confirmation status (confirmed or ruled out cases). Data were analyzed with Stata software version 11.0 (StataCorp LP, College Station, TX, USA). Maps were drawn with ArcGIS version 10.1 (Esri, Redlands, CA, USA).

During January 2007–July 2012, a total of 3,220 suspected cases of yellow fever were reported to IPB (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/6/13-0671-Techapp1.pdf). Suspected cases were reported in all provinces but mostly in Bangui (32.7%) and in neighboring Ombella M'Poko Province (24.7%) (online Technical Appendix Figure 2). Median age of patients with suspected cases was 21 years (interquartile range 10–30 years) and 57.5% were men. Only 21.9% reported having been vaccinated against yellow fever virus within the previous decade (Table 1).

Mean time to confirmation was 16.6 days (95% CI 16.2–16.9 days). Mean delay was 9.9 days (95% CI 9.5–10.2 days) for blood sample collection, 1.5 days (95% CI

¹Current affiliation: European Hospital Georges Pompidou and Descartes University, Paris, France.

Author affiliations: Institut Pasteur, Bangui, Central African Republic (A. Rachas, E. Nakouné, J. Bouscaillou, B. Selekon, M. Kazanji); Institut Pasteur, Paris, France (A. Rachas, J. Bouscaillou, J. Paireau, A. Fontanet); Ministry of Health, Bangui, (D. Senekian); and Conservatoire National des Arts et Métiers, Paris (A. Fontanet)

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1.4–1.6 days) for field storage, 1.1 days (95% CI 1.0–1.1 days) for transportation, and 4.2 days (95% CI 4.1–4.3 days) for testing. Detection of yellow fever (Figure 1, panel A) and delay for blood collection (Figure 1, panels B–E) were shortest in 2009.

Mean time until blood collection was shortest in Bangui (14.8 days) and surrounding areas and longest in Mbomou (26.2 days) (Figure 2, panel A). Mean delay for blood sample collection was shorter in eastern and northern regions of the country and longer in central and western regions (Figure 2, panel B). Mean length of transportation varied along a west–east gradient and ranged from 0.1 days in Bangui to 4.9 days in Haut Mbomou (Figure 2, panel D). A longer period to blood sample testing for the eastern part of the country was related to longer times of field storage and transportation (Figure 2, panels C, D). These areas are least accessible because of distance and

security concerns. Transportation time was longer in the southwestern region despite its relative proximity to Bangui. Mean time for testing remained stable at ≈4–5 days (Figure 2, panel E).

Province of residence and year of onset were associated with a shorter period to diagnosis ($p < 0.001$) (Table 2). Time to blood sample testing increased with patient age ($p < 0.001$). No association was found between other characteristics and time required to confirm cases.

A total of 55 yellow fever cases were confirmed and 11 case-patients died. Of the confirmed case-patients, 22 (40.0%) were 15–24 years of age, 35 (63.6%) were male, and for 13 (23.6%) date of vaccination against yellow fever virus was unknown. Age and history of vaccination differed between patients who had confirmed yellow fever cases and those who did not (Table 1). Timelines were similar for confirmed and ruled out cases.

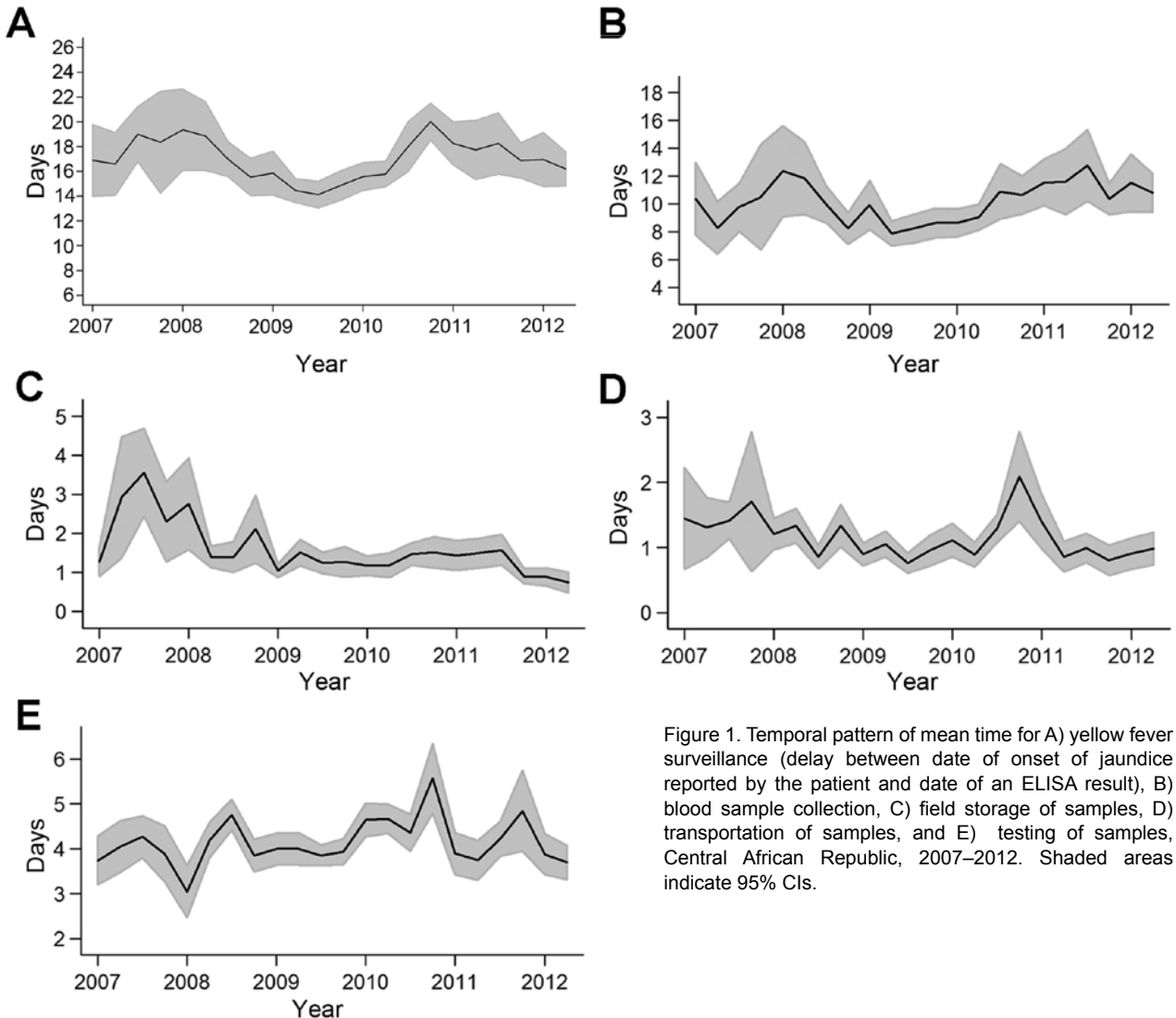


Figure 1. Temporal pattern of mean time for A) yellow fever surveillance (delay between date of onset of jaundice reported by the patient and date of an ELISA result), B) blood sample collection, C) field storage of samples, D) transportation of samples, and E) testing of samples, Central African Republic, 2007–2012. Shaded areas indicate 95% CIs.

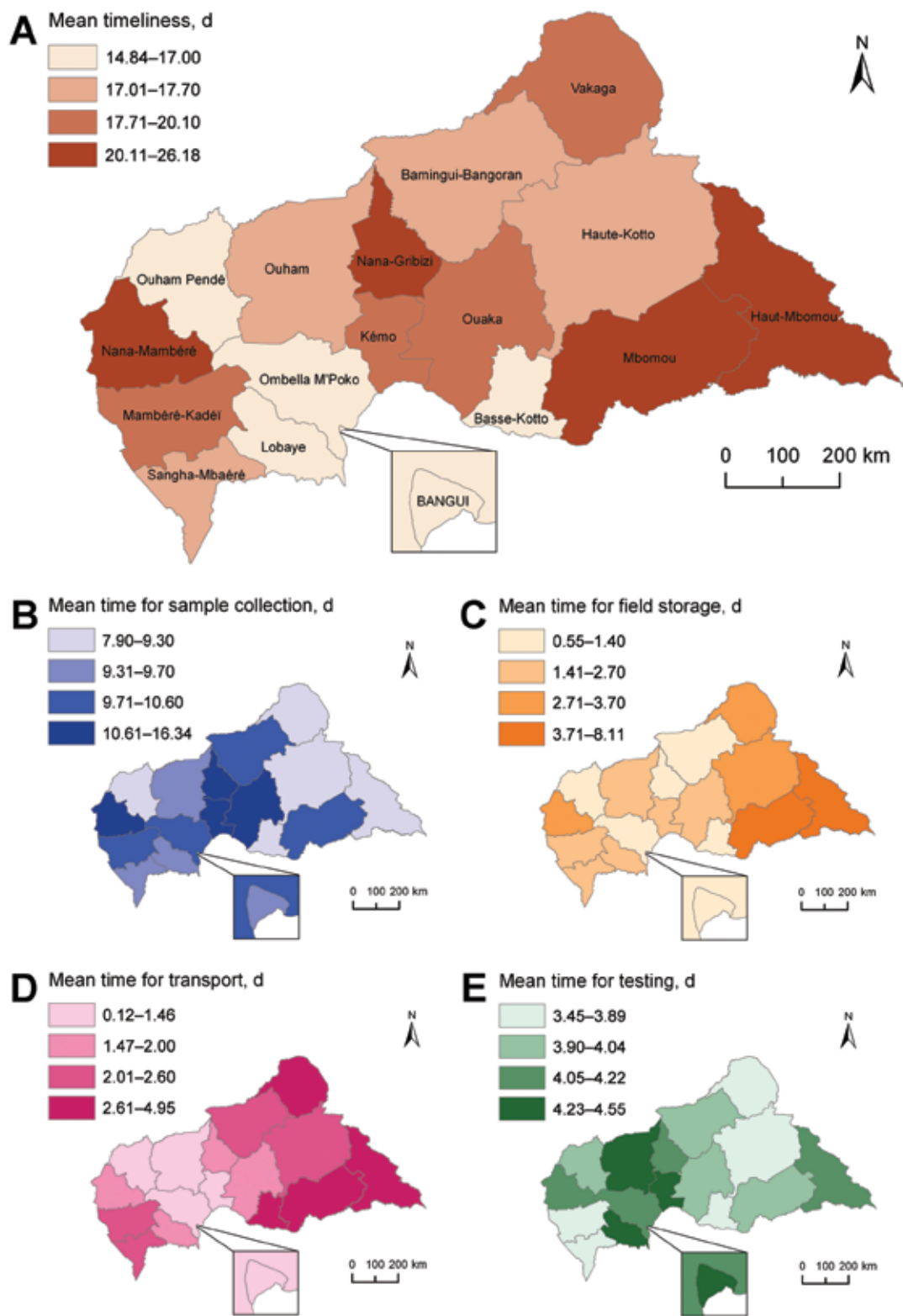


Figure 2. Spatial pattern of mean time for A) yellow fever surveillance (delay between date of onset of jaundice reported by the patient and date of an ELISA result), B) mean time for blood sample collection, C) mean time for field storage of samples, D) mean time for transportation of samples, and E) mean time for testing of samples, by province, Central African Republic, 2007–2012.

Table 1. Characteristics of 3,220 case-patients with suspected yellow fever, Central African Republic, 2007–2012*

| Characteristic | Suspected case-patients, n = 3,220 | Confirmed case-patients, n = 55 | Ruled out case-patients, n = 3,165 | p value |
|----------------------------------|---------------------------------------|------------------------------------|---------------------------------------|---------|
| Age, y | | | | |
| <14 | 1,017 (31.7) | 9 (16.4) | 1,008 (32.0) | NA |
| 15–24 | 882 (27.5) | 22 (40.0) | 860 (27.3) | NA |
| 25–34 | 673 (21.0) | 16 (29.1) | 657 (20.9) | NA |
| ≥35 | 633 (19.8) | 8 (14.6) | 625 (19.8) | 0.020 |
| Median (IQR) | 21 (10–30) | 23 (17–28) | 21 (10–31) | 0.32 |
| Sex | | | | |
| M | 1,851 (57.5) | 35 (63.6) | 1,816 (57.4) | NA |
| F | 1,366 (42.5) | 20 (36.4) | 1,346 (42.6) | 0.36 |
| Onset during rainy season | | | | |
| No | 1,446 (45.4) | 27 (50.0) | 1,419 (45.3) | NA |
| Yes | 1,738 (54.6) | 27 (50.0) | 1,711 (54.7) | 0.50 |
| Province of residence | | | | |
| Bangui | 1,053 (32.7) | 11 (20.0) | 1,042 (32.9) | NA |
| Ombella M'Poko | 794 (24.7) | 30 (54.5) | 764 (24.1) | NA |
| Lobaye | 189 (5.9) | 5 (9.1) | 184 (5.8) | NA |
| Sangha Mbaéré | 20 (0.6) | 0 | 20 (0.6) | NA |
| Mambéré Kadéi | 43 (1.3) | 0 | 43 (1.4) | NA |
| Nana Mambéré | 40 (1.2) | 0 | 40 (1.3) | NA |
| Ouham Péndé | 178 (5.5) | 1 (1.8) | 177 (5.6) | NA |
| Ouham | 111 (3.5) | 0 | 111 (3.5) | NA |
| Nana Grigbizi | 71 (2.2) | 0 | 71 (2.2) | NA |
| Kemo | 88 (2.7) | 0 | 88 (2.8) | NA |
| Bamingui Bangoran | 23 (0.7) | 0 | 23 (0.7) | NA |
| Ouaka | 188 (5.8) | 1 (1.8) | 187 (5.9) | NA |
| Basse Kotto | 178 (5.3) | 4 (7.3) | 174 (5.5) | NA |
| Vakaga | 33 (1.0) | 0 | 33 (1.0) | NA |
| Haute Kotto | 114 (3.5) | 3 (5.5) | 111 (3.5) | NA |
| Mbomou | 78 (2.4) | 0 | 78 (2.5) | NA |
| Haut Mbomou | 19 (0.6) | 0 | 19 (0.6) | NC |
| Vaccination against yellow fever | | | | |
| Never | 2,301 (71.5) | 28 (50.9) | 2,273 (71.8) | NA |
| >10 y ago | 160 (5.0) | 3 (5.5) | 157 (5.0) | NA |
| ≤10 y ago | 705 (21.9) | 11 (20.0) | 694 (21.9) | NA |
| Unknown date | 54 (1.7) | 13 (23.6) | 41 (1.3) | <0.001 |

*Values are no. (%) except as indicated. NA, not applicable; IQR, interquartile range; NC, not computable.

Conclusions

This study shows that in the Central African Republic, a country with limited health care and transportation facilities, confirmation of a yellow fever case takes 2–3 weeks. Approximately 10 days (60% of the delay) are required from the onset of symptoms to blood collection.

A fast time to case confirmation was observed in 2009, which was largely caused by a decrease in time required for blood collection, could be related to greater public awareness resulting from a yellow fever outbreak in Ombella M'Poko and Lobaye (5) and from a large outbreak of hepatitis E. This study showed an unexpected association between younger age and more rapid time to diagnosis, which was caused mainly by a shorter delay until blood collection, suggesting that parents have their children tested earlier. These 2 results indicate that the delay to blood collection could be reduced by better awareness of the population to the need for testing when they have symptoms compatible with yellow fever.

The 4–5 days of delay until testing is below the threshold of 7 days recommended by WHO. However, it could be decreased by implementation of a method of yellow fever

detection (Bioplex Technology; BioRad Laboratories, Hercules, CA, USA), which is now in progress at IPB.

The need for confirmation by PRNT might delay vaccination because of time necessary for transportation of samples to Dakar and testing. The Central African Republic is landlocked and surrounded by countries to which yellow fever is endemic (6). Thus, PRNT for this region could be implemented at IPB, where biosafety level 3 facilities are functional. This factor could improve timeliness of diagnosis for rapid introduction of prevention measures.

Delays in reporting cases observed in this study are consistent with time between onset of symptoms of yellow fever in index case-persons and in persons infected by them, which is estimated to be ≈2 weeks (7). However, reasonable time for reporting is difficult to define. A comparison of delays found in this study with those in other countries would be helpful.

Acknowledgments

We thank the WHO office in Bangui for providing funds for transportation of samples to the laboratory and diagnostic reagents and the IPB for providing diagnostic tests.

Table 2. Factors associated with timeliness of yellow fever surveillance, Central African Republic, 2007–2012*

| Factor | Mean timeliness, d | Adjusted time ratio (95% CI) | p value† |
|----------------------------------|--------------------|------------------------------|----------|
| Age, y | | | |
| <14 | 15.3 | 1.00 | NA |
| 15–24 | 16.5 | 1.13 (1.08–1.18) | NA |
| 25–34 | 16.8 | 1.15 (1.09–1.21) | NA |
| ≥35 | 18.6 | 1.21 (1.15–1.27) | <0.001 |
| Sex | | | |
| M | 16.6 | 1.00 | NA |
| F | 16.5 | 1.01 (0.98–1.05) | 0.43 |
| Onset during rainy season | | | |
| No | 16.7 | 1.00 | NA |
| Yes | 16.5 | 1.01 (0.97–1.04) | NA |
| Province of residence | | | |
| Bangui | 14.8 | 1.00 | NA |
| Ombella M'Poko | 15.7 | 1.07 (1.02–1.12) | NA |
| Lobaye | 16.8 | 1.18 (1.09–1.28) | NA |
| Sangha Mbaéré | 17.6 | 1.17 (0.93–1.46) | NA |
| Mambéré Kadéi | 18.0 | 1.21 (1.03–1.41) | NA |
| Nana Mambéré | 20.7 | 1.43 (1.22–1.67) | NA |
| Ouham Péndé | 15.5 | 1.10 (1.01–1.19) | NA |
| Ouham | 17.0 | 1.19 (1.08–1.32) | NA |
| Nana Grigbizi | 22.9 | 1.56 (1.39–1.77) | NA |
| Kemo | 19.4 | 1.35 (1.21–1.51) | NA |
| Bamingui Bangoran | 17.5 | 1.24 (1.01–1.53) | NA |
| Ouaka | 19.2 | 1.34 (1.24–1.46) | NA |
| Basse Kotto | 16.5 | 1.19 (1.10–1.29) | NA |
| Vakaga | 20.1 | 1.38 (1.16–1.65) | NA |
| Haute Kotto | 17.4 | 1.25 (1.13–1.39) | NA |
| Mbomou | 26.2 | 1.86 (1.66–2.09) | NA |
| Haut Mbomou | 23.9 | 1.72 (1.37–2.15) | <0.001 |
| Vaccination against yellow fever | | | |
| Never | 16.6 | 1.00 | NA |
| >10 y ago | 16.9 | 1.01 (0.93–1.09) | NA |
| ≤10 y ago | 16.3 | 1.01 (0.96–1.05) | NA |
| Unknown date | 16.1 | 1.06 (0.92–1.23) | 0.85 |
| Year of onset | | | |
| 2007 | 17.8 | 1.00 | NA |
| 2008 | 17.3 | 1.02 (0.95–1.09) | NA |
| 2009 | 14.7 | 0.91 (0.85–0.98) | NA |
| 2010 | 16.8 | 1.04 (0.97–1.12) | NA |
| 2011 | 17.8 | 1.10 (1.02–1.18) | NA |
| 2012 | 16.6 | 1.05 (0.96–1.15) | <0.001 |

*Adjusted time ratios are from a parametric survival model assuming log-normal distribution of the event times. A time ratio represents a relative increase in time between 2 groups. Timeliness was defined as the delay between the date of onset of jaundice (reported by the patient) and the date of ELISA result. NA, not applicable.

†By global Wald test.

Dr Rachas is an assistant professor at the European Hospital Georges Pompidou, Paris France. His primary research interest is the epidemiology of communicable diseases.

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Address for correspondence: Mirdad Kazanji, Institut Pasteur de Bangui, BP 923, Bangui, Central African Republic; email: mirdad.kazanji@pasteur.fr

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Fatal Monkeypox in Wild-Living Sooty Mangabey, Côte d'Ivoire, 2012

Aleksandar Radonić, Sonja Metzger,
Piotr Wojtek Dabrowski,
Emmanuel Couacy-Hymann, Livia Schuenadel,
Andreas Kurth, Kerstin Mätz-Rensing,
Christophe Boesch, Fabian H. Leendertz,
and Andreas Nitsche

We isolated a monkeypox virus from a wild-living monkey, a sooty mangabey, found dead in Taï National Park, Côte d'Ivoire, in March 2012. The whole-genome sequence obtained from this isolate and directly from clinical specimens showed its close relationship to monkeypox viruses from Western Africa.

Among the poxviruses are several species of orthopoxviruses (OPVs) that are pathogenic to humans, including monkeypox virus (MPXV) and variola virus (VARV). MPXV was first discovered in laboratory captive monkeys in Copenhagen in 1958 (1). After the eradication of VARV during the 1970s, MPXV became the highest pathogenic OPV infection in humans. On the basis of epidemiologic and sequence data, strains of MPXV can be assigned to a West African or a Congo Basin clade; viruses from the Congo Basin clade show more pronounced illness, death, viremia, and human-to-human transmission than do strains from the West African clade (2,3).

Serologic studies showed that monkeys from Africa have OPV antibodies, but no natural case of MPXV has been reported in wild-living primates (4,5). The only MPXV isolate obtained from an animal in the wild was from a Thomas's rope squirrel (*Funisciurus anerythrus*) caught in Democratic Republic of the Congo in 1985 (6). Here we describe natural MPXV infection in a sooty mangabey (*Cercocebus atys*) found dead in Taï National Park (TNP), Côte d'Ivoire.

Author affiliations: Robert Koch Institute, Berlin, Germany (A. Radonić, S. Metzger, P.W. Dabrowski, L. Schuenadel, A. Kurth, F.H. Leendertz, A. Nitsche); Laboratoire de la Pathologie Animale, Bingerville, Côte d'Ivoire (E. Couacy-Hymann); Deutsches Primatenzentrum, Göttingen, Germany (K. Mätz-Rensing); and Max-Planck-Institute for Evolutionary Anthropology, Leipzig, Germany (C. Boesch)

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The Study

During a long-term program to monitor deaths in wild-life, an infant mangabey was found dead in the TNP in March 2012. The body did not show any apparent injuries, and the animal had died relatively recently, as indicated by the presence of blowfly eggs but absence of maggots. Multiple skin lesions typical of MPXV infection occurred as dark red crusts 5–7 mm in diameter, partly confluent, which were disseminated over the body. Extremities were mainly affected; fewer lesions were seen on the belly and none on the back.

A full necropsy was performed under high-level safety measures, and samples of all organs and blood were collected and preserved in liquid nitrogen and 10% buffered formalin (7). Histologic analysis of the skin showed eosinophilic inclusion bodies, suggesting that an OPV infection had caused the ulcers. Severe bacterial secondary infection of the ulcers also was observed, as well as bacteremia that might have contributed to the pathologic changes and death.

DNA was extracted from different tissues, and quantitative PCR for OPV DNA (rpo18) and a cellular target (c-myc) were performed as described (8). We found a high viral DNA load in relation to cellular DNA in all tissues, except muscle, indicating a systemic infection, with particularly high loads in a skin lesion and from a throat swab sample (Table). An immunofluorescence assay performed on MPXV-infected cells showed titers of 320 for IgG and 80 for IgM, indicating an acute OPV infection.

Virus from skin tissue was propagated in HEP-2 cells, infected cells were harvested, and DNA was extracted. We performed library preparation and sequencing on an Ion Torrent PGM with an Ion PGM Sequencing 200 Kit (Life Technologies, Darmstadt, Germany) (average read length = 93 bases). Sequences were analyzed by using Geneious (Biomatters, Auckland, NZ). At first, a de novo assembly from PGM fastq-data was conducted, followed by mapping of the resulting contigs to all 11 MPXV genomes published

Table. Results of quantitative PCR from tissues of a wild-living sooty mangabey (*Cercocebus atys*), Taï National Park, Côte d'Ivoire, March 2012*

| Tissue | C _t OPV rpo18 | C _t c-myc | ΔC _t , rpo18–c-myc |
|-------------|--------------------------|----------------------|-------------------------------|
| Spleen | 32.0 | 22.1 | –9.9 |
| Lung | 34.3 | 27.8 | –6.5 |
| Kidney | 28.2 | 18.9 | –9.3† |
| Skin | 16.9 | 26.9 | 9.9 |
| Liver | 30.3 | 20.0 | –10.3† |
| Heart | 32.2 | 25.0 | –7.2 |
| Intestine | 32.2 | 31.1 | 0.1 |
| Muscle | ND | ND | ND |
| Thymus | 15.8 | 19.7 | 3.9† |
| Throat swab | 21.5 | 28.4 | 6.9† |
| Lymph node | 24.1 | 20.2 | –3.9† |

*Virus DNA (rpo18) was quantified in relation to cellular c-myc DNA in 1 μL DNA; higher values indicate higher virus loads in a respective tissue. C_t, cycle threshold; OPV, orthopoxvirus; ND, OPV DNA not detectable. †5 μL used in PCR.

in the National Center for Biotechnology Information to obtain orientation and to form a consensus sequence. The reads were mapped again to the consensus sequences to identify assembly errors. These efforts resulted in a single 197,571-bp genome. Additionally, DNA preparations from the mangabey's skin and throat swab specimens were subjected directly to an Illumina HiSeq 1500 (San Diego, CA, USA), sequencing 150 + 150 bases (paired end). The genome could also be assembled to 190,562 bp by using Illumina data only (2.9% of the 285 million Illumina reads could be used for the MPXV assembly, compared with 45% of the 3.5 million reads from the PGM), indicating that the viral genome can be assembled by sequencing specimens of high viral load directly without previous virus propagation.

Finally, we obtained a 12,395-fold coverage of the MPXV genome from Illumina data and a 732-fold coverage for data from the PGM. Eighty-four percent of the reads from the skin sample could be mapped to *Staphylococcus aureus*. These data provide evidence of the usability of direct Illumina sequencing for metagenomic analysis. Combining gained sequence data showed a full genome of MPXV-TNP with 200,035 bp (GenBank accession no. KJ136820).

All known MPXV genomes, including MPXV-TNP, and the genome of cowpox virus GRI-90 (used as outgroup) were truncated to include the sequence information between the first and last coding region for further phylogenetic analysis (MrBayes, v. 3.2.1 (<http://mrbayes.sourceforge.net/>), with gaps as binary model). MPXV-TNP is closely related to a human MPXV isolate detected in the neighboring country of Liberia in 1970 (Figure 1). Together with other isolates from this geographic region, MPXV-TNP belongs to the West African clade of MPXV (2,9). Additionally, protein sequences of MPXV-TNP were more

similar to those of sequences from the West African clade than to those of the Congo Basin clade (Figure 2).

Sequence comparison of West African and Congo Basin MPXVs by other researchers showed individual genes that are conserved across the 2 clades and are speculated to be responsible for the different pathogenicity of the viruses (2,3). For example, we analyzed the sequence of the immunomodulatory protein IL-1 β receptor (B14R), which moderated the severity of vaccinia virus infection in a mouse model (10). MPXV-TNP encodes a further truncated sequence variant of the IL-1 β protein (confirmed by Sanger sequencing) that is unique among known MPXV strains. Unfortunately, the effects of IL-1 β receptor fragmentation on functionality and on virulence of MPXV is not known (11).

Conclusions

The case described here suggests that mangabeys can be fatally infected with MPXV in nature and have high viral loads found in various tissues. Phylogenetic analysis showed that the newly identified MPXV-TNP strain is closely related to MPXV isolated from humans in Liberia and Sierra Leone in 1970 and can be assigned to the less virulent West African clade of MPXV, which seems surprising. A possible explanation is that MPXV disease progression appears to be rare in wild-living monkeys, and this case was the first observed in both regions: Central Africa, with more lethal strains, and West Africa, with less lethal strains. Also, the mangabey was only a few weeks of age and might not have had a fully developed immune system, and this factor and its secondary bacterial infection were likely to have contributed to disease severity. Moreover, individual immunologic defects cannot be ruled out. Nevertheless, very few monkeys are under systematic observation by humans, and infection of humans with the MPXV-TNP strain cannot be ruled out,

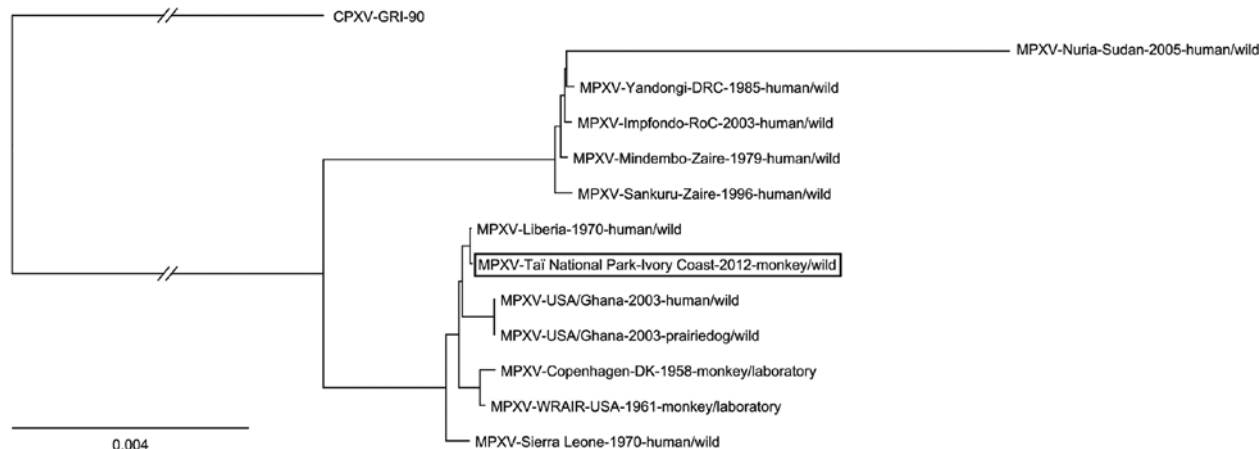


Figure 1. Phylogenetic position of the MPXV-TNP isolate (black box) from a wild-living sooty mangabey (*Cercocebus atys*), March 2012, within the West African clade. MPXV-TNP is closely related to the strain isolated from a human in Liberia in 1970. Calculated with MrBayes (with gaps) as binary model (<http://mrbayes.csit.fsu.edu>). MPXV, monkeypox virus; TNP, Taï National Park (Côte d'Ivoire). Scale bar indicates nucleotide substitutions per site.

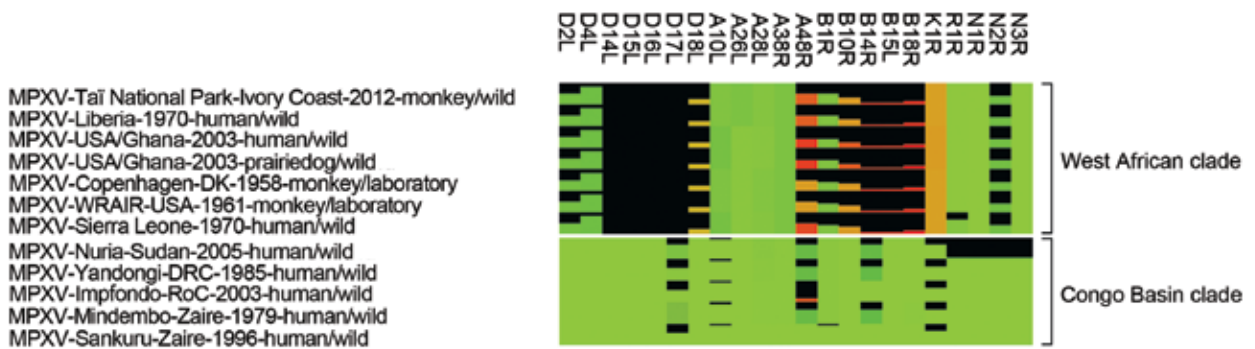


Figure 2. Heat map of MPXV proteins with rather low conservation. Shown is the comparison of protein length and identity. The degree of protein truncation is represented as a black bar. The differences in protein identity of the remainder of the proteins are represented by color gradation ranging from green (100% protein identity) to brown (\approx 50% protein identity) to red (0% protein identity). Only proteins with protein length or identity $<$ 95% are shown. Protein names are based on MPXV-Sankuru-Zaire-1996. MPXV, monkeypox virus.

even though no cases in humans were recognized in this area during the same period.

This case demonstrates that wild primates can serve as indicators for specific pathogens in certain regions (12). The local human population hunts and eats potential reservoir species, such as rodents, and therefore follow-up investigations of human infections and the prevalence of MPXV in reservoir species are needed to pinpoint the zoonotic risk posed by MPXV in the area. Additionally, studying the role of MPXV infections in wild nonhuman primates could enhance understanding of the natural history of this virus.

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Dr Radonić is a research scientist at the Robert Koch Institute. His primary research focus is the implementation, development, validation, and diagnostic applications of next-generation sequencing approaches.

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Address for correspondence: Andreas Nitsche, Centre for Biological Threats and Special Pathogens, Highly Pathogenic Viruses, Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany; email: nitschea@rki.de

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Human Infection with MERS Coronavirus after Exposure to Infected Camels, Saudi Arabia, 2013

Ziad A. Memish, Matthew Cotten, Benjamin Meyer, Simon J. Watson, Abdullah J. Alsahafi, Abdullah A. Al Rabeeah, Victor Max Corman, Andrea Sieberg, Hatem Q. Makhdoom, Abdullah Assiri, Malaki Al Masri, Souhaib Aldabbagh, Berend-Jan Bosch, Martin Beer, Marcel A. Müller, Paul Kellam, and Christian Drosten

We investigated a case of human infection with Middle East respiratory syndrome coronavirus (MERS-CoV) after exposure to infected camels. Analysis of the whole human-derived virus and 15% of the camel-derived virus sequence yielded nucleotide polymorphism signatures suggestive of cross-species transmission. Camels may act as a direct source of human MERS-CoV infection.

Middle East respiratory syndrome coronavirus (MERS-CoV) was identified in 2012 in a cell culture taken from a patient who died of pneumonia in Saudi Arabia (1). Since 2012, at least 187 laboratory-confirmed human cases of MERS-CoV infection, most resulting in respiratory tract illness, have been reported to the World Health Organization; 97 of these cases were fatal. Known cases have been directly or indirectly linked to countries in the Arabian Peninsula (2). Dromedary camels across and beyond the region

Author affiliations: Al Faisal University, Riyadh, Saudi Arabia (Z.A. Memish); Global Centre for Mass Gatherings Medicine, Riyadh (Z.A. Memish, A.A. Al Rabeeh); Ministry of Health, Riyadh (Z.A. Memish, A.A. Al Rabeeh, A. Assiri, M. Al Masri); Wellcome Trust Sanger Institute, Hinxton, UK (M. Cotten, S.J. Watson, P. Kellam); University of Bonn Medical Centre Institute of Virology, Bonn, Germany (B. Meyer, V.M. Corman, A. Sieberg, S. Aldabbagh, M.A. Müller, C. Drosten); Regional Health Directorate, Jeddah, Saudi Arabia (A.J. Alsahafi); Jeddah Regional Laboratory, Jeddah (Hatem Q. Makhdoom); Utrecht University, the Netherlands (B.-J. Bosch); Friedrich-Loeffler-Institut, Institute for Virus Diagnostics, Greifswald-Insel Riems, Germany (M. Beer); and University College London, London, UK (P. Kellam)

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show high rates of antibodies against MERS-CoV (3–7), and viral RNA has been detected in camels in different countries (8,9). In 1 instance, a camel and 2 humans caring for the camel were found to be infected with viruses that were highly similar but distinct within 4,395 nt of the camel-derived virus sequence, including several phylogenetically informative nucleotide changes (10). To investigate possible camel–human virus transmission, we analyzed an infection with MERS-CoV in a man after he had contact with an infected camel.

The Study

On November 3, 2013, the Ministry of Health of Saudi Arabia was notified of a suspected case of MERS-CoV infection in a 43-year-old male patient at King Abdulaziz University Hospital in Jeddah. The patient had cared for ill camels in his herd of 9 animals starting in early October, when the patient noted respiratory signs of illness with nasal discharge in several animals; he continued caring for the sick animals until October 27, the day of onset of his own illness. The patient cared for the animals for ≈3 hours per day 3 days per week, applying herbal remedies to the animals' snouts and nostrils. He did not clean the stables or milk the animals, but he routinely consumed raw, unpasteurized camel milk from the herd.

Presence of MERS-CoV RNA in the patient was confirmed at Jeddah Regional Laboratory by using reverse transcription PCR (RT-PCR) targeting the *upE* and *orfA* gene fragments (11,12). Respiratory swab specimens yielded detectable signal after 28 RT-PCR cycles, indicative of an approximate viral load of 350,000 RNA copies per sample. A nearly complete viral genome was obtained (Jeddah_1_2013; GenBank accession no. KJ556336), confirming the presence of a typical MERS-CoV whose closest relatives were in the Riyadh_3 clade, as defined in (2) (phylogeny shown in online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/6/14-0402-Techapp1.pdf).

To identify potential sources of infection, on November 9, the Ministry of Health investigated 5 close household contacts and the animal attendant on a farm owned by the patient. Nasopharyngeal swab samples were taken and tested at Jeddah Regional Laboratory by using RT-PCR. Deep nasal swab specimens were taken on the same day from 3 of the 9 camels at the farm. Testing of all samples by RT-PCR using the *upE* assay (11,12) did not detect MERS-CoV RNA in any of the human patients, but 1 of the 3 camels (camel G) tested positive (cycle threshold [C_t] = 33). On November 13, nasal swab samples were obtained from all 9 animals. *upE* RT-PCR results were positive for camel G (C_t = 38) and a second camel (camel B; C_t = 39).

Samples from November 13 and a small remaining amount of RNA extract from camel G from November 9 were sent to the Sanger Institute in Cambridge, UK, and

confirmation of reactivity ($C_t \approx 38$) was obtained for pooled samples with the *upE* assay from camel G but not for camel B. The result for camel G was confirmed at the Institute of Virology in Bonn, Germany, for the same samples by using real-time RT-PCRs targeting the *upE* and 1A diagnostic target regions.

A sequence of $\approx 15\%$ of the camel-derived genome was determined from 8 RT-PCR fragments, 2 of them partially overlapping (4,608 nt total) (13). Phylogenetic analyses supported the conclusion that transmission occurred between camel and patient, but no direction was implied (e.g. camel to human vs. human to camel; online Technical Appendix Figure 1). The human- and camel-derived sequences shared a signature of single nucleotide polymorphisms that occurred in no other known MERS-CoV sequences (Figure). Single nucleotide exchanges occurred at nt positions 21945 and 29662; these exchanges might have

arisen during virus transmission, as described (10). However, because of the low RNA concentration in the samples, reamplification of the material and investigation of possible PCR-based mutations could not be done.

A serum sample taken from camel G during the initial investigation on November 9 was tested by recombinant immunofluorescence assay (IFA) as described (6,14) and showed reactivity to MERS-CoV (titer 320). To investigate signs of recent MERS-CoV infection in the group of camels, we obtained blood samples at short intervals from all 9 animals during November 14–December 9, 2013. All samples were tested by ELISA against recombinant MERS-CoV spike antigen domain S1 fused to human Fc fragment, using a formulation as described (15). Serum samples from all 9 animals showed reactivity to the MERS-CoV antigen; serum samples from control animals showed no reactivity (online Technical Appendix Figure 2, panel A).

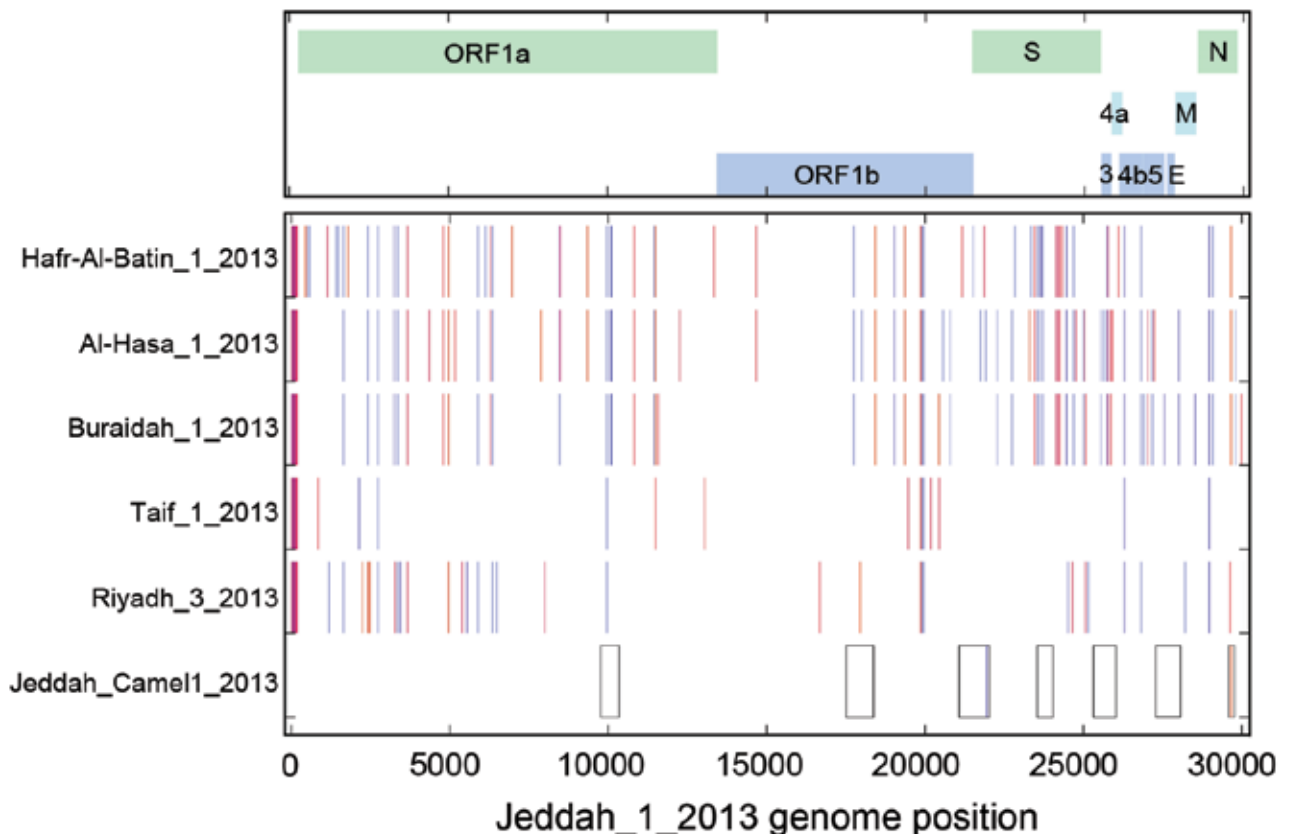


Figure. Direct comparison of the Middle East respiratory syndrome coronavirus (MERS-CoV) Jeddah_1_2013 genome sequence, Jeddah_Camel1_2013 fragments (boxes at bottom), and representative genomes of other clade viruses: 2 additional genomes from the Riyadh_3 clade, Riyadh_3_2013 and Taif_1_2013; and representative genomes from the Al-Hasa and Hafr-Al-Batin_1 and Buraidah_1 clades. A map of the MERS-CoV genome with the major open reading frames (ORFs) indicated is shown at the top. Nucleotide differences for other genomes from Jeddah_1_2013 are shown by vertical colored bars: orange, change to A; red, change to T; blue, change to G; violet, change to C. Gaps in all full-genome sequences are indicated in gray. Positions according to the MERS-CoV genome EMC/2012: fragment 1, 9767–10354; fragment 2, 17507–18394; fragment 3, 21089–22046; fragment 4, 23569–24059; fragment 5, 25349–26056; fragment 6, 27276–28095; fragment 7, 29596–29757. The sequences reported here have been deposited in GenBank (accession nos. KJ556337–KJ556340; others are pending).

ELISA signals were constant over time in most animals, but a small, yet visible, change of signal over the observation period was noted for camels B and G. To clarify the reasons for this putative signal increase, the first and last serum samples (obtained on November 14 and December 9) from all animals were re-tested by IFA. As summarized in the Table, camels B and G showed 4-fold increases of titer for the paired first and last serum samples. In serologic tests that rely on 2-fold endpoint titrations, a titer increase ≥ 2 dilution steps is considered a significant sign of recent acute infection. For additional confirmation of rises of titer, sequential samples from camels B and G were compared with sequential samples from camels E and I by using IFA with endpoint titration. These data confirmed the increases of titers for camels B and G (online Technical Appendix Figure 2, panel B).

Because bovine CoV occurs in camels, we tested for antibodies against bovine CoV in camels B and G to exclude potential cross-reactions. Using IFA (6), we found no bovine CoV antibodies in camel G, but camel B showed rising bovine CoV antibody titers (Table). To obtain further differentiation, we performed neutralization assays against MERS-CoV and bovine CoV (7). Titers in serum samples from November 14 and December 9, respectively, were 160 and 320 for camel B and 160 and 160 for camel G. None of the animals showed serum neutralization against bovine CoV.

Conclusions

These data add to recent findings showing high similarity of MERS-CoVs carried by humans and camels (8,10), supporting the hypothesis that human MERS-CoV infection may be acquired directly from camels. In addition, both animals that showed signs of recent infection were juvenile, which provides further support to previous findings that mainly young animals are infected by MERS-CoV (7,8). Given the synchronized parturition pattern of dromedary camels, with birthing in the

winter months, an increase of epizootic activity might be expected after some latency during the first half of each year.

Our data provide particular insight into the timing of infections and transmission. Antibody titers rose and viral RNA concentrations were already on the decline in the camels while the patient was hospitalized with acute symptoms. Assuming a time before appearance of antibodies of 10–21 days, at least some of the camels would have been actively infected during middle to late October, when some animals showed signs of respiratory illness and the patient acquired his infection. Nevertheless, we cannot rule out other infectious causes of the animals' upper respiratory signs. Also, because of the retrospective nature of this investigation, we cannot rule out the possibility of a third source of MERS-CoV infection for camels and humans.

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Dr Memish is Deputy Minister for Public Health, Ministry of Health; director of the WHO Collaborating Center for Mass Gathering Medicine; and professor at Alfaisal University College of Medicine, Riyadh, Kingdom of Saudi Arabia. His research interest is MERS coronavirus.

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Table. Reciprocal immunofluorescence titers for MERS and bovine CoV in sequential serum samples from 9 camels, Jeddah, Saudi Arabia, 2013*

| Camel | Age | Anti-MERS CoV titers | | Anti-bovine CoV titers | |
|-------|------|----------------------|--------|------------------------|-------------|
| | | Nov 14 | Dec 9 | Nov 14 | Dec 9 |
| A | 13 y | 40,960 | 81,920 | | |
| B | 3 mo | 640 | 2,560 | 320 | 1,280–2,560 |
| C | 12 y | 20,480 | 20,480 | | |
| D | 9 y | 40,960 | 40,960 | | |
| E | 13 y | 5,120 | 5,120 | | |
| F | 8 y | 40,960 | 40,960 | | |
| G† | 8 mo | 640 | 2,560 | <10 | <10 |
| H | 8 mo | 40,960 | 40,960 | | |
| I | 2 y | 5,120 | 5,120 | | |

*MERS, Middle East respiratory syndrome; CoV, coronavirus.

†For camel G, a sample taken on November 9 was also available and yielded an immunofluorescence titer of 320.

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Addresses for correspondence: Ziad A. Memish, Ministry of Health, Riyadh 11176, Saudi Arabia; email: zmemish@yahoo.com; or Christian Drosten, Institute of Virology, University of Bonn Medical Centre, Sigmund Freud Str 25, 53105 Bonn, Germany; email: drosten@virology-bonn.de

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Sequential Gastroenteritis Episodes Caused by 2 Norovirus Genotypes

Gabriel I. Parra and Kim Y. Green

We investigated sequential episodes of acute norovirus gastroenteritis in a young child within an 11-month period. The infections were caused by 2 distinct genotypes (GII.4 and GII.6). Failure to achieve cross-protective immunity was linked to absence of an enduring and cross-reactive mucosal immune response, a critical consideration for vaccine design.

Noroviruses are major pathogens associated with acute gastroenteritis in persons of all ages. It is estimated that each year in developing countries, noroviruses are responsible for up to 200,000 deaths of children <5 years of age (1). Moreover, in the United States, because of the successful implementation of vaccination against rotaviruses, noroviruses have emerged as the leading cause of severe gastroenteritis requiring medical intervention among infants and young children (2).

Noroviruses are genetically diverse, and differences in the major capsid protein (VP1) have led to their classification into 6 genogroups (GI–GVI) and ≈30 genotypes. Noroviruses from genogroups GI, GII, and GIV infect humans; worldwide, GII.4 is the most prevalent genotype (3–5). Expression of VP1 results in self-assembly of virus-like particles that have been used to examine structural and antigenic differences among genotypes (3,6–8). However, lack of an *in vitro* cell culture system has hindered the ability to establish serotype differences by neutralization. Initial evidence for the existence of at least 2 distinct norovirus serotypes came from early studies among volunteers; these studies showed that infection with Norwalk or Hawaii viruses (representing GI and GII, respectively) did not induce cross-protection (9). Evidence also exists for the periodic emergence of new GII.4 strain variants that cause large global epidemics, possibly driven by escape from herd immunity (5,10). Further understanding of the natural history of these viruses is needed to establish the potential role of genotypic and antigenic variation in vaccine development.

Author affiliation: National Institutes of Health, Bethesda, Maryland, USA

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The Study

On January 15, 2012, a 13-month-old girl enrolled in a childcare center in Rockville, Maryland, experienced 3 episodes of vomiting within 1 hour, after which she had diarrhea or loose stools for ≈1 week. Within 24 hours after this child's onset of symptoms, 2 family members reported multiple episodes of vomiting and diarrhea that lasted >3 days. Because several children and teachers at the childcare center reported similar symptoms, parents of children enrolled at the childcare center were alerted to the possibility of a gastroenteritis outbreak.

The patient reported here was subsequently enrolled in National Institutes of Health clinical study NCT01306084, after receipt of informed consent from the mother. Fecal samples were collected from the child and examined for norovirus RNA by reverse transcription PCR. Viral RNA was detected for 4 weeks after the onset of symptoms, and viral RNA quantification reached up to 1.7×10^8 genome copies/g feces. Sequence and phylogenetic analyses of VP1 from the virus (designated norovirus Hu/GII.4/RockvilleD1/2012/U.S.) showed that it grouped within the newly emerging virus GII.4 Sydney_2012 cluster (Figure 1, panel A) (11).

On December 10, 2012, a gastroenteritis outbreak occurred in a different childcare center in Bethesda, Maryland, at which the same child (now 24 months of age) was enrolled. The child experienced vomiting, diarrhea, and fatigue that lasted ≈2 days, and a similar disease pattern developed in a family member 24 hours after the onset of the child's symptoms. Fecal samples were again positive for norovirus with 5.3×10^9 genome copies/g of feces, and viral RNA was detected up to 3 weeks after disease onset. Phylogenetic and sequence analyses revealed a GII.6 norovirus (designated norovirus Hu/GII.6/BethesdaD1/2012/U.S.), most closely related to GII.6 noroviruses detected in Miami (Florida, USA) and Texas (USA) in 1994 and 1997, respectively (Figure 1, panel A).

Norovirus strains GII.4 and GII.6 differed by ≈38% in VP1 sequences; most amino acid sequence variation occurred in the capsid protruding domain (29%; 163/556). Alignment of the VP1 sequences from the 2 strains in this study showed several gaps; each strain bore discrete regions of amino acid insertions or deletions that differed from those in the other strain. The GII.6 VP1 (547 aa long) contained 3 insertions at positions 296 (11 residues), 310 (2 residues), and 344 (3 residues); GII.4 VP1 (540 aa long) did not contain these insertions. The same alignment showed GII.4 VP1 insertions at positions 190 (1 residue), 373 (1 residue), and 390 (7 residues) (Figure 2, Appendix, wwwnc.cdc.gov/EID/article/20/6/13-1627-F2.htm). Of note, most gaps in the alignment of the VP1 sequences were present in or near recently described GII.4 epitopes (7,12), thereby suggesting that these residues might play a role in defining the antigenic specificity of the 2 genotypes.

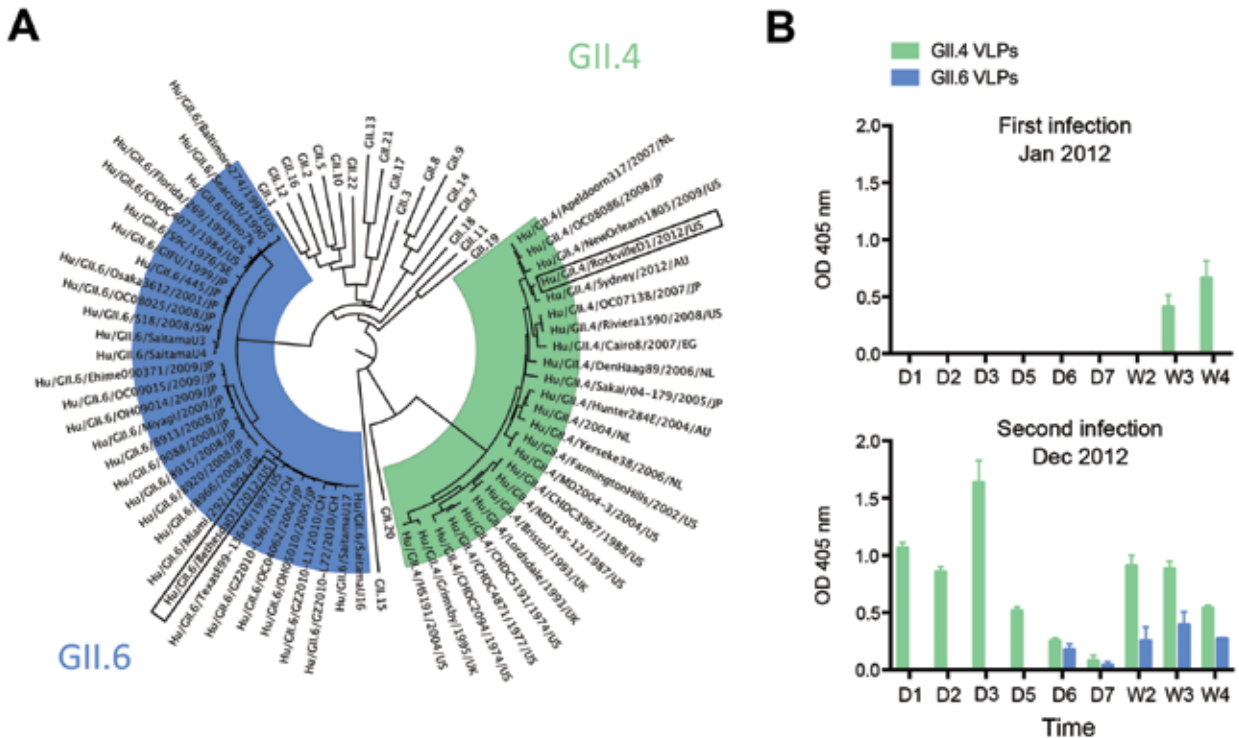


Figure 1. Characterization of norovirus detected in stool samples and levels of local IgA responses for each infection. A) Phylogenetic tree of the major capsid protein (VP1) region from representative norovirus strains from each of the 22 genotypes within strain GII. Representative strains from each GII.4 and GII.6 cluster were compared with the strains reported in this article (boxed). For each strain, the name/year/country of isolation are shown. B) Levels of IgA in feces collected during the first (GII.4) and (GII.6) second infections. ELISA plates were coated with 1 µg/mL of each virus-like particle (VLP). Fecal samples were collected daily (D) and weekly (W), diluted to 1:500 in phosphate-buffered saline (pH 7.4), and tested for the presence of IgA with a polyclonal anti-human IgA conjugate. The experiment was performed twice in duplicate wells. Bars represent mean; error bars represent SEM. OD, optical density.

To understand the absence of immunity to the second norovirus and to gain insight into the specificity of the mucosal immune response after sequential norovirus infections, we developed virus-like particles from both noroviruses. We used the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA) and baculovirus-infected Sf-9 cells for virus-like particle production as described elsewhere (7). The corresponding virus-like particles were used to test for IgA in feces with an ELISA that used polyclonal goat anti-human IgA conjugated with horseradish peroxidase (1:2,000 dilution; KPL, Gaithersburg, MD, USA) as the detector antibody and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; KPL) as the substrate. During the child's first infection with norovirus GII.4, virus-specific IgA was not detectable until 3 weeks after infection (Figure 1, panel B). At the onset of symptoms during the second infection with norovirus GII.6, a rapid anamnestic response specific for GII.4 virus-like particles developed. The GII.6-specific IgA response was detected at day 6 after infection, and IgA was detectable in feces until the last daily collection at 4 weeks

(Figure 1, panel B). Examination of feces for norovirus-specific IgG after the second infection showed a similar anamnestic response that endured throughout the period examined (data not shown).

The child's clinical history, young age, and absence of early detectable norovirus-specific fecal IgA suggest that she experienced a primary infection with GII.4 norovirus in the first childcare center outbreak. During the second infection with GII.6 norovirus, a rapid anamnestic GII.4-specific IgA response developed and persisted up to 4 weeks as the slower primary GII.6-specific IgA response developed ≈1 week after symptom onset. Follow-up fecal samples collected at 14 weeks after the second infection contained little or no detectable levels of either GII.6- or GII.4-specific IgA, indicating an eventual decline in mucosal IgA titers against each genotype (data not shown).

Previous data from human volunteer studies suggest that homologous immunity to Norwalk virus (genotype GI.1) lasts from 2 months to 2 years (13) and that a rapid and specific mucosal IgA response (likely anamnestic) was a correlate of protection (14). The data presented here are

consistent with the development of a genotype-specific, short-lived mucosal IgA response to norovirus infection that, when stimulated anamnesticly, might provide little or no protection against other norovirus genotypes.

Conclusions

This study shows that a young child can experience 2 episodes of acute gastroenteritis caused by distinct norovirus genotypes (GII.4 and GII.6) within 1 year. Reinfection with distinct genotypes can commonly occur in younger persons, as recently demonstrated in a longitudinal study of norovirus infection in infants and young children in Peru (15). Genotypes within the 2 major genogroups of human noroviruses might represent distinct serotypes. The mechanisms of enduring norovirus immunity in the development of cross-protective and effective vaccines need to be elucidated.

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Dr Parra is a visiting fellow at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. His research interests include epidemiology, immunology, and vaccine development against gastrointestinal viral infections.

Dr Green is chief of the Caliciviruses Section of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Her research is directed toward the prevention and control of acute gastrointestinal disease caused by noroviruses.

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Address for correspondence: Gabriel I. Parra and Kim Y. Green, Room 6318, Bldg 50, Caliciviruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 50 South Dr, Bethesda, MD 20892, USA; email: parrag@niaid.nih.gov and kgreen@niaid.nih.gov

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Species H Rotavirus Detected in Piglets with Diarrhea, Brazil, 2012

Bruna L.D. Molinari, Elis Lorenzetti,
Rodrigo A.A. Otonel, Alice F. Alfieri,
and Amauri A. Alfieri

We determined nucleotide and deduced amino acid sequences of the rotavirus gene encoding viral protein 6 from 3 fecal samples collected from piglets with diarrhea in Brazil, 2012. The analyses showed that the porcine rotavirus strains in Brazil are closely related to the novel species H rotavirus.

Rotaviruses (RVs) form a genus of the family *Reoviridae* and are a common cause of viral gastroenteritis in humans and animals (1). The RV genome consists of 11 segments of double-stranded RNA that encode 6 structural viral protein (VP1–4, VP6, and VP7) and 6 nonstructural proteins (NSP1–6) (1). RVs have been classified into 7 species, which are also known as groups, termed A–G, on the basis of the antigenicity and genetic characteristics of VP6 (2,3). *Rotavirus* A (RVA) infections cause severe diarrhea in infants and young children worldwide but can also infect adults, other mammals, and birds (1). *Rotavirus* B (RVB) infections were first associated with severe diarrhea in adults (4) and have also been detected in cattle, pigs, sheep, and rats (5–7).

In addition to the 7 established species of RV, Matthijnssens et al. (2) recently proposed the new *Rotavirus* H (RVH) on the basis of VP6 sequence analysis. This species includes the following: the novel adult diarrhea RV strain (ADRV-N) isolated from specimens collected during an outbreak of gastroenteritis among adults during 1997 in China (8); strain J19, identified during the same outbreak in China in 1997 (9); the human *Rotavirus* B219, detected in a sporadic case of diarrhea in Bangladesh during 2002 (10,11); and the porcine RV strain SKA-1 that was isolated from a pig with diarrhea in Japan (12).

In this study, we determined the VP6 nucleotide sequence for 3 RV-positive fecal samples obtained from piglets with diarrhea in Brazil during 2012. A comparative

analysis with other VP6 genes showed that the porcine RV strain from Brazil is closely related to the novel RVH.

A molecular study of RVB infection on a pig farm in Mato Grosso do Sul in the Central-West region of Brazil was performed during an outbreak of diarrheal illness in 2012. A total of 59 diarrheic fecal specimens were collected from 59 piglets whose ages ranged from 12 to 35 days, and the presence of RV was investigated by using polyacrylamide gel electrophoresis (13). Eight samples that showed RVB dsRNA pattern (4:2:2:3) and 2 that showed polyacrylamide gel electrophoresis inconclusive results were subjected to reverse transcription PCR (RT-PCR) by using the primer pair described by Gouvea et al. (14), which were designed to amplify a partial fragment of the NSP2 gene of RVB. All 10 samples were positive for RVB on the basis of the amplification of the 434-bp target fragment. To amplify larger fragments of the distinctive RVB NSP2 gene, we submitted the 10 fecal samples to RT-PCR using the primer pairs NSP2–1 F/R (993 bp), and NSP2–2 F/R (938 bp) as described by Suzuki et al. (15). Products of 993 bp expected for amplification reaction with a NSP2–1 primer pair were obtained for 7 of the samples. The remaining 3 samples (BR59, BR60, and BR63), from 35-day-old piglets, did not generate the expected fragments with any of the primer pairs. However, because of an unexpected annealing of the NSP2–2 primer pair in RT-PCR, shorter (\approx 750 bp) amplicons were generated for these 3 samples.

The nonspecific amplification products of the 3 samples were purified and sequenced with NSP2–2 forward and reverse primers by using the BigDye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI3500). Similarity searches were performed with sequences deposited in GenBank by using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Of note, the highest nucleotide identities were obtained for the VP4 genes of the RVH strains SKA-1 (89%), B219 (72%), and J19 (70%). The VP4 nucleotide sequence alignment of the RVH SKA-1 strain and the nonspecific product was performed from the nucleotide positions 1792–2433 by using MEGA (v. 6) (www.mega-software.net/).

To confirm the similarity of the samples BR59, BR60, and BR63 with RVH, we performed an additional set of RT-PCRs using 2 new primer pairs designed on the basis of the complete sequence of the VP6 gene of the porcine RVH strain SKA-1 (12) (Table 1). Phylogenetic analysis and nucleotide distance calculations were performed by using MEGA (v. 6) and BioEdit (v. 7.0.8.0) software (www.mbio.ncsu.edu/bioedit/bioedit.html).

The pairwise comparisons of the VP6 nucleotide and inferred amino acid sequences of the 3 specimens

Author affiliation: Universidade Estadual de Londrina, Londrina, Parana, Brazil

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Table 1. Oligonucleotide primers designed from the VP6 gene of the SKA-1 rotavirus strain for reverse transcription PCR sequence analysis, Brazil, 2012*

| Primer | Sequence (5'→3') | Nucleotide position |
|------------|-----------------------|---------------------|
| VP6/RVN-1F | TGCTACAAGTGACCCACAAGG | 11–31 |
| VP6/RVN-1R | GCCATCTTTCCAGTGGCTCT | 581–600 |
| VP6/RVN-2F | ACCAGGTGGAGCAACAAACA | 529–548 |
| VP6/RVN-2R | CAGTGCCTGACCAGATCTCA | 1225–1244 |

*VP, viral protein.

revealed 100% nt and aa identities among them. In contrast, the BR59, BR60, and BR63 sequences showed <36% nt identity (<13.5% aa identity) with cogent sequences of *Rotavirus* A, C, D, and F, and 49.7%–51.6% nt identity (35.8%–38.3% aa identity), respectively, with RVB and RVG. The specimens had relatively high identities with RVH (71.7%–85.5% and 75.7%–96.9% at the nt and aa levels, respectively). The highest identity was shared with the VP6 gene of the porcine RVH SKA-1 strain. (Table 2) The phylogenetic tree (Figure) inferred from the VP6 sequences was separated into distinct phylogenetic clusters representative of RV species. The BR59, BR60, and BR63 samples grouped closest to the RVH species. Although they segregated in a different branch, they clearly were within the RVH cluster.

Conclusions

The 3 rotavirus samples, BR59, BR60, and BR63, detected in diarrheic fecal specimens from 35-day-old piglets from the same farm shared 100% nt and aa identities of their VP6 gene sequences, suggesting that the specimens represented the same local rotavirus strain. Although initial RT-PCR results by using RVB-specific NSP2 primers suggested that these samples were RVB, sequence analysis of the VP6 gene showed that they are different from RVB.

Matthijnsens et al. (2) proposed a 53%-aa cutoff value for VP6 to be used for the differentiation of distinct RV species. The 3 rotavirus samples included in this study showed a VP6 aa identity ranging 76.1% (human strain) to 96.9% (porcine strain) when compared with RVH and are thus considered to belong to the novel species RVH. Prior to this study, 1 porcine RVH strain (SKA-1), detected in Japan, had been reported (2,12).

The VP6 nucleotide and amino acid sequences of BR59, BR60, and BR63 samples did not show high identities with VP6 sequences from RVs A, C, D, and F, but showed a moderate level of relatedness to VP6 sequences of species RVB, in agreement with previous reports (10,12). Of note, BR59, BR60, and BR63 sequences shared high similarities with an RVG strain found in chickens, in both nt (51.6%) and aa (38.3%) levels, compared with RVB. This similarity is also evident in the phylogenetic tree, in which these 3 samples cluster closer to RVG and RVB than to the other species of RV.

Our study reports the detection of a porcine RVH from the Americas. One porcine strain, SKA-1, was previously isolated in Japan; there have been no other reports of porcine RVH. Very little information is available regarding this new RV species, strains of which infect humans and piglets. To date, strains of RVH have been detected in China, Bangladesh, Japan, and now in Brazil. The scarcity of molecular and epidemiologic information on these viruses results from lack of appropriate diagnostic tools. Extensive epidemiologic studies are needed to determine the worldwide dissemination and prevalence of this rotavirus species and its effects on diarrheal diseases.

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Table 2. Comparison of VP6 gene sequences (nt 24–1221) of porcine RV samples BR59, BR60, and BR63 with sequences from representative RV strains of different species, Brazil, 2012*†

| Species | Rotavirus | |
|---------|-------------------------|---------------------|
| | Strain (species origin) | % Identity, nt (aa) |
| A | KU (human) | 35.6 (11) |
| | UK (bovine) | 35.2 (11.2) |
| | OSU (porcine) | 34.7 (11.7) |
| B | ADRV (human) | 51.4 (35.8) |
| | CAL-1 (human) | 50.7 (36.8) |
| | Bang 373 (human) | 51 (36.3) |
| | DB176 (bovine) | 49.7 (37.9) |
| | IDIR (murine) | 50.2 (37.4) |
| C | Bristol (human) | 35 (8.7) |
| | Toyama (bovine) | 34.5 (9) |
| | Cowden (porcine) | 34.7 (8.5) |
| D | 05V0049 (chicken) | 35.2 (13.2) |
| F | 03V0568 (chicken) | 35.5 (10) |
| G | 03V0567 (chicken) | 51.6 (38.3) |
| H | ADRV-N (human) | 72.3 (76.4) |
| | B219 (human) | 71.7 (76.2) |
| | J19 (human) | 72.3 (75.7) |
| | SKA-1 (porcine) | 85.5 (96.9) |

*VP, viral protein; RV, rotavirus.

†The exact position of the 1,197 fragments of the porcine samples BR59, BR60, and BR63 VP6 gene entered into the phylogenetic comparison was based on the RVH SKA-1 VP6 complete gene. GenBank accession nos.: BR59 (KF021619), BR60 (KF021620), BR63 (KF021621), KU (AB022768), UK (X53667), OSU (AF317123), ADRV (M55982), CAL-1 (AB037931), Bang 373 (AY238389), DB176 (GQ358713), IDIR (M84456), Bristol (X59843), Toyama (AB738416), Cowden (M94157), 05V0049 (GU733448), 03V0568 (HQ403603), 03V0567 (HQ403604), ADRV-N (AY632080), B219 (DQ168033), J19 (DQ113902), and SKA-1 (AB576626).

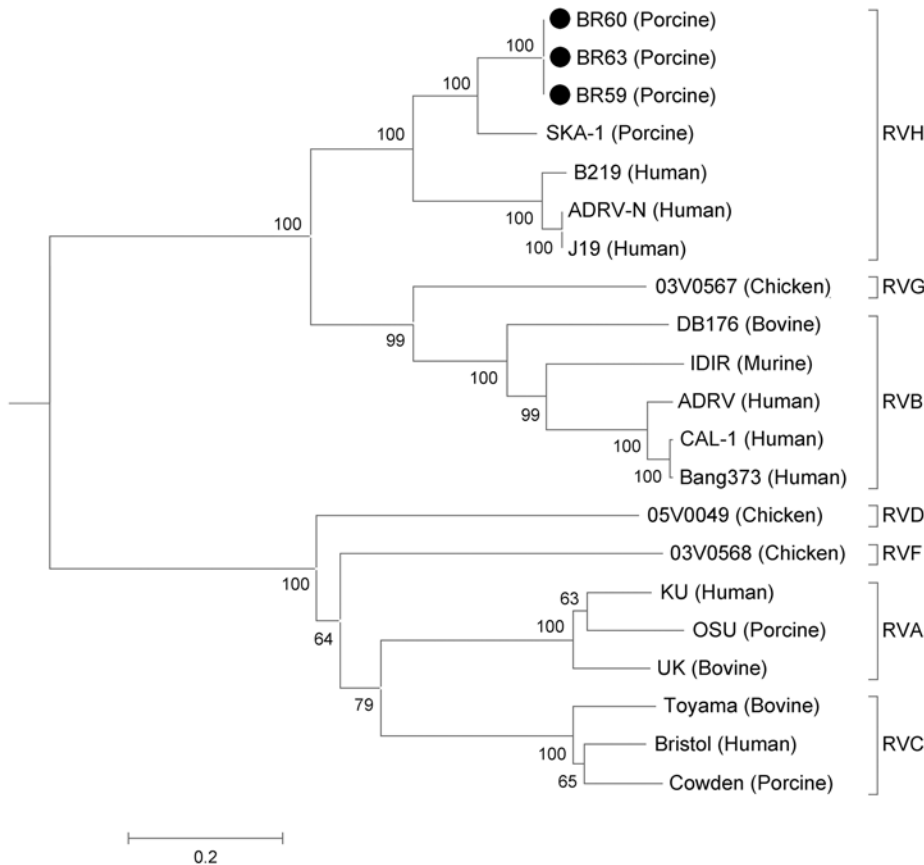


Figure. Phylogenetic tree showing the inferred evolutionary relationships among representative rotavirus (RV) strains belonging to species A, B, C, D, F, G, and H, as well as the samples BR59, BR60, and BR63 based on a 1,197-bp fragment of the viral protein 6 (VP6) gene. The tree was constructed by using the neighbor-joining method and the Kimura 2-parameter nucleotide substitution model. Bootstrapping was statistically supported with 1,000 replicates. Scale bar indicates nucleotide substitutions per site. The VP6 gene sequences of the following strains were obtained from the GenBank database (accession nos.): BR59 (KF021619), BR60 (KF021620), BR63 (KF021621), KU (AB022768), UK (X53667), OSU (AF317123), ADRV (M55982), CAL-1 (AB037931), Bang373 (AY238389), DB176 (GQ358713), IDIR (M84456), Bristol (X59843), Toyama (AB738416), Cowden (M94157), 05V0049 (GU733448), 03V0568 (HQ403603), 03V0567 (HQ403604), ADRV-N (AY632080), B219 (DQ168033), J19 (DQ113902), and SKA-1 (AB576626).

Ms Molinari is a graduate student at Universidade Estadual de Londrina, Brazil. Her research interest is the genomic characterization of rotavirus.

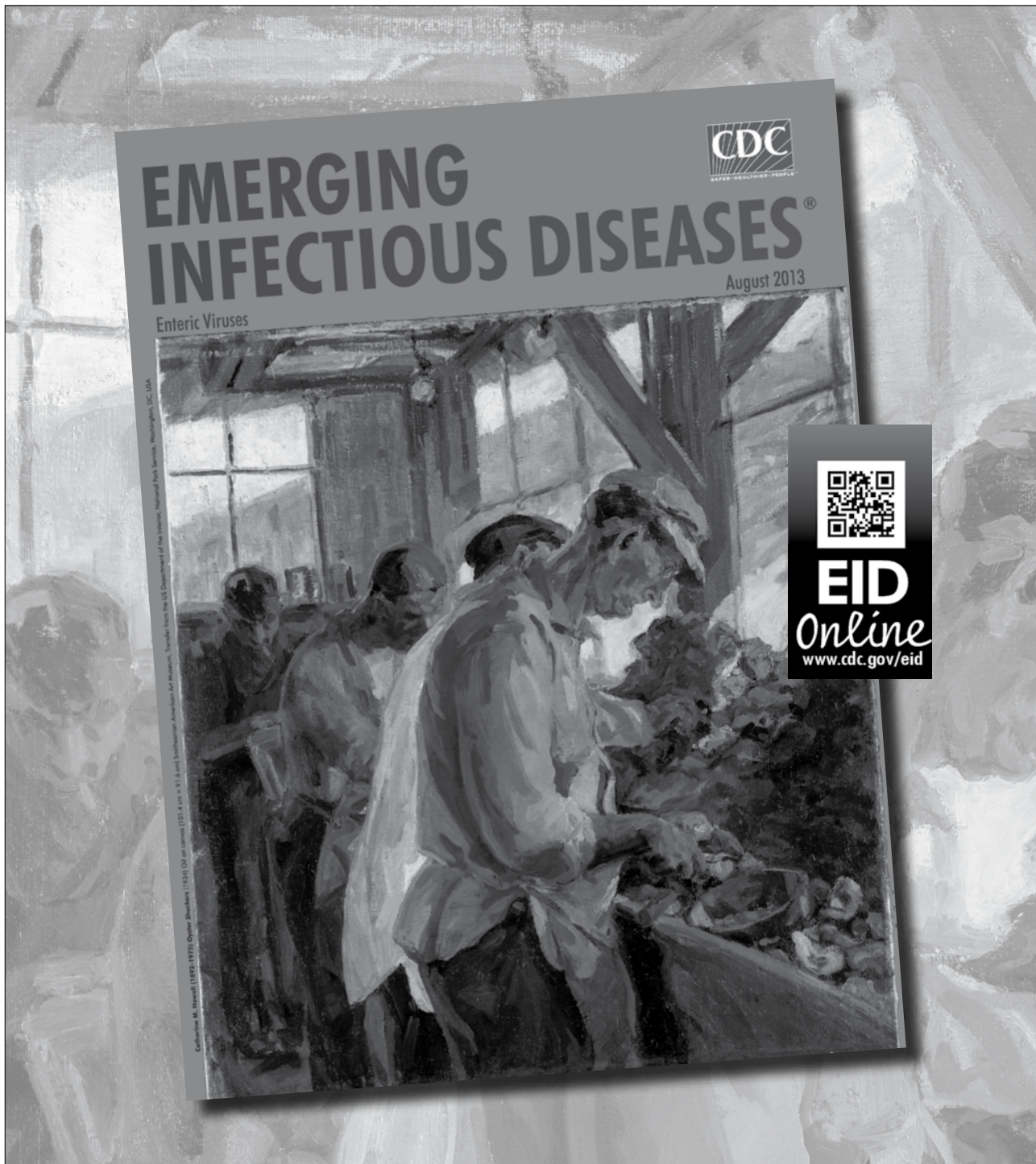
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Address for correspondence: Amauri A. Alfieri, Laboratory of Animal Virology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina, P.O. Box 10011, 86057-970, Londrina, Parana, Brazil; email: alfieri@uel.br



Iatrogenic Meningitis Caused by *Neisseria sicca/subflava* after Intrathecal Contrast Injection, Australia

Damoon Entesari-Tatafi, Mohammad Bagherirad, Doreen Quan, and Eugene Athan

We report a case of invasive *Neisseria sicca/subflava* meningitis after a spinal injection procedure during which a face mask was not worn by the proceduralist. The report highlights the importance of awareness of, and adherence to, guidelines for protective face mask use during procedures that require sterile conditions.

Neisseria sicca/subflava is a known commensal bacterium of the upper respiratory tract and has rarely been found to cause meningitis, endocarditis, or bacteremia (1,2). There is limited literature describing the clinical course and optimal management of iatrogenic meningitis caused by *N. sicca/subflava*. Infections of the central nervous system caused by this organism occur rarely; most reported cases are in the pediatric population (3,4). In the literature describing illness in adults, 4 cases of *N. sicca* meningitis are described, 1 of which was an iatrogenic case: a complication of ventriculostomy (5). Of 2 case reports of iatrogenic *N. subflava* meningitis (6,7) 1 case occurred 48 hours after intrathecal injection in a young immunocompetent female patient (7).

Iatrogenic meningitis is a well-documented complication of lumbar puncture and carries an estimated mortality of ~35% extrapolated from a US data review (8). Most cases occur after catheter insertion or injection into the intrathecal space, but infection related to diagnostic lumbar puncture is less common. The most frequently identified causative organisms in samples are *Streptococcus salivarius*, *Streptococcus viridans* and other α -hemolytic streptococci, *Staphylococcus aureus*, and *Pseudomonas* spp. (9).

Multiple case reports of iatrogenic meningitis associated with nonuse of face masks prompted a review of the evidence by the Healthcare Infection Control Practices Advisory Committee, which advises US Health and Human

Services. The result was a recommendation for the routine use of face masks for clinicians placing a catheter or injecting material into the epidural or spinal space, which was included in the guideline, 2007 Safe Injection Practices to Prevent Transmission of Infection to Patients (10). After subsequent outbreaks, the US Centers for Disease Control and Prevention released a clinical reminder in 2011 (11).

The Case

We report the case of an independent 78-year-old man with low back pain and mild lower limb weakness in whom iatrogenic meningitis and associated bacteremia developed after a computed tomography myelogram. The procedure was performed on June 21, 2013, on an outpatient basis in the radiology department of Geelong Hospital, a teaching tertiary hospital. The patient's medical history included atrial fibrillation that was managed by a permanent pacemaker, which precluded the use of magnetic resonance imaging. Other conditions in his medical history included hypertension, gout, rash after penicillin exposure, and moderate chronic obstructive pulmonary disease that did not require long-term prednisone.

The patient underwent fluoroscopy-guided spinal injection of 10 mL of iohexol 300 mg/mL contrast medium from a single-dose vial prepared by a nurse in accordance with Centers for Disease Control and Prevention guidelines (10). The procedure was performed at the level of lumbar disc space 4–5 by using a 22-gauge spinal needle. Aseptic measures included the use of sterile gloves, gown, drapes, and adequate skin antisepsis by the proceduralist. However, in conflict with hospital policy, a face mask was not used. The procedure was prolonged because the patient's challenging anatomy required multiple passes. The opening pressure was normal; the patient showed no signs of complications immediately post-procedure and was discharged after 4 hours of observation.

The patient came to the emergency department within 18 hours of the procedure after onset of confusion, severe headache, neck pain, nausea, vomiting, and fever. The patient had a deteriorating conscious state; the examination showed no additional remarkable findings. Specifically, there was no meningism, photophobia, or focal neurologic deficit. The puncture site was not inflamed; no other source of infection was identified.

The patient's condition was investigated by using a septic screen, including blood cultures and a diagnostic lumbar puncture. Subsequently, treatment with intravenous ceftazidime, vancomycin, and dexamethasone were commenced for presumed iatrogenic meningitis. The initial investigations showed a leukocyte count of $16.1 \times 10^9/L$ and a C-reactive protein level of 9.8 mg/L that increased to 185 mg/L within 24 hours of the patient's return. The diagnostic lumbar puncture revealed turbid cerebrospinal fluid

Author affiliation: Barwon Health, Geelong, Victoria, Australia

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consisting of $4,260 \times 10^6/L$ leukocytes, 99% polymorphonuclear leukocytes, $270 \times 10^6/L$ erythrocytes, and levels of 3.0 g/L protein, and 1.7 mmol/L glucose.

Initial microscopic examination of cerebrospinal fluid revealed a gram-positive coccus; on review, intracellular diplococci were identified, suggesting an undercolored specimen that likely represented *Neisseria* species. Cefazidime was discontinued and intravenous ceftriaxone, 2 g twice daily, was initiated; vancomycin was stopped on confirmation of *N. sicca/subflava* infection. A single set of blood cultures initiated on admission were also positive for this bacterial species, but subsequent blood cultures were negative. The final isolates of *N. sicca/subflava* from blood culture and cerebrospinal fluid were penicillin resistant but ceftriaxone sensitive.

The patient required 24 hours of management in the intensive care unit because of profound confusion and severe agitation. He improved substantially during a period of 7 days in the hospital and was transitioned from intravenous ceftriaxone to a 7-day course of oral ciprofloxacin, 500 mg twice daily, to be completed after discharge. On discharge, the patient had improved to his baseline level of cognitive function.

The proceduralist who performed the spinal injection denied upper respiratory tract symptoms, but admitted to not using a face mask because of unawareness of the hospital lumbar puncture protocol mandating face mask use when conducting all lumbar punctures. A nasopharyngeal swab specimen confirmed that the proceduralist was a carrier of *N. sicca/subflava* who had an identical antibacterial drug resistance pattern to that identified in the case-patient. Molecular typing of the organism could not be performed because the patient's isolate had been discarded.

Review by the infection control team identified the low level of awareness of and adherence to the hospital protocol for wearing face masks as a contributory factor. No other cases of iatrogenic meningitis could be traced to the proceduralist.

Conclusions

We describe the clinical course of iatrogenic meningitis caused by *N. sicca/subflava* with associated bacteremia after a spinal injection procedure. The suspected mechanism of transmission in this case is contamination of the sterile field or equipment by oropharyngeal secretions caused by nonuse of a face mask by a carrier of this organism. The prolonged and technically difficult nature of the procedure likely contributed to contamination by increasing exposure. This hypothesis is supported by the isolation of *N. sicca/subflava* with an identical antimicrobial resistance pattern in a swab sample from the proceduralist's nasopharynx. An alternative mechanism could involve oropharyngeal secretions from assistant staff or contamination of the contrast

medium for spinal injection, although the latter is less likely because it was prepared in accordance with guidelines.

In this case, nonadherence to face mask use standards resulted from lack of awareness by the clinician. To improve clinician awareness after this event, the infection control unit of the hospital updated its lumbar puncture protocol. The protocol mandated the use of face masks for all lumbar punctures, and it was disseminated to all clinical areas where lumbar punctures were performed.

We believe that the best method for promotion of face mask use is making face masks available in preparatory areas and procedure rooms and requiring that all lumbar punctures are performed with the use of a face mask. We also believe there is a need for a system that maintains vigilance. This could include documentation of face mask use for all lumbar punctures and intermittent auditing.

In conclusion, *N. sicca/subflava*, an organism that is harmless in the human oropharynx, can cause invasive infection in immunocompetent adults when introduced directly into the subarachnoid space. Prevention is essential; thus, wearing of face masks should be mandatory for all personnel present during lumbar punctures and all other sterile procedures, and compliance should be monitored.

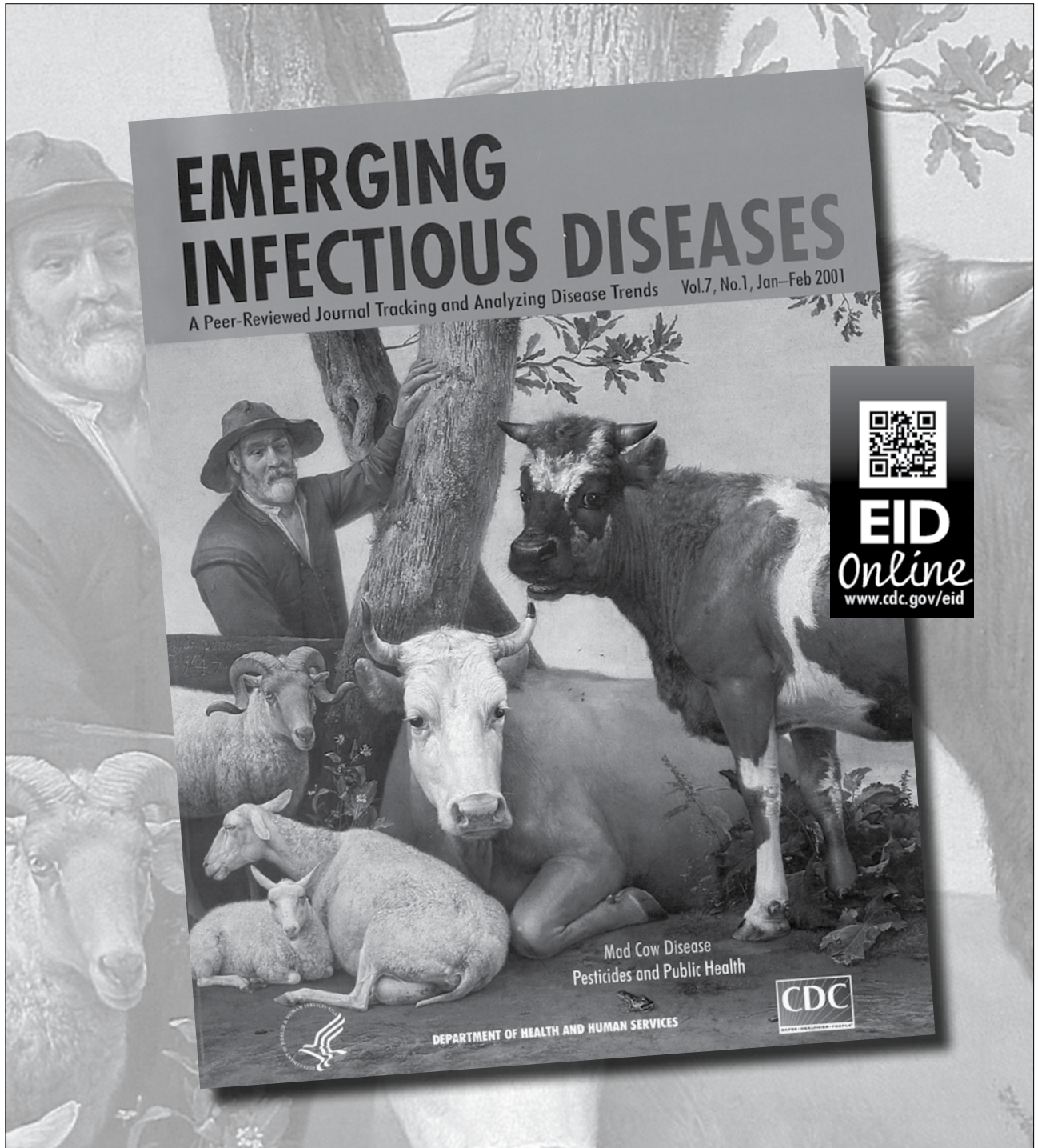
Dr Entesari-Tatafi is an advanced trainee in adult acute care medicine (general medicine) and a dual trainee in intensive care medicine at Barwon Health, Geelong, Australia. His primary research interests are clinical audit and improvement.

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Address for correspondence: Damoon Entesari-Tatafi, Department of General Medicine, Barwon Health, Bellerine Street, Geelong, Victoria 3220, Australia; email: dentej2@hotmail.com



Identification of Possible Virulence Marker from *Campylobacter jejuni* Isolates

James W. Harrison, Tran Thi Ngoc Dung,
Fariha Siddiqui, Sunee Korbrisate,
Habib Bukhari, My Phan Vu Tra,
Nguyen Van Minh Hoang, Juan Carrique-Mas,
Juliet Bryant, James I. Campbell,
David J. Studholme, Brendan W. Wren,
Stephen Baker, Richard W. Titball,
and Olivia L. Champion

A novel protein translocation system, the type-6 secretion system (T6SS), may play a role in virulence of *Campylobacter jejuni*. We investigated 181 *C. jejuni* isolates from humans, chickens, and environmental sources in Vietnam, Thailand, Pakistan, and the United Kingdom for T6SS. The marker was most prevalent in human and chicken isolates from Vietnam.

Campylobacter species are the principal bacterial cause of human foodborne enterocolitis worldwide (1). Despite the global significance of *C. jejuni* as a leading cause of diarrheal disease (2), the mechanisms of pathogenesis of *C. jejuni* are not well understood. Research on *Campylobacter* epidemiology has largely been conducted in high-income countries and therefore may not be representative of global patterns.

Recently, a novel class of protein translocation system was identified in gram-negative bacteria. This system, named the type-6 secretion system (T6SS), has been found to play roles in pathogen–pathogen and host–pathogen interactions and has a major effect on virulence in a range of pathogens, including *Vibrio cholerae* (3–6) (reviewed

in 7,8). A functional T6SS was recently identified in *C. jejuni* (9,10) and found to have several roles in virulence, influencing cell adhesion, cytotoxicity toward erythrocytes, and colonization of mice (9,10). However, it is unknown whether T6SS changes the effects of these pathogens in human infection.

In this study, we aimed to determine whether presence of T6SS in *C. jejuni* is potentially a marker associated with more severe human disease. Moreover, because human infection with *C. jejuni* is often associated with contact with poultry, we investigated whether poultry harbor *C. jejuni* that possess T6SS.

The Study

To partially address bias toward study of *C. jejuni* strains from high-income countries and the under-representation of strains from Asia in previous studies, we previously sequenced the genomes of 12 clinical isolates of *C. jejuni* from Asia: 4 from Thailand, 3 from Pakistan, and 5 from Vietnam (J. Harrison, unpub. data; Figure 1). We noted that 8 (67%) of these isolates possessed a cluster of genes homologous to the recently described T6SS (Figure 1). This finding was in contrast to findings regarding previously sequenced *C. jejuni* genomes; only 10 (14%) of 71 previously sequenced *C. jejuni* strains possessed an apparently intact T6SS gene cluster (Figure 1; full listing of genomes is in online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/6/13-0635-Techapp1.pdf). Several other strains from our study and previously sequenced strains contained ≥ 1 T6SS genes but not a complete T6SS cluster. Figure 1 shows the presence and absence of each T6SS gene in each available genome sequence (J. Harrison, unpub. data) and the previously sequenced strains. A nonrandom distribution of T6SS can be seen across the phylogenetic diversity of *C. jejuni*; T6SS is limited to certain clades, and degeneration of the T6SS gene cluster apparently occurs in parallel within several of those clades (Figure 1).

Our genome sequencing analysis indicated that strains possessing a complete T6SS cluster could be distinguished by the presence of the *hcp* gene (Figure 1) (9,10). Therefore, we used *hcp* as a proxy for determining the presence of a functional T6SS in 181 *C. jejuni* isolates from chickens, humans, and environmental sources (collections of the Oxford University Clinical Research Unit and the University of Exeter; online Technical Appendix Table 2). We designed and used a multiplex PCR (online Technical Appendix Table 3) to screen for the presence of *hcp* in these isolates; the conserved *C. jejuni* housekeeping gene, *gltA*, was used as a positive control.

Of the 181 isolates, 28 originated from chickens in the United Kingdom and 21 from chickens in Vietnam. The *hcp* gene was found significantly more often in isolates

Author affiliations: University of Exeter, Exeter, UK (J.W. Harrison, D.J. Studholme, R.W. Titball, O.L. Champion); The Hospital for Tropical Diseases, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam (T.T.N. Dung, M.P.V. Tra, N.V.M. Hoang, J. Carrique-Mas, J. Bryant, J.I. Campbell, S. Baker); Comsats University, Islamabad, Pakistan (F. Siddiqui, H. Bukhari); Mahidol University, Bangkok, Thailand (S. Korbrisate); University of Oxford, Oxford, UK (J. Carrique-Mas, J. Bryant, J.I. Campbell, S. Baker); and London School of Hygiene and Tropical Medicine, London, UK (B.W. Wren)

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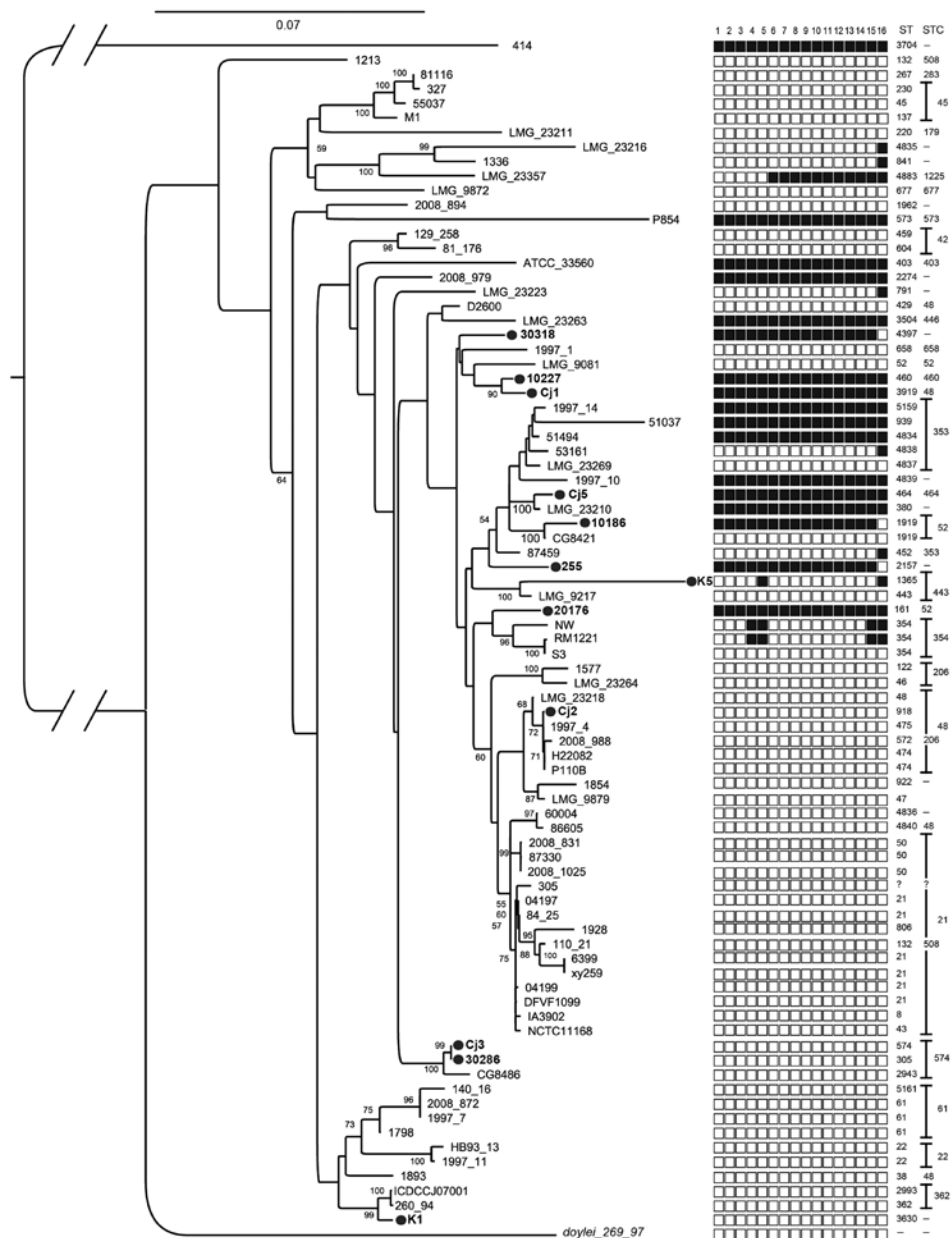


Figure 1. Distribution of the type-six secretion system (T6SS) marker across the phylogenetic diversity of *Campylobacter jejuni* strains, as determined by multilocus sequence analysis. We generated a maximum-likelihood tree from concatenated nucleotide alignments of 31 housekeeping genes; nucleotide sequences were aligned by using MUSCLE (www.drive5.com/muscle) and masked by using GBLOCKS (<http://molevol.cmima.csic.es/castresana/Gblocks.html>). Maximum-likelihood analysis was done by using the GTR model in PhyML (<http://code.google.com/p/phyml/>). Numbers on nodes denote bootstrap values (1,000 bootstrap replicates); values <50 are not shown. Black circles indicate strains whose genomes were sequenced in this study (GenBank accession nos. AUUQ00000000, AUUP00000000, AUUO00000000, AUUN00000000, AUUM00000000, AUUL00000000, AUUK00000000, AUUJ00000000, AUUI00000000, ARWS00000000, AUUH00000000, AUUG00000000). We inferred the presence/absence of each of the T6SS genes on the basis of TBLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch) searches against the predicted proteins sequences from *C. jejuni* strain 414 (National Center for Biotechnology Information reference sequence no. NZ_CM000855). Presence or absence of each gene is indicated by a black or white square, respectively, for each strain: column 1, *hcp*; column 2, *icmF_1*; column 3, *icmF_2*; column 4, *vasK*; column 5, *FHA*; column 6, *vasF*; column 7, *vasE*; column 8, *vasD*; column 9, *impA*; column 10, *impD*; column 11, *impC*; columns 12 and 13, conserved hypotheticals; column 14, *vasA*; column 15, *vasB*; column 16, *vgrg*. The sequence type (ST) and ST complex (STC) columns represent global multilocus sequence types as described by the Oxford multilocus sequence typing scheme (<http://pubmlst.org>). ?, unknown ST; -, isolate could not be allocated to a specific ST or STC. Scale bar indicates nucleotide substitutions per site. Further details of the isolates are provided in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/20/6/13-0635-Techapp1.pdf).

from Vietnam (15 [71.4%] isolates) than in those from the United Kingdom (1 [3.5%] isolate) ($p < 0.01$ by 2-sample Z-test; online Technical Appendix Figure 1). An additional 38 of the isolates were from humans in the United Kingdom and 33 from humans in Vietnam; again, the *hcp* gene was significantly more prevalent in isolates from Vietnam (20 [60.6%] isolates) than those from the United Kingdom (1 [2.6%] isolate) ($p < 0.01$ by 2-sample Z-test; online Technical Appendix Figure 2).

We also found that patients infected with *hcp*-positive *C. jejuni* experienced bloody diarrhea more commonly than those infected with *hcp*-negative *C. jejuni*. For the 36 isolates for which detailed clinical data on patients were available, 6 (31.6%) of 19 patients in Vietnam who were infected with *hcp*-positive *C. jejuni* had bloody diarrhea, compared with 1 (5.9%) of 17 patients infected with *hcp*-negative *C. jejuni* ($p < 0.05$ by 2-sample Z-test) (Figure 2). These results suggest a potential correlation between T6SS and bloody diarrhea, a serious clinical manifestation of the infection that results in higher rates of hospitalization and greater need for treatment with antimicrobial drugs (11). Moreover, *Campylobacter*-related septicemia developed in the 1 patient in the United Kingdom who was infected with a T6SS-positive strain (11). These data suggest that infection with the *C. jejuni* T6SS genotypic strains is associated with more severe disease. However, for sample bias to be ruled out, a comprehensive study is required in which the prevalence of T6SS is measured in *C. jejuni* samples from patients with mild and severe forms of infection.

We found a number of *C. jejuni* strains from humans and poultry that possessed the T6SS cluster, although some strains showed a slightly modified gene order (online Technical Appendix Table 1 and Figure 3). However, most (61 [85.9%] of 71) of the previously sequenced *C. jejuni* isolates lacked a complete T6SS gene cluster (Figure 1); this finding might explain why T6SS was not discovered in *C. jejuni* sooner. Conversely, our PCR-based study frequently identified the *hcp* marker in isolates from Thailand, Pakistan, and Vietnam (Table). We cannot be certain that all of the isolates with the *hcp* marker possessed a complete and functional T6SS gene cluster, but the *hcp* gene is consistently associated with the presence of a complete T6SS cluster in all available sequenced *C. jejuni* genomes (Figure 1). This correlation lends confidence to the use of *hcp* as a proxy.

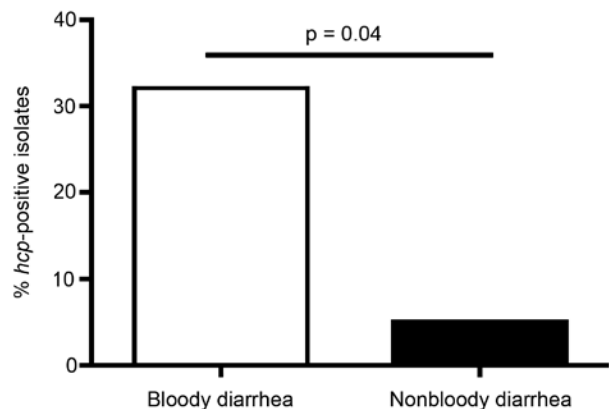


Figure 2. Percentage of *hcp*-positive *Campylobacter jejuni* strains isolated from patients in Vietnam who had bloody diarrhea and nonbloody diarrhea. Patients who were hospitalized because of *C. jejuni* infection were scored for the presence of bloody diarrhea or nonbloody diarrhea, and presence of the *hcp* type-six secretion system (T6SS) marker in strains isolated from the patients was determined. Of patients with bloody diarrhea, 32% were infected with *hcp*-positive strains; of patients with nonbloody diarrhea, 5% were infected with *hcp*-positive strains.

Poultry are a well-documented reservoir of human *Campylobacter* infection (12). We found that *Campylobacter* strains harboring the *hcp* marker were significantly associated with chickens in Asia. Large numbers of poultry are imported into North America and Europe from low-income countries, including Thailand (13). This process could introduce T6SS-positive *Campylobacter* genotypes into the food chains of the importing countries, posing a potential emerging threat to public health.

Conclusions

Our results suggest that the T6SS may be more prevalent in *C. jejuni* in Vietnam, Pakistan, and Thailand than in the United Kingdom. Furthermore, our results suggest that *hcp* may be a marker associated with severe human disease caused by *C. jejuni* infection in Vietnam, although there is no evidence that the association is causal. Chickens imported from these countries could be a source of *hcp*-positive strains and may have the potential to cause severe human infection.

Table. Overview of *Campylobacter jejuni* strains containing type-six secretion system genetic marker *hcp*, by country and isolate source

| Isolate source | No. <i>hcp</i> -positive strains/total no. strains (%) | | | | |
|----------------|--|--------------|-------------|------------|---------------|
| | United Kingdom | Vietnam | Pakistan | Thailand | Total |
| Human | 1/38 (2.6) | 20/33 (60.6) | 2/13 (15.4) | 1/3 (33.3) | 24/87 (27.6) |
| Chicken | 1/28 (3.9) | 15/21 (71.4) | 1/2 (50) | 0 | 17/51 (33.3) |
| Other | 5/26 (19.2) | 1/14 (7.1) | 1/3 (33.3) | 0 | 7/43 (16.3) |
| Total | 7/92 (7.6) | 36/68 (54.4) | 4/18 (22.2) | 1/3 (33.3) | 48/181 (26.5) |

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Mr Harrison is a PhD student at the University of Exeter under the supervision of D.S. His research focuses on using bioinformatic methods to investigate the comparative genomics of emerging diseases and plant-associated microbes.

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Address for correspondence: Olivia L. Champion, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK; email: O.L.Champion@exeter.ac.uk

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Gastroenteritis Outbreaks Caused by a DS-1-like G1P[8] Rotavirus Strain, Japan, 2012–2013

Seiji P. Yamamoto, Atsushi Kaida, Hideyuki Kubo, and Nobuhiro Iritani

Rotavirus A (RVA) genotype G1P[8], a hallmark of the Wa-like strain, typically contains only genotype 1 genes. However, an unusual RVA G1P[8] with genotype 2 genes was recently detected in Japan. We determined the complete genomic constellation of this RVA. Our findings suggest that mixed RVAs may be more competitive than once thought.

It is estimated that rotavirus A (RVA), the leading cause of severe gastroenteritis in children worldwide, causes >500,000 deaths among children each year, primarily in developing countries (1). Genes of viral protein (VP) 7 and VP4 form the basis of a dual classification system that defines the RVA G- and P-types, respectively. Five G-types (G1–4 and G9) and 3 P-types (P[4], P[6], and P[8]) represent most of the G-P-combined RVA strains (2). RVAs are classified on the basis of a system that assigns a specific type to each of the 11 RNA gene segments, according to established nucleotide percentage cutoff values (3).

Two well-known RVA prototype strains are Wa (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) and DS-1 (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2). RVAs G1P[8], G3P[8], G4P[8], and G9P[8] are pure Wa genogroup members because they have a Wa-like constellation (Gx-P[x]-I1-R1-C1-M1-A1-N1-T1-E1-H1) composed of genotype 1 genes; G2P[4] is a pure DS-1 genogroup member because it has a DS-1-like constellation (Gx-P[x]-I2-R2-C2-M2-A2-N2-T2-E2-H2) composed of genotype 2 genes (4). The segmented nature of RVA genomes enables them to undergo gene reassortment during co-infection in 1 cell, leading to the emergence of progeny viruses containing mixed segments from ≥ 2 different parental strains. However, some human RVA G/P-types have a purely Wa-like or a DS-1-like genome constellation. Mixed viruses are rarely detected and have a low prevalence, even if they emerge; thus, it is

Author affiliation: Osaka City Institute of Public Health and Environmental Sciences, Osaka, Japan

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believed that mixed viruses may be less fit than parental strains and unable to compete with them (5).

We identified and characterized a prevalent genotype G1P[8] RVA with genotype 2 genes. The RVA was detected during rotavirus gastroenteritis outbreaks in Japan.

The Study

During 2009–2013, a total of 21 RVA-associated gastroenteritis outbreaks occurred among children ≤ 7 years of age in Osaka City, the third largest city in Japan; 20 of the outbreaks were in nursery schools and 1 was in a primary school (Table 1). To determine which outbreaks were associated with RVA, we tested fecal samples from children who became ill during the outbreaks. An RVA-associated outbreak was defined as a gastroenteritis (vomiting and/or diarrhea) outbreak among ≥ 8 children who were epidemiologically linked, among whom ≥ 2 had RVA-positive ELISA results by our testing. Analyses to determine epidemiologic links included age, outbreak setting, person-to-person virus transmission, and date of symptom onset.

We extracted viral RNA from RVA-positive 10% fecal suspensions and converted the RNA to cDNA by using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and RVA-specific primers (6). PCR was performed with Ex Taq DNA Polymerase (TaKaRa Bio Inc., Shiga, Japan); the products were used for direct sequencing with a BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific). RotaC v2.0 was used for RVA genotyping (7), and nucleotide alignment was confirmed by using ClustalW (www.clustal.org/) in MEGA5.2 (8). MEGA5.2 was used to identify the optimal evolutionary model that best fit each sequence dataset and to construct maximum-likelihood trees (8).

All detected RVA strains were sequenced and genotyped for 3 regions: VP7 (877 bp), including antigenic epitopes 7-1a, 7-1b, and 7-2 (9); VP4 (656 bp), including antigenic epitopes 8-1 to 8-4 (10); and VP6 (1,132 bp). Only 1 RVA genotype was detected in each outbreak (Table 1). G1-P[8]-I1, the most common Wa-like genotype worldwide (4), was detected in a few outbreaks each year during 2010–2013. However, an unusual RVA genotype, G1-P[8]-I2, first identified in April 2012, was detected in 6 (66.7%) of the 9 RVA outbreaks in 2012 alone. Moreover, infections caused by genotype G1-P[8]-I2 were detected in 6 (85.7%) of the 7 RVA outbreaks in 2013. Phylogenetic analysis based on the VP7, VP4, and VP6 regions showed that 12 genotype G1-P[8]-I2 strains were closely related to each other (>99% nt identity) (Figure). These data suggest that G1-P[8]-I2 originated from 1 parental strain and became the prevalent genotype in RVA-associated outbreaks in Osaka City during 2012–2013.

We selected 6 strains for study. The strains had been isolated during outbreaks HC12012, HC12016, HC12022,

Table 1. Description of RVA-associated outbreaks in Osaka City, Japan, during 2009–2013*

| Outbreak year, month | Outbreak no. | No. patients | No. specimens collected/ no. positive for RVA | RVA genotype |
|----------------------|--------------|--------------|--|--------------|
| 2009 | | | | |
| April | HC09022 | 24 | 6 (2) | G9-P[8]-I1 |
| 2010 | | | | |
| April | HC10032† | 19 | 1 (1) | G1-P[8]-I1 |
| December | HC10062 | Unknown | 2 (2) | G2-P[4]-I2 |
| 2011 | | | | |
| April | HC11027 | 20 | 3 (2) | G1-P[8]-I1 |
| May | HC11030 | 18 | 3 (2) | G9-P[8]-I1 |
| 2012 | | | | |
| March | HC12010 | 18 | 3 (3) | G1-P[8]-I1 |
| April | HC12012 | 26 | 5 (3) | G1-P[8]-I2 |
| April | HC12013 | 41 | 5 (2) | G1-P[8]-I2 |
| April | HC12014† | 15 | 5 (1) | G9-P[8]-I1 |
| April | HC12016† | 18 | 3 (1) | G1-P[8]-I2 |
| April | HC12018† | 23 | 3 (1) | G1-P[8]-I2 |
| April | HC12020 | 32 | 2 (2) | G1-P[8]-I2 |
| May | HC12021 | 29 | 3 (2) | G1-P[8]-I1 |
| May | HC12022 | 18 | 2 (2) | G1-P[8]-I2 |
| 2013 | | | | |
| March | HC13027 | 33 | 5 (4) | G1-P[8]-I1 |
| May | HC13037 | 26 | 5 (2) | G1-P[8]-I2 |
| May | HC13040 | 8 | 2 (2) | G1-P[8]-I2 |
| May | HC13041† | 26 | 1 (1) | G1-P[8]-I2 |
| May | HC13043 | 21 | 5 (2) | G1-P[8]-I2 |
| May | HC13048 | 11 | 2 (2) | G1-P[8]-I2 |
| May | HC13049 | 15 | 5 (3) | G1-P[8]-I2 |

*All outbreaks occurred in nursery schools, except outbreak no. HC10062 (Dec 2010), which occurred in a primary school. RVA, rotavirus A.

†Only 1 sample had test results positive for RVA but the outbreak was confirmed on the basis of epidemiologic data from hospital clinics showing that other patients had RVA-positive test results.

each of which occurred at the beginning, middle, or end of an RVA G1-P[8]-I2 outbreak period in 2012, and during outbreaks HC13037, HC13043, and HC13049, each of which occurred at the beginning, middle, or end of an RVA G1-P[8]-I2 outbreak period in 2013. We determined the complete genome constellations of the 6 strains on the basis of partial sequences of the other 8 RNA gene segments. Genotyping of all 11 RNA gene segments showed that the 6 strains each had 9 genotype 2 genes: G1-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2 (DS-1-like G1P[8]). As a representative of DS-1-like G1P[8] strains, HC12016/2012/G1P[8] (hereafter referred to as strain HC12016) was used for analysis of nearly full-length sequences of all 11 RNA gene segments. Strain HC12016 (listed in Table 2) was deposited in the DNA Data Bank of Japan as RVA/Human-wt/JPN/HC12016/2012/G1P[8] under accession nos. AB848004–AB848014.

In addition, we performed a BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search of each RNA gene segment of strain HC12016. The results showed that the VP7 and VP4 genes of RVA/Human-wt/USA/2007719635/2007/G1P[8] (hereafter referred to as strain 2007719635), a pure Wa genogroup member, shared the highest identity with strain HC12016 (Table 2). In contrast, 4 pure DS-1 genogroup strains contained ≥ 1 gene showing the highest identity, but none of those strains shared $>98\%$ identity in all 9 genes (i.e., VP6, VP1–VP3, and nonstructural protein [NSP] 1–5) (Table 2).

It should be noted that while our manuscript was under review, G1P[8] strains that had 3 genotype 2 genes (VP6, NSP4, and NSP5) were reported from a different location in Japan (Okayama Prefecture) (11); these strains were also called DS-1-like G1P[8]. The genes of these strains showed 98.8%–100.0% identity with strain HC12016 genes (Table 2).

Conclusions

We determined the full genomic constellation of the DS-1-like G1P[8] strain that emerged in 2012. Our results suggest that this strain, detected in Osaka City, seems to be an intergenogroup reassortant between a G1P[8] strain (Wa genogroup) and a pure member of the DS-1 genogroup. Five genes (VP7, VP4, VP6, NSP4, and NSP5) of the DS-1-like G1P[8] strains from Osaka City were closely related to those of DS-1-like G1P[8] strains from Okayama Prefecture (11), indicating that the strains may have originated from the same parental strain. DS-1-like G1P[8] was prevalent in Osaka City and Okayama Prefecture around the same time (11); thus, because of its rapid spread, the strain should be monitored further.

The extent of rotavirus vaccination in Japan has varied, so the level of vaccine coverage in the country is unknown. Thus, it is unclear whether the emergence of strain DS-1-like G1P[8] was caused by vaccine pressure. The introduction of rotavirus vaccines must be followed by full genomic

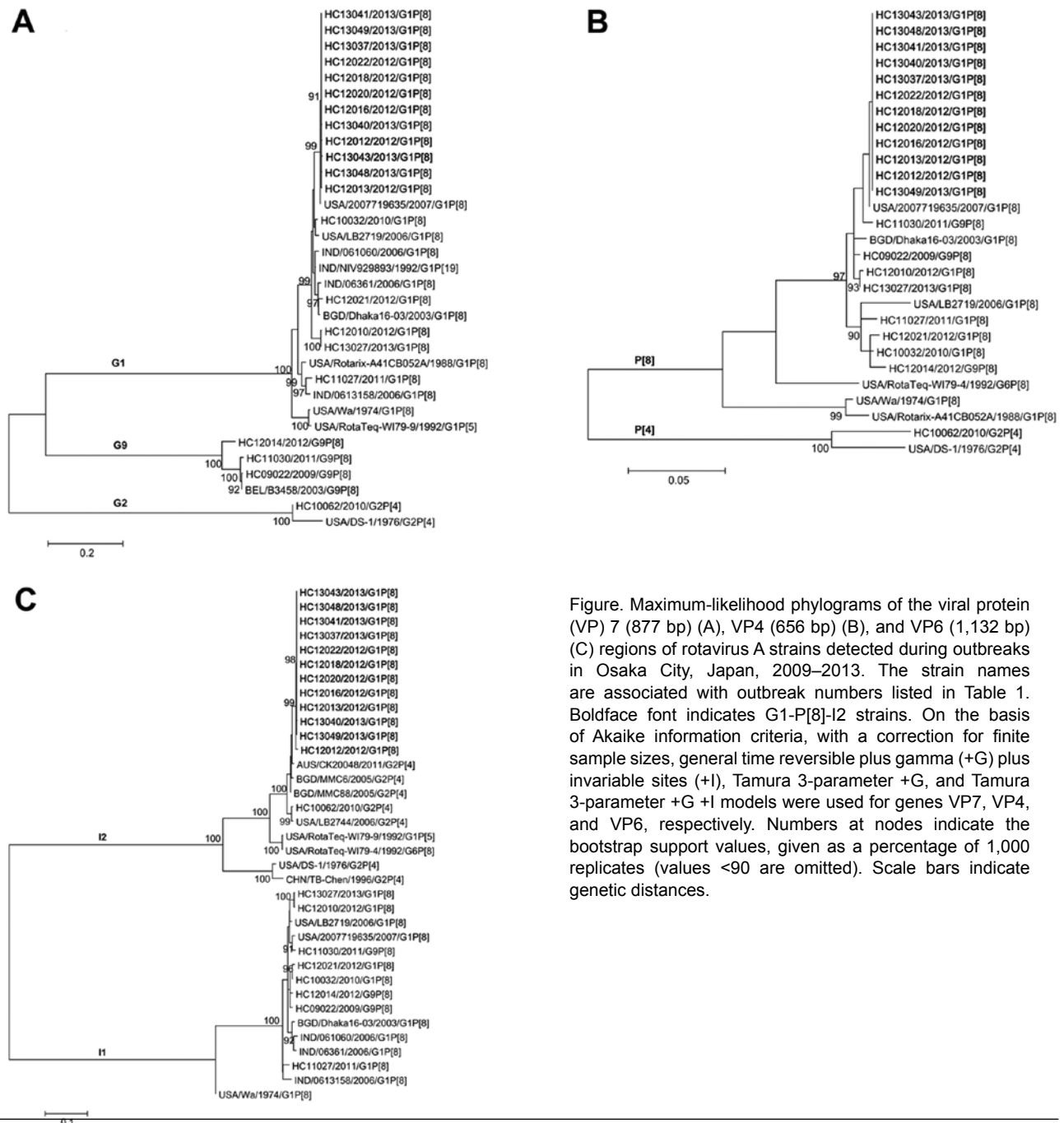


Figure. Maximum-likelihood phylogenetic trees of the viral protein (VP) 7 (877 bp) (A), VP4 (656 bp) (B), and VP6 (1,132 bp) (C) regions of rotavirus A strains detected during outbreaks in Osaka City, Japan, 2009–2013. The strain names are associated with outbreak numbers listed in Table 1. Boldface font indicates G1-P[8]-I2 strains. On the basis of Akaike information criteria, with a correction for finite sample sizes, general time reversible plus gamma (+G) plus invariable sites (+I), Tamura 3-parameter +G, and Tamura 3-parameter +G +I models were used for genes VP7, VP4, and VP6, respectively. Numbers at nodes indicate the bootstrap support values, given as a percentage of 1,000 replicates (values <90 are omitted). Scale bars indicate genetic distances.

analysis of RVA so that reassortants between wild-type and vaccine strains can be monitored. Such analyses may also lead to the discovery of new wild-type reassortants with unusual genome constellations and provide us with plausible explanations for rotavirus epidemics.

It is unclear why some RVA G/P-types have pure genogroup genomic constellations (5). Although the whole VP7 and VP4 amino acid sequences of strains HC12016 and 2007719635 are identical (data not shown), strain HC12016 contains genes that encode DS-1-like

proteins. In contrast, strain 2007719635 is a pure Wa genogroup member, which suggests that G1P[8] type outer capsid proteins encoded by strain HC12016 are compatible with Wa-like and DS-1-like backbones. In other words, the DS-1-like G1P[8] seems to retain its fitness, even though it is the progeny of a reassortment event between viruses of different genogroups. Further studies using DS-1-like G1P[8] strains may provide key insights into preferred combinations of RVA genes derived from different genotypes.

Table 2. Nucleotide sequence identity of various RVA strains to RVA DS-1-like G1P[8] strain HC12016, which was detected during an outbreak in Osaka City, Japan, 2012*

| Strain name | Genogroup | % Nucleotide identity by gene segment | | | | | | | | | | |
|-------------------|-----------|---------------------------------------|------------------|------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------------|------------------|
| | | VP7 | VP4 | VP6 | VP1 | VP2 | VP3 | NSP1 | NSP2 | NSP3 | NSP4 | NSP5 |
| Wa† | Wa | 91.96 | 90.30 | 79.73 | 79.94 | 80.92 | 77.14 | 75.14 | 81.59 | 78.83 | 81.19 | 81.69 |
| 2007719635‡ | Wa | <u>99.60</u> | <u>99.44</u> | 79.73 | 80.07 | 80.41 | 77.14 | 75.16 | 82.88 | 78.62 | 80.41 | 84.29 |
| DS-1§ | DS-1 | 72.56 | 87.22 | 87.68 | 90.17 | 93.93 | 93.06 | 92.60 | 85.93 | 94.65 | 89.34 | 92.91 |
| CK20048¶ | DS-1 | 72.96 | 87.09 | <u>98.82</u> | <u>99.25</u> | <u>99.30</u> | 87.82 | <u>99.02</u> | <u>99.26</u> | 97.38 | <u>98.28</u> | <u>99.24</u> |
| CK20030# | DS-1 | 72.76 | 87.43 | <u>98.74</u> | <u>99.41</u> | 97.77 | 87.70 | <u>99.09</u> | <u>98.84</u> | 97.59 | <u>98.28</u> | 97.13 |
| CK20038** | DS-1 | 75.18 | 87.09 | 96.00 | 96.78 | <u>99.41</u> | <u>98.66</u> | 96.65 | 97.57 | <u>99.27</u> | 88.71 | 98.94 |
| PA130†† | DS-1 | 73.24 | 87.26 | <u>98.59</u> | <u>98.97</u> | 97.53 | 87.53 | <u>98.60</u> | <u>98.62</u> | 97.27 | <u>98.43</u> | 97.13 |
| Okayama strains‡‡ | DS-1 | <u>99.78–100</u> | <u>99.90–100</u> | <u>99.84–100</u> | | | | | | | <u>98.75–99.69</u> | <u>99.70–100</u> |

*Strain HC12016 is RVA/Human-wt/JPN/HC12016/2012/G1P[8] (G1-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2). The various strains included in this table are genetically related to strain HC12016, and of the strains tested, these showed the highest sequence identity to strain HC12016 in each gene segment. Underlining indicates the highest identity to strain HC12016, excluding DS-1-like G1P[8] strains. Dark gray indicates >99% identity to strain HC12016, excluding DS-1-like G1P[8] strains. Light gray indicates >98% identity to strain HC12016, excluding DS-1-like G1P[8] strains. RVA, rotavirus A; VP, viral protein; NSP, nonstructural protein.

†RVA/Human-tc/USA/Wa/1974/G1P[8] (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1).

‡RVA/Human-wt/USA/2007719635/2007/G1P[8] (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1).

§RVA/Human-tc/USA/DS-1/1976/G2P[4] (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2).

¶RVA/Human-wt/AUS/CK20048/2011/G2P[4] (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2).

#RVA/Human-wt/AUS/CK20030/2006/G2P[4] (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2).

**RVA/Human-wt/AUS/CK20038/2008/G6P[4] (G6-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2).

††RVA/Human-wt/ITA/PA130/2010/G2P[4] (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2).

‡‡RVA/Human-wt/JPN/OH3385/2012/G1P[8], RVA/Human-wt/JPN/OH3493/2012/G1P[8], RVA/Human-wt/JPN/OH3506/2012/G1P[8], and RVA/Human-wt/JPN/OH3625/2012/G1P[8] (G1-P[8]-I2-Rx-Cx-Mx-Ax-Nx-Tx-E2-H2), which were detected in Okayama Prefecture, Japan (11).

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Dr Yamamoto is a research scientist at Osaka City Institute of Public Health and Environmental Sciences. His research interests include the molecular biology and molecular epidemiology of gastroenteritis viruses.

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Address for correspondence: Seiji P. Yamamoto, Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan; email: seij-yamamoto@city.osaka.lg.jp

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Dengue Virus Type 3, South Pacific Islands, 2013

Van-Mai Cao-Lormeau, Claudine Roche, Didier Musso, Henri-Pierre Mallet, Tenneth Dalipanda, Alfred Dofai, Francisco Nogareda, Eric J. Nilles, and John Aaskov

After an 18-year absence, dengue virus serotype 3 reemerged in the South Pacific Islands in 2013. Outbreaks in western (Solomon Islands) and eastern (French Polynesia) regions were caused by different genotypes. This finding suggested that immunity against dengue virus serotype, rather than virus genotype, was the principal determinant of reemergence.

In contrast to circulation in countries in Southeast Asia, where dengue is hyperendemic and ≤ 4 dengue virus (DENV) serotypes might co-circulate, it is rare for >1 DENV serotype to sustainably circulate in any South Pacific island country or territory. The pattern of single-serotype predominance has been historically observed in the entire South Pacific region (Figure 1); 1 serotype circulates for 4–5 years before being displaced by another serotype, i.e., DENV-3 (1989–1996), DENV-2 (1996–2000), DENV-1 (2001–2009), and DENV-4 (2008–2009) (1–5). Because DENV-3 had not circulated in the South Pacific Islands since 1996, a large nonimmune human population susceptible to infection with this serotype was present, and it had been suggested that this serotype would reemerge in ≈ 2012 (4).

In January 2013, an outbreak of DENV-3 infections was reported in the Solomon Islands (6). Two months later, DENV-1 and DENV-3 infections were identified in patients in French Polynesia. DENV-3 isolated in the Solomon Islands belonged to genotype I, and DENV-3 isolated in French Polynesia belonged to genotype III. This finding is an example of dengue outbreaks in the South Pacific

Islands caused by introductions of multiple genotypes of the same DENV serotype into susceptible populations in the South Pacific Islands (2), in this instance, genotype I from Southeast Asia and genotype III from South America.

The Study

A dengue outbreak was reported by clinicians at the National Referral Hospital in Honiara, Solomon Islands, in January 2013. By July, $>6,000$ cases of suspected dengue had been reported, and 7 deaths were attributed to this outbreak. Serum samples from 3,141 patients were tested for DENV nonstructural protein 1 (NS1) and IgM against DENV (Dengue Duo, Standard Diagnostics Inc., Suwon, South Korea), and 1,220 (39%) samples were positive. DENV-3 was isolated by cell culture from 4 NS1-positive and IgM-negative samples by the World Health Organization Collaborating Centre for Arbovirus Reference and Research (Brisbane, Queensland, Australia). Ten additional NS1-positive samples were positive by reverse transcription PCR (RT-PCR) for DENV-3 when tested at the Institut Louis Malardé (Papeete, Tahiti, French Polynesia) (6).

In the first week of March 2013, DENV-3 was detected in French Polynesia in 2 patients in the same family cluster, 1 of whom had returned with a fever from Cayenne, French Guiana, 2 weeks earlier. Patients infected with DENV-1 were also detected in French Polynesia in February 2013. By July 2013, a total of 1,326 suspected dengue cases had been reported, of which 258 were laboratory confirmed by NS1 ELISA (Bio-Rad, Hercules, CA, USA), IgM ELISA (Bio-Rad), or RT-PCR. Serotype identification confirmed 170 DENV-1 infections, 73 DENV-3 infections, and 1 coinfection with both virus serotypes.

Envelope genes from DENV-3 isolated in the Solomon Islands and French Polynesia were amplified by using RT-PCR and sequenced as described (7–10). The 2 sequenced DENV-3 isolates from the Solomon Islands were obtained in February 2013 from patients who lived in the capital (Honiara), where the outbreak occurred. The 5 sequenced DENV-3 isolates from French Polynesia were obtained at different times and distances from the initially detected case. One virus (PF13/120213) was obtained in February from the initial family cluster in Mahina, Tahiti; 2 viruses (PF13/280213 and PF13/260313) were obtained in February and March from patients who lived in Mahina; 1 virus (PF13/040313) was obtained in March from a patient who lived in Papeete, Tahiti; and 1 virus (PF13/230413) was obtained in April from a patient who lived on Moorea Island.

All sequenced DENV-3 strains isolated in the Solomon Islands belonged to genotype I, and all sequenced DENV-3 strains isolated in French Polynesia belonged to genotype III (Figure 2, Appendix, wwwnc.cdc.gov/EID/article/20/6/13-1413-F2.htm). Phylogenetic analyses

Author affiliations: Institut Louis Malardé, Papeete, Tahiti, French Polynesia (V.-M. Cao-Lormeau, C. Roche, D. Musso); Direction de la Santé, Papeete (H.-P. Mallet); Ministry of Health and Medical Services, Honiara, Solomon Islands (T. Dalipanda); National Referral Hospital, Honiara (A. Dofai); World Health Organization, Suva, Fiji (F. Nogareda, E.J. Nilles); and Queensland University of Technology, Brisbane, Queensland, Australia (J. Aaskov)

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Figure 1. South Pacific Region showing the study areas (Solomon Islands and French Polynesia) where patient samples were tested for dengue virus type 3.

suggested that the recent DENV-3 outbreak in the Solomon Islands resulted from introduction of a strain from Indonesia that might have been circulating unrecognized in Papua New Guinea for 4–5 years. Previous reemergence of DENV-4 into the Pacific Islands might have resulted from introduction of a strain from Indonesia into the Solomon Islands (4,5,11).

In contrast, epidemiologic and phylogenetic evidence suggested that the outbreak of DENV-3 infection in French Polynesia resulted from introduction of virus from South America (French Guiana). Before 1990, most dengue outbreaks in the South Pacific region were caused by DENV that originated in Latin America. However, since that time most outbreaks have been caused by DENV from Southeast Asia (1–4,12,13). Recent transmission of DENV-3 from South America into French Polynesia is a timely reminder of the ongoing risk for DENV infection from a region in which DENV transmission is becoming hyperendemic. It remains to be seen whether, in the South Pacific, genotypes I and III of DENV-3 will maintain independent

areas of transmission, they will co-circulate in the same localities, one will become dominant, or populations of the South Pacific Island nations are large enough for these sorts of dynamics to occur.

Conclusions

As anticipated by Li et al. (4), reemergence of DENV-3 in the South Pacific Islands in 2013 supports the contention that birth and immigration rates (1.2% and 1.1%, respectively, in French Polynesia in 2010) in these island nations provide sufficient susceptible hosts for DENV to cause an epidemic every 4–5 years and for a given DENV serotype to reappear every 12–15 years. Moreover, the finding that recent DENV-3 outbreaks were caused by multiple genotypes supports observations made during outbreaks of DENV-1 infection in 2001–2004 (2). These observations also suggest that the principal determinant of DENV reemergence in the South Pacific Islands is herd immunity, rather than the genotype of the DENV being introduced.

Genotype III of DENV-3 is believed to have originated in Sri Lanka, spread to eastern Africa in the mid-1980s, and then to South America in the mid-1990s (14). The discovery that DENV-3 genotype III has recently been introduced into the South Pacific Islands from South America indicates that this lineage might be the first DENV lineage to have circumnavigated the globe. Although the reemergence of DENV-3 in the South Pacific Islands in 2013 was anticipated, the reemergence of DENV-1 in New Caledonia in 2012 (15) and in French Polynesia in 2013 was not anticipated. Reports of co-circulation of multiple DENV serotypes are becoming more frequent in the South Pacific region and might reflect consequences of incomplete vector control (i.e., sufficient to prevent a major dengue outbreak but also preventing sufficient DENV transmission for development of effective herd immunity). Whether DENV-3 genotype I, genotype III, or both genotypes, will displace DENV-1 is not known.

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Dr Cao-Lormeau is a research scientist at the Institut Louis Malardé, Papeete, Tahiti, French Polynesia. Her research focuses on the genetic evolution of dengue viruses in Pacific Island countries, in particular, dengue virus intrahost genetic diversity.

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Address for correspondence: Van-Mai Cao-Lormeau, Institut Louis Malardé, PO Box 30, 98713 Papeete, Tahiti, French Polynesia; email: mlormeau@ilm.pf

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Novel Human Bufavirus Genotype 3 in Children with Severe Diarrhea, Bhutan

Takaaki Yahiro, Sonam Wangchuk,
Kinlay Tshering, Purushotam Bandhari,
Sangay Zangmo, Tshering Dorji,
Karchung Tshering, Takashi Matsumoto,
Akira Nishizono, Maria Söderlund-Venermo,
and Kamruddin Ahmed

We identified a new genotype of bufavirus, BuV3, in fecal samples (0.8%) collected to determine the etiology of diarrhea in children in Bhutan. Norovirus GII.6 was detected in 1 sample; no other viral diarrheal pathogens were detected, suggesting BuV3 as a cause of diarrhea. This study investigates genetic diversity of circulating BuVs.

In 2012, a novel parvovirus, bufavirus (BuV), was discovered in fecal samples of children with diarrhea in Burkina Faso (1). The virus belongs to the species primate protoparvovirus 1 of the genus *Protoparvovirus* (2). BuV has a single-stranded DNA genome and encodes nonstructural protein 1 (NS1) and viral structural proteins 1 and 2 (VP1 and VP2). Two genotypes, BuV1 and BuV2, have been described; the highly diverse capsid gene indicates the possibility of further genotypes of this virus (1).

One research group, which used PCR to test fecal samples collected in 3 countries, had previously found various proportions of specimens positive for BuV: 4 of 98 (4%) in Burkina Faso, 1 of 63 (1.6%) in Tunisia, and none of 100 in Chile (1). Fecal samples from Tunisia were from children with acute flaccid paralysis; samples from Burkina Faso and Chile were from children with diarrhea. It is not known whether BuV is pathogenic in humans.

Author affiliations: Faculty of Medicine, Oita University, Yufu, Japan (T. Yahiro, T. Matsumoto, A. Nishizono, K. Ahmed); Ministry of Health, Thimphu, Bhutan (S. Wangchuk, S. Zangmo, T. Dorji, K. Tshering); Jigme Dorji Wangchuk National Referral Hospital, Thimphu (K. Tshering); Mongar Regional Referral Hospital, Mongar, Bhutan (P. Bandhari); and University of Helsinki, Helsinki, Finland (M. Söderlund-Venermo)

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Bhutan is a small landlocked country between India and China; an estimated 23% of the people of Bhutan live below the poverty line (3). The population is mainly concentrated in the capital, Thimphu (altitude 2,248–2,648 m), and is otherwise sparsely distributed throughout the country. Diarrhea is a major cause of illness and death among children in Bhutan. Irrespective of severity, hospitalization, or causative agents, the annual morbidity rate for children <5 years of age with diarrhea is 168.8/1,000 (17%) (4); however, the etiology of diarrhea in this country has not been studied in detail. We conducted this study to investigate the genetic diversity of circulating BuVs and to clarify the public health significance of BuV in Bhutan.

The Study

As part of a project to identify viral etiology of diarrhea, 393 fecal samples were collected from children <5 years of age with watery diarrhea attending 2 hospitals in Bhutan. During February 2010–January 2012, 381 fecal samples (109 in 2010, 185 in 2011, and 87 in 2012) were collected from hospitalized patients or outpatients of Jigme Dorji Wangchuk National Referral Hospital. This hospital mainly serves the population of Thimphu and is the only national reference hospital in the country. In April 2010, 12 more samples were obtained from children hospitalized in Mongar Regional Referral Hospital. This hospital mainly serves the population of Mongar and is the reference hospital for the eastern part of the country. The Research Ethics Board of Health in Bhutan approved this study.

Viral genomic DNA was extracted from fecal samples by using the QIAamp Viral RNA Mini Kit (QIAGEN Sciences, Valencia, CA, USA) according to the manufacturer's instructions. The presence of BuV was determined by nested PCR targeting the NS1 region (1). For whole-genome sequencing of BuV3, primers were constructed from consensus regions after aligning the whole-genome sequences of BuV1. BuV-positive samples were tested for norovirus, bocavirus, adenovirus, astrovirus, salivirus, co-savirus, and aichivirus by PCR, and for rotavirus by enzyme immunoassay (5–8). No tests for diarrheic bacteria were done on these samples. PCR amplicons were directly sequenced to confirm the findings and to compare the sequences. Nucleotide sequencing was performed by using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA USA) according to the manufacturer's instructions. Multiple sequence alignment was done by using MUSCLE (9) and the phylogenetic tree was constructed by using the neighbor-joining method by MEGA 5 (10). A bootstrap analysis of 1,000 replicates was done to find the significance of branching.

Three of 393 samples (0.8%) were positive for BuV DNA. The overall proportion of positive samples of BuV in Thimphu was 0.5% (2 samples); 1 (0.9%) was detected

Table 1. Demographic information and tested diarrheagenic viruses in the stool samples of hospital inpatients with bufavirus-positive diarrhea, Bhutan, 2010–2011*

| ID no. | Age, mo/ sex | Underlying condition | Sample collection date | Hospital | Other viruses tested for | | | | | | | |
|---------|--------------|---------------------------|------------------------|----------|--------------------------|-----|-----|-----|--------|-----|-----|-----|
| | | | | | RV | ADV | BCV | ASV | NRV | SLV | CSV | ACV |
| BTN-63 | 18/M | None | 2010 Apr 26 | MRR | - | - | - | - | - | - | - | - |
| BTN-109 | 1/M | None | 2010 Nov 2 | JDWNR | - | - | - | - | - | - | - | - |
| BTN-310 | 31/F | Ventricular septal defect | 2011 Dec 12 | JDWNR | - | - | - | - | G II.6 | - | - | - |

*ID, identification; RV, rotavirus; ADV, adenovirus; BCV, bocavirus; ASV, astrovirus; NRV, norovirus; SLV, salivirus; CSV, cosavirus; ACV, aichivirus; -, negative; MRR, Mongar Regional Referral Hospital; JDWNR, Jigme Dorji Wangchuk National Referral Hospital.

in 2010 and the other (1.1%) in 2011. No samples from Thimphu were BuV-positive in 2012. In addition, 1 sample (8.3%) from Mongar was BuV-positive. The demographic data of these patients are shown in Table 1. In 1 of the 3 BuV-positive fecal samples, norovirus GII.6 was also detected; in the other 2 samples, the enteric viruses tested for were not detected.

The near-complete genome sequences of the 3 BuVs collected in Bhutan were obtained. The lengths of the open reading frames of NS1, VP1, and VP2 of the Bhutanese BuV genes were different from those of BuV1 and BuV2 (Table 2). Phylogenetic analyses, with a bootstrap value of 100, of the deduced amino acid sequence of NS1, VP1 (Figure), and VP2 genes showed that the Bhutanese BuVs consistently formed a cluster different from the genotypes BuV1 and BuV2. The NS1, VP1, and VP2 of the Bhutanese isolates showed $\approx 99\%$ nucleotide and amino acid identities among themselves. Compared with BuV1, the respective NS1, VP1, and VP2 nt (amino acid) identities of the Bhutanese BuVs were 96% (95%–96%), 83% (77%–78%), and 79%–80% (73%), and compared with BuV2, 96% (95%), 80% (71%), and 76% (65%). Phylogenetic analysis and identity data support the finding of a new genotype, BuV3, in Bhutan. Similar to BuV1 and BuV2, all isolates collected in Bhutan contained an ATP- or GTP-binding Walker loop, phospholipase A₂, 2 conserved replication initiator motifs, and a glycine-rich sequence; they also had the same splice sites. In the Bhutanese BuVs, 3 tandem repeats, TAGTTGATAAGT, TAGTTTATAAGT, and TAGTTTATAAAT, in the 3' untranslated region (UTR) occurred at a frequency of 0, 6, or 3 times, and 0 or 1 times, respectively (Table 2). These

results show that BuV3 3'UTR differs from 3'UTR of the other 2 genotypes.

Conclusion

Genetic analyses of BuVs from Bhutan revealed a new genotype that may cause severe diarrhea in children. The absence of other diarrhea-causing viruses tested for in 2 of the 3 samples containing BuV3 supports a possible role for this parvovirus in diarrhea in infants; however, direct proof of causation is necessary (11,12). A more extensive testing for other known pathogens by using virochip microarrays, metagenomics, and direct testing for pathogenic bacteria will help determine whether BuVs alone can cause diarrhea.

Phylogenetic analyses and identity data indicate that BuV3 is a new genotype. The identity data between BuV3 and other genotypes are comparable to the data found by Phan et al., wherein genotypes BuV1 and BuV2 showed 95% identity in the NS1 region, but only 72% identity in the capsid region (1). In BuV1, the 3 tandem repeats each occurred with a frequency of 3 or 2 times, once, and once, respectively. This differs substantially from that of BuV3 found in Bhutan, further revealing the molecular characteristics of the new genotype.

In conclusion, we have detected BuV DNA in the feces of children with severe diarrhea in Bhutan. Additional work is needed to determine this is indeed a human causal pathogen of diarrhea or of any illness. Identification of BuV3 provides evidence of a more diverse population of BuVs than previously documented. Whether this novel genotype is circulating globally or only in Bhutan requires further investigation.

Table 2. Comparison of the length of the nucleotide sequences of nearly complete genome, 5' UTR, NS1, VP1, VP2, and 3' UTR of strains of different bufavirus genotypes, Bhutan, 2010–2012*†

| Strain | Genotype | Total | 5' UTR | NS1 | VP1 | VP2 | 3' UTR | TAGTTGATAAGT | TAGTTTATAAGT | TAGTTTATAAAT |
|---------|----------|-------|--------|-------|-------|-------|--------|--------------|--------------|--------------|
| BTN-63 | BuV3 | 4,733 | ND | 2,022 | 2,133 | 1,719 | 224 | 0 | 3 | 0 |
| BTN-109 | BuV3 | 4,734 | ND | 2,022 | 2,133 | 1,719 | 225 | 0 | 6 | 0 |
| BTN-310 | BuV3 | 4,766 | ND | 2,022 | 2,133 | 1,719 | 259 | 0 | 3 | 1 |
| BF.96 | BuV1 | 4,912 | 18 | 2,016 | 2,124 | 1,710 | 400 | 3 | 1 | 1 |
| BF.86 | BuV1 | 4,822 | 1 | 2,016 | 2,124 | 1,710 | 327 | 2 | 1 | 1 |
| BF.7 | BuV1 | 4,822 | 1 | 2,016 | 2,124 | 1,710 | 327 | 3 | 1 | 1 |
| BF.39 | BuV2 | 4,562 | ND | 2,022 | 2,124 | 1,710 | 62 | ND | ND | ND |

*UTR, untranslated region; NS, nonstructural; VP, viral protein; ND, no data: sequence could not be determined.

†Only partial sequence of 3' UTR regions have been achieved in all strains. Nucleotide sequence of 5' UTR of BuV strains from samples from Bhutan could not be determined.

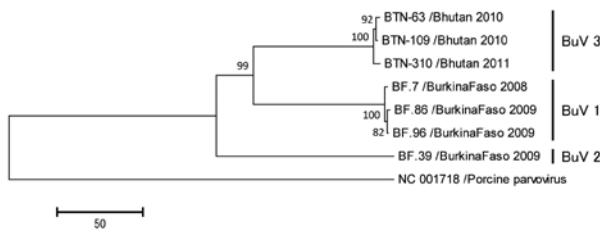


Figure. Phylogenetic tree of the viral protein 1 (VP1) of bufaviruses, constructed by using deduced amino acid sequences by neighbor-joining method. The full length open reading frames of VP1 genes were used to deduce amino acid sequences. Porcine parvovirus strain NC 001718 was used as an out-group. The numbers adjacent to the nodes represent the bootstrap values, and values <50% are not shown. Scale bar indicates the genetic distances expressed as amino acid substitutions per site. Accession numbers for DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank are AB847987, AB847988, and AB847989 for strains BTN-63, BTN-109, and BTN-310, respectively.

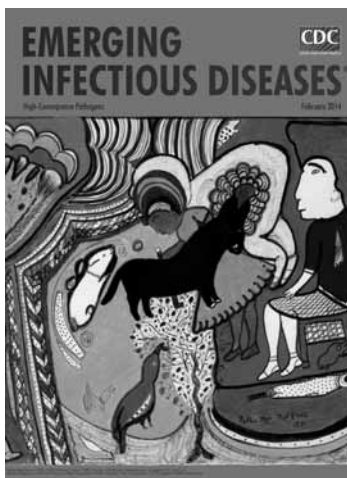
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Mr Yahiro is a PhD student at the Department of Microbiology, Faculty of Medicine, Oita University, in Yufu, Japan. His research interest is emerging infectious diseases.

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Address for correspondence: Kamruddin Ahmed, Faculty of Medicine, Research Promotion Institute, Oita University, Yufu 879-5593, Oita, Japan; email: ahmed@oita-u.ac.jp



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Novel Phlebovirus with Zoonotic Potential Isolated from Ticks, Australia

Jianning Wang,¹ Paul Selleck,¹ Meng Yu,¹ Wendy Ha, Chrissy Rootes, Rosemary Gales, Terry Wise, Sandra Cramer, Honglei Chen, Ivano Broz, Alex Hyatt, Rupert Woods, Brian Meehan, Sam McCullough, and Lin-Fa Wang

Recently discovered tick-borne phleboviruses have been associated with severe disease and death among persons in Asia and the United States. We report the discovery of a novel tick phlebovirus in Tasmania State, Australia, that is closely related to those zoonotic viruses found in Asia and North America.

Viruses in the family *Bunyaviridae* can infect animals and plants (1). The family is composed of 5 genera: *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, *Hantavirus*, and *Tospovirus* (2). The genus *Phlebovirus* contains known disease agents of animals, including humans, that can be carried by different vectors (e.g., phlebotomine sandflies, mosquitoes, and ticks) (3). In 2009, an outbreak of an acute febrile illness, known as severe fever with thrombocytopenia syndrome (SFTS), occurred in China. During an investigation of the outbreak, a previously unknown bunyavirus from the tick *Haemaphysalis longicornis* was identified as the causative agent of SFTS (4). SFTS virus (SFTSV) has since been shown to be responsible for >150 human infections in 16 Chinese provinces and to have an associated death rate of ≈12% (5,6).

In June 2009, in northwestern Missouri, United States, 2 men from 2 geographically distant farms were

hospitalized for fever, fatigue, diarrhea, thrombocytopenia, and leukopenia. Both men had been bitten by ticks 5–7 days before the onset of symptoms. A virus was isolated from the leukocytes of each patient and later identified as a novel phlebovirus by next-generation sequencing. The 2 viruses were highly related (98%, 95%, and 99% sequence identity for the small, medium, and large viral genome segments, respectively), indicating that the men were independently infected with the same virus. This new virus, named Heartland virus (HRTV), was most closely related to, but clearly distinct from, the SFTSV detected in China (7).

Subsequent to these disease events in China and the United States, fatal SFTSV infections were reported in humans in Japan (8) and Korea (9). Because similar viruses and human infections have been detected in 2 well-separated continents (i.e., Asia and North America), it is tempting to hypothesize that similar viruses may also exist in tick populations on other continents. We report the isolation and characterization of a phlebovirus from ticks in Australia that is similar to SFTSV and HRTV.

The Study

In 2002, a disease outbreak occurred in a shy albatross (*Thalassarche cauta*) colony on Albatross Island, a small island in the Hunter Island Group in northwestern Tasmania, Australia. The disease in the albatrosses was characterized by weight loss and death. Serum samples and ticks (*Ixodes euryptidis*) from healthy and affected birds were sent to the CSIRO (the Commonwealth Scientific and Industrial Research Organization) Australian Animal Health Laboratory in Geelong, Victoria, Australia, for diagnostic investigation. Several laboratory tests were conducted to detect potential viral pathogens involved in the disease event.

The ELISA or hemagglutination inhibition assay was used to test serum samples (N = 38) for antibodies against avian influenza virus, infectious bursal disease virus, fowlpox virus, and Newcastle disease virus. Neutralizing antibodies for Newcastle disease virus (titer of 64) were detected in 1 serum sample; otherwise, no antibodies against any of these suspected agents were detected.

Pooled tick homogenates (N = 5) were cultured on chicken embryos, chicken embryo fibroblast and skin cells, and Vero cells. No virus was detected in chicken embryos or in any of the chicken cell lines used. In contrast, cytopathic effect was observed on the third passage in Vero cell cultures inoculated with 2 tick pools (1 collected from diseased birds and the other from healthy birds).

Electron microscopy examination revealed morphologically similar viral particles from both cultures; the morphologic features resembled those of bunyaviruses

Author affiliations: Commonwealth Scientific and Industrial Research Organisation, Geelong, Victoria, Australia (J. Wang, P. Selleck, M. Yu, W. Ha, C. Rootes, T. Wise, S. Cramer, H. Chen, I. Broz, A. Hyatt, B. Meehan, S. McCullough, L.-F. Wang); Department of Primary Industries, Parks, Water and Environment, Hobart, Tasmania, Australia (R. Gales); Australian Wildlife Health Network, Mosman, New South Wales, Australia (R. Woods); and Duke–National University of Singapore Graduate Medical School, Singapore (L.-F. Wang)

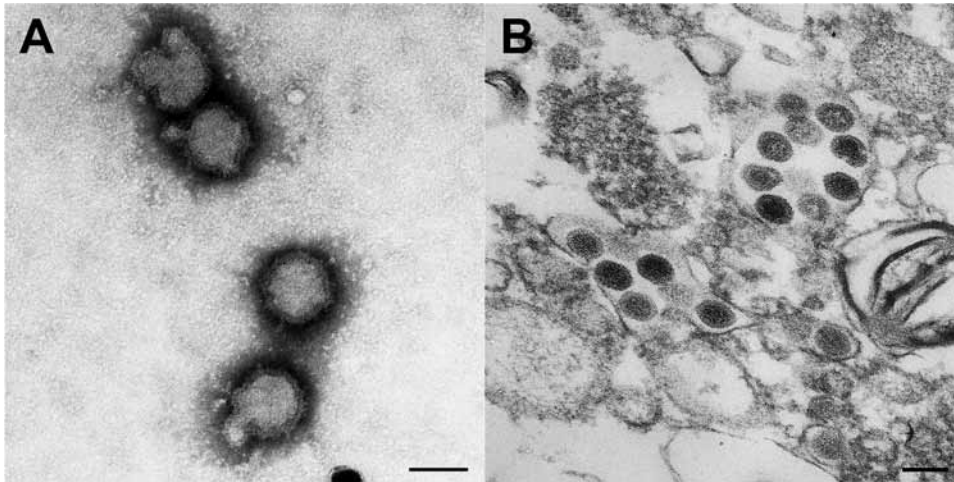


Figure 1. Electron microscopic examination results of a newly isolated virus, tentatively named Hunter Island Group virus, isolated from ticks collected from shy albatrosses, Tasmania, Australia. A) Negative-contrast staining of virions. B) Thin section of infected Vero cells showing the presence of viral particles. Original magnification $\times 100,000$; scale bars represent 100 nm.

(Figure 1). However, testing with antibodies against a range of known bunyaviruses (orthobunyavirus, phlebovirus, and nairovirus) did not reveal any cross-reactivity. PCR analysis using bunyavirus-specific primers (10) also failed to detect any specific sequence from this newly isolated virus.

The investigation was put on hold until 2011 when the Australian Animal Health Laboratory established its next-generation sequencing capability. RNA extracted from partially purified virus grown in Vero cells was used for random PCR amplification. The resulting products were used for library preparation and subsequently sequenced by using the 454 GS FLX system (454 Life Sciences, Branford, CT, USA) according to standard protocols (11,12). In total, 6.9 Mb of sequence, consisting of 23,617 reads, was obtained and assembled into 722 contigs.

BLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis at the nucleotide level showed no sequence identity to any of the known viruses in GenBank (data not shown). However, a protein-based BLASTX (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search yielded 10 contigs showing protein sequence identity to SFTSV and some degree of sequence identity to several other members of the family *Bunyaviridae*. The virus was tentatively named Hunter Island Group

virus (HIGV) after the location where the tick samples were obtained.

On the basis of the partial sequences derived from next-generation sequencing, primers were designed to obtain the rest of the genome sequences by using gap-filling PCR and 5' and 3' RACE methods previously developed by our group (13). The complete genome sequences of the large, medium, and small segments have been deposited in GenBank under accession numbers KF848980, KF848981, and KF848982, respectively.

The Table summarizes the genetic and sequence features of the genome segments and deduced proteins of HIGV and several selected phleboviruses. From the sequence comparison, it is evident that HIGV is a new member of the genus *Phlebovirus* and that it is most closely related to the newly discovered zoonotic members of the genus, SFTSV and HRTV. This finding was further confirmed by phylogenetic analysis based on nucleotide and protein sequences of all 3 genome segments (representative trees shown in Figure 2). Because HIGV was isolated from ticks collected from healthy and diseased birds we believed it was unlikely that the virus was the causative agent for the disease in the shy albatrosses. To further confirm this, we conducted ELISA and Western blot analysis using a recombinant HIGV nucleocapsid protein, which was

Table. Summary of genomic and sequence features of Hunter Island Group virus and other selected phleboviruses*

| Virus | Large segment | | Medium segment | | Small segment | | |
|-------|------------------|------------------------------|------------------|------------------------|------------------|--------------------------------|---------------------------------|
| | Segment size, nt | Polymerase protein size, aa† | Segment size, nt | Glycoprotein size, aa† | Segment size, nt | Nucleocapsid protein size, aa† | Nonstructural protein size, aa† |
| HIGV | 6,368 | 2,085 | 3,328 | 1,063 | 1,694 | 233 | 267 |
| HRTV | 6,368 | 2,084 (66) | 3,427 | 1,076 (53) | 1,772 | 245 (57) | 299 (28) |
| SFTSV | 6,368 | 2,084 (65) | 3,378 | 1,073 (54) | 1,744 | 245 (58) | 293 (31) |
| BHAV | 6,333 | 2,082 (35) | 3,304 | 1,068 (26) | 1,867 | 247 (39) | 313 (17) |
| UUKV | 6,423 | 2,103 (35) | 3,229 | 1,008 (23) | 1,720 | 254 (27) | 273 (20) |
| RVFV | 6,404 | 2,092 (34) | 3,885 | 1,197 (21) | 1,692 | 245 (35) | 265 (18) |

*HIGV, Hunter Island Group virus; HRTV, Heartland virus; SFTSV, severe fever with thrombocytopenia syndrome virus; BHAV, Bhanja virus; UUKV, Uukuniemi virus; RVFV, Rift Valley fever virus.

†For proteins, the % amino acid sequence identity with cognate proteins of HIGV is shown in parentheses.

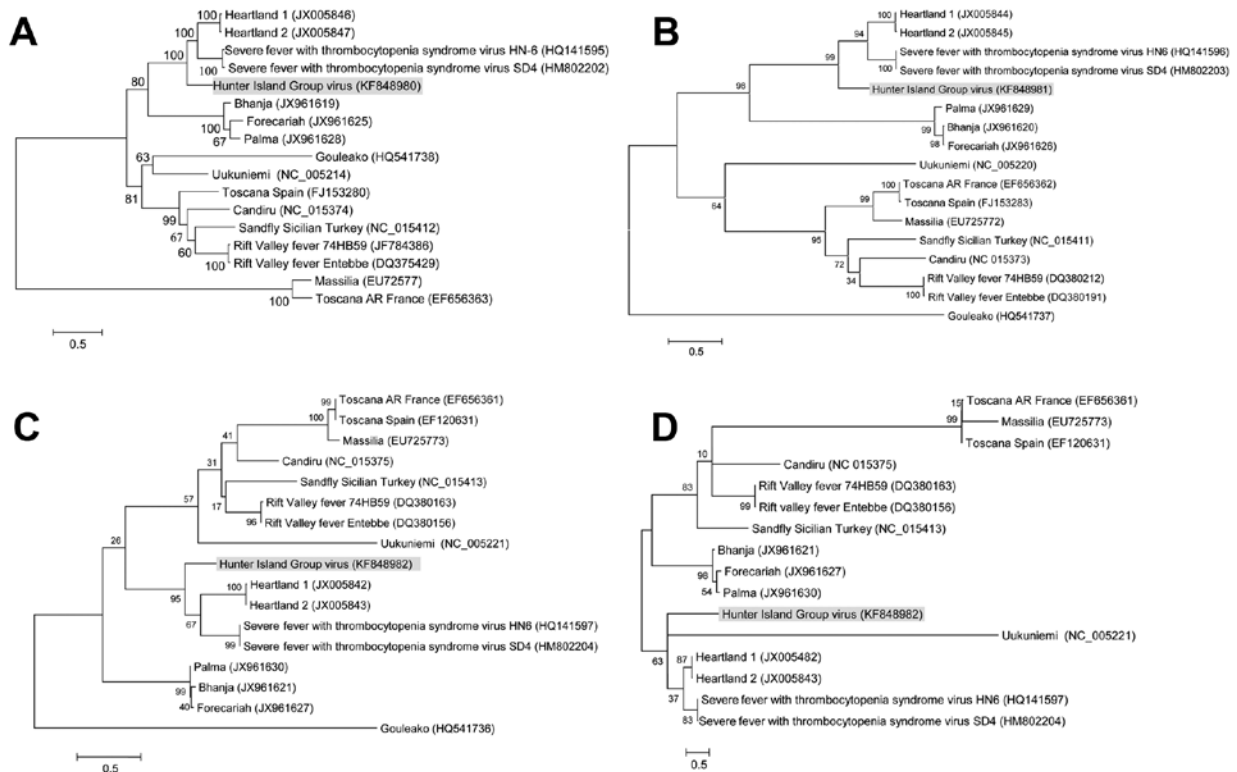


Figure 2. Phylogenetic trees of recently isolated bunyaviruses based on amino acid sequences of the polymerase protein (A) encoded by the large segment, the membrane glycoprotein polyprotein (B) encoded by the medium segment, and the nucleocapsid protein (C) and the nonstructural protein (D) encoded by the small segment of selected bunyaviruses. Maximum-likelihood trees were constructed by using MEGA5 (www.megasoftware.net/) with bootstrapping at 1,000 replicates. GenBank accession numbers are within parentheses next to the virus names. Scale bars indicate nucleotide substitutions per site.

expressed and purified from *Escherichia coli* by using previously described methods (14,15). Of the 38 serum samples tested, none produced positive readings in ELISA or Western blot. In addition, none were positive when tested by HIGV-specific quantitative PCR targeting the polymerase gene of the large segment.

Conclusions

We identified a novel tick-borne phlebovirus, HIGV, during the investigation of a disease outbreak among shy albatrosses in Tasmania. Genetic characterization showed that the virus is closely related to 2 newly discovered tick-borne zoonotic phleboviruses (SFTSV and HRTV) that were responsible for severe disease and death in humans in 4 separate countries in Asia and North America. However, with the current data alone, the particular disease event in the shy albatrosses could not be attributed to HIGV.

The findings from this study demonstrate the key role that a vigilant pathogen investigation has in any diagnostic assessment. The study findings also suggests that zoonotic phleboviruses genetically related to SFTSV, HRTV, and HIGV may be widely distributed in different parts of the world and that heightened international surveillance is

needed to fully understand and appreciate the public health risk from these emerging viruses.

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Dr Wang is a research scientist in charge of the molecular diagnostics group at CSIRO Australian Animal Health Laboratory. His research interests include novel molecular diagnostics and pathogen discovery.

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Address for correspondence: Lin-Fa Wang, CSIRO Animal, Food and Health Science, Australian Animal Health Laboratory, PO Bag 24, Geelong, Victoria 3220, Australia; email: linfa.wang@csiro.au

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New Hepatitis E Virus Genotype in Camels, the Middle East

Patrick C.Y. Woo,¹ Susanna K.P. Lau,¹
 Jade L.L. Teng,¹ Alan K. L. Tsang,¹
 Marina Joseph, Emily Y.M. Wong, Ying Tang,
 Saritha Sivakumar, Jun Xie, Ru Bai,
 Renate Wernery, Ulrich Wernery,
 and Kwok-Yung Yuen

In a molecular epidemiology study of hepatitis E virus (HEV) in dromedaries in Dubai, United Arab Emirates, HEV was detected in fecal samples from 3 camels. Complete genome sequencing of 2 strains showed >20% overall nucleotide difference to known HEVs. Comparative genomic and phylogenetic analyses revealed a previously unrecognized HEV genotype.

Hepatitis E virus (HEV) belongs to the family *Hepeviridae* and genus *Hepevirus*. Among humans worldwide, HEV is the most common cause of acute viral hepatitis. The disease is generally self-limiting, but mortality rates are high among pregnant women and young infants. Chronic HEV infection is a problem for immunocompromised patients, such as those who have received a solid organ transplant and those with HIV infection. In addition to humans, HEV has been found in the other mammals: pigs, boar, deer, rodents, ferrets, rabbits, mongoose, bats, cattle, sheep, foxes, minks, and horses (1–3). Among the 4 known HEV genotypes, HEV1 and HEV2 infect only humans; whereas, HEV3 and HEV4 can infect humans, pigs, and other mammals. Human infections with HEV3 and HEV4 have been associated with consumption of raw or undercooked pork or game meat (4). Traditionally, HEV infection is mainly transmitted through water contaminated with infected feces. Since water supplies and sanitary infrastructures have been improved, animals have become a major source of human HEV infection. We detected HEV in fecal samples from dromedary camels in the Middle East.

Author affiliations: State Key Laboratory of Emerging Infectious Diseases, Hong Kong, China (P.C.Y. Woo, S.K.P. Lau, K.-Y. Yuen); The University of Hong Kong, Hong Kong (P.C.Y. Woo, S.K.P. Lau, J.L.L. Teng, A.K.L. Tsang, E.Y.M. Wong, Y. Tang, J. Xie, R. Bai, K.-Y. Yuen); and Central Veterinary Research Laboratory, Dubai, United Arab Emirates (M. Joseph, S. Sivakumar, R. Wernery, U. Wernery)

The Study

As part of a molecular epidemiology study, 203 fecal samples from 203 adult dromedaries (*Camelus dromedarius*) were submitted to the Central Veterinary Research Laboratory in Dubai, United Arab Emirates, over a 7-month period (January–July 2013). RNA extraction and reverse transcription were performed, as described, to detect other positive-sense single-stranded RNA viruses (5,6). Screening for HEV was performed by PCR amplification of a 284-bp fragment of open reading frame (ORF) 2 in HEV; specific primers used were 5'-TTTATTCTC-GTCCAGTCGTTTC-3' and 5'-GTCAGTGGAG-GACCCATATGT-3', designed from sequence information from our metagenomic study (P.C.Y. Woo et al., unpub. data). PCR was performed according to previously described conditions (7); annealing temperature were set at 50°C. DNA sequencing and quantitative real-time reverse transcription PCR were also performed as described (8). Using strategies we have reported for other positive-sense single-stranded RNA viruses, we performed complete-genome sequencing on 2 HEV-positive samples (5,6). Comparative genomic analysis was performed as described (9). Phylogenetic analysis was conducted in MrBayes5D version 3.1.2 (www.fifthdimension.jp/products/mrbayes5d/) by using an optimal substitution model with 1 million Markov chain Monte Carlo generations; sampling was conducted every 100 generations with a burn-in of 25,000. The substitution model was selected on the basis of the corrected Akaike information criterion by ProtTest version 2.4 (<http://darwin.uvigo.es/software/prottest.html>).

Reverse transcription PCR for a 284-bp fragment in ORF2 of this HEV, which we named dromedary camel HEV (DcHEV), was positive for 3 fecal samples; viral loads were 3.7×10^5 , 4.5×10^5 , and 3.2×10^7 copies/mL. Complete-genome sequence data for 2 DcHEV strains (GenBank accession nos. KJ496143–KJ496144) revealed that the genome size was 7,220 bases and had a G+C content of 55% (Table). Overall, the DcHEV genomes differed from all other HEVs by >20% nt (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/20/6/14-0140-Techapp1.pdf).

The DcHEV genome contained 3 major ORFs (Table; Figure 1). ORF1 polyprotein contained motifs consistent with a methyltransferase, a peptide containing a Y domain, a papain-like cysteine protease, a peptide with a hypervariable region (HVR), a helicase, and an RNA-dependent RNA polymerase. Also present in DcHEV were conserved sequences TLYTRTWS and RLLXTPDG, which bound the HVR of HEV1–4 and of 2 recently discovered wild boar HEV strains (10, 11) but not the HVR

¹These authors contributed equally to this article.

Table. Comparison of genomic organization of HEV genotypes and isolates*

| HEV (GenBank accession no.) | Genome length, nt | GC content, % | 5' UTR, nt | ORF1, aa | ORF2, aa | ORF3, aa | 3' UTR, nt |
|--|-------------------|---------------|------------|----------|----------|----------|------------|
| DcHEV-178C (KJ496143)† | 7,220 | 55.1 | 39 | 1,698 | 660 | 113 | 66 |
| DcHEV-180C (KJ496144)‡ | 7,219 | 54.4 | 39 | 1,698 | 660 | 113 | 66 |
| HEV1 (M73218) | 7,194 | 58.1 | 27 | 1,693 | 660 | 114 | 65 |
| HEV2 (M74506)‡§ | ≥7,170 | 56.5 | NA | 1,691 | 659 | 114 | 74 |
| HEV3 (AB089824) | 7,244 | 55.3 | 25 | 1,709 | 660 | 113 | 72 |
| HEV4 (AJ272108) | 7,232 | 54.4 | 25 | 1,707 | 658 | 112 | 68 |
| Rabbit HEV (FJ906895)‡ | 7,283 | 55.5 | 26 | 1,722 | 660 | 113 | 71 |
| Germany rat HEV (GU345042) | 6,948 | 57.8 | 10 | 1,636 | 644 | 102 | 65 |
| Vietnam rat HEV (JX120573) | 6,927 | 56.6 | 10 | 1,629 | 644 | 102 | 65 |
| Ferret HEV (JN998606) | 6,841 | 53.8 | 12 | 1,596 | 654 | 108 | 65 |
| Wild boar HEV novel unclassified genotype (AB602441) | 7,246 | 57.0 | 25 | 1,709 | 660 | 112 | 70 |
| Bat HEV (JQ001749) | 6,767 | 51.8 | 33 | 1,580 | 637 | 137 | 77 |
| Avian HEV genotype 1 (AM943647)§ | ≥6,627 | 55.1 | NA | ≥1,531 | 606 | 87 | 123 |
| Avian HEV genotype 2 (AY535004) | 6,654 | 55.5 | 24 | 1,531 | 606 | 87 | 127 |
| Avian HEV genotype 3 (AM943646)§ | ≥6,631 | 55.6 | NA | 1,532 | 606 | 87 | 126 |
| Avian HEV novel unclassified genotype (JN997392)§ | ≥6,543 | 55.7 | NA | ≥1,515 | 606 | 87 | NA |
| Cutthroat trout HEV (HQ731075) | 7,269 | 49.7 | 100 | 1,707 | 634 | 225 | 76 |

*HEV, hepatitis E virus; UTR, untranslated region; ORF, open reading frame; DcHEV HEV from dromedary camel; NA, not available because of incomplete genome.

†Assuming the third AUG of ORF2 is the start codon.

‡Assuming the third AUG of ORF3 is the start codon.

§Near-complete genome.

of ferret, rat, bat, avian or cutthroat trout HEVs. A conserved motif, (T/V)SGFSS(D/C)F(S/A)P, immediately preceding the HVR of only HEV3 and HEV4 was present in DcHEV as VSGFSSDFAP. The relative excess of proline and serine observed in the HVR of all other HEVs was also observed for DcHEV. For DcHEV strain 178C, ORF2 began at nt 5172, similar to what is found for HEV4 and wild boar HEV, with an insertion of a single nucleotide (U) at nt 5146, and ended at nt 7154, encoding a capsid protein of 660 aa (online Technical Appendix Figure) (11–13). As for DcHEV strain 180C, because of the lack of the U insertion as in HEV1, HEV2, and HEV3, ORF2 began at nt 5171 (online Technical Appendix Figure). For DcHEV strain 178C, similar to HEV4 and the 2 recently discovered wild boar HEV strains (11), ORF3 began at nt 5161 (online Technical Appendix Figure) and ended at nt 5502, encoding a small phosphoprotein of 113 aa with a multifunctional C-terminal region. As for DcHEV strain 180C, because of the lack of the U insertion as in HEV1, HEV2, and HEV3, ORF3 began at nt 5160. The conserved *cis*-reactive element (UGAAUAACAUGU) located upstream of ORF2 and ORF3 in both strains might serve as promoter for the synthesis of the subgenomic mRNA for these 2 ORFs.

Phylogenetic trees constructed by using ORF1, ORF2, ORF3, and concatenated ORF1/ORF2 excluding the HVR showed that DcHEV was clustered with different HEVs in different phylogenetic trees (Figure 2). For ORF1 and concatenated ORF1/ORF2 excluding the HVR, DcHEV was clustered with HEV3; but for ORF2 and ORF3, DcHEV was clustered with HEV1 and HEV2. Recombination analysis performed by using bootscan revealed no obvious and definite site of recombination, similar to what we

observed in previous studies for other viruses (14), although different regions of the DcHEV genome might be more similar to different genotypes of HEV (data not shown).

Conclusions

We discovered HEV in dromedaries from the Middle East and named the virus DcHEV. In a recent study conducted in Dubai, HEV accounted for 40% of cases of acute hepatitis in humans (15). Although HEV is a major pathogen in the Middle East, sequence data for HEVs in the Arabian Peninsula are limited. The study reported here revealed that 1.5% of the adult dromedary fecal samples showed evidence of DcHEV RNA. Because humans come in close contact with dromedaries, our finding of DcHEV in dromedaries indicates a previously unknown potential reservoir and source of HEV infection for humans.

Comparative genomic and phylogenetic analyses showed that DcHEV probably represents a previously unrecognized HEV genotype. The conserved motif preceding the HVR in ORF1 resembled those found in HEV3, HEV4, and the 2 recently discovered wild boar HEV strains. Although phylogenetically ORF1 of DcHEV was clustered with HEV3, ORF2 and ORF3 of DcHEV were clustered with HEV1 and HEV2. Of note, ORF2 and ORF3 of the 2 DcHEV strains with complete genomes sequenced in this study resembled those of different HEV genotypes. The presence of a U insertion downstream to the second possible start codon for ORF2 (AUG2) in DcHEV strain 178C resembled the presence of a U insertion in HEV4 and wild boar HEV, leading to 3 possible start codons for its ORF2 but 1 possible start codon for its ORF3; whereas, the lack of this U insertion downstream to AUG2 in DcHEV strain 180C resembled the lack of U insertions in HEV1, HEV2 and HEV3, leading

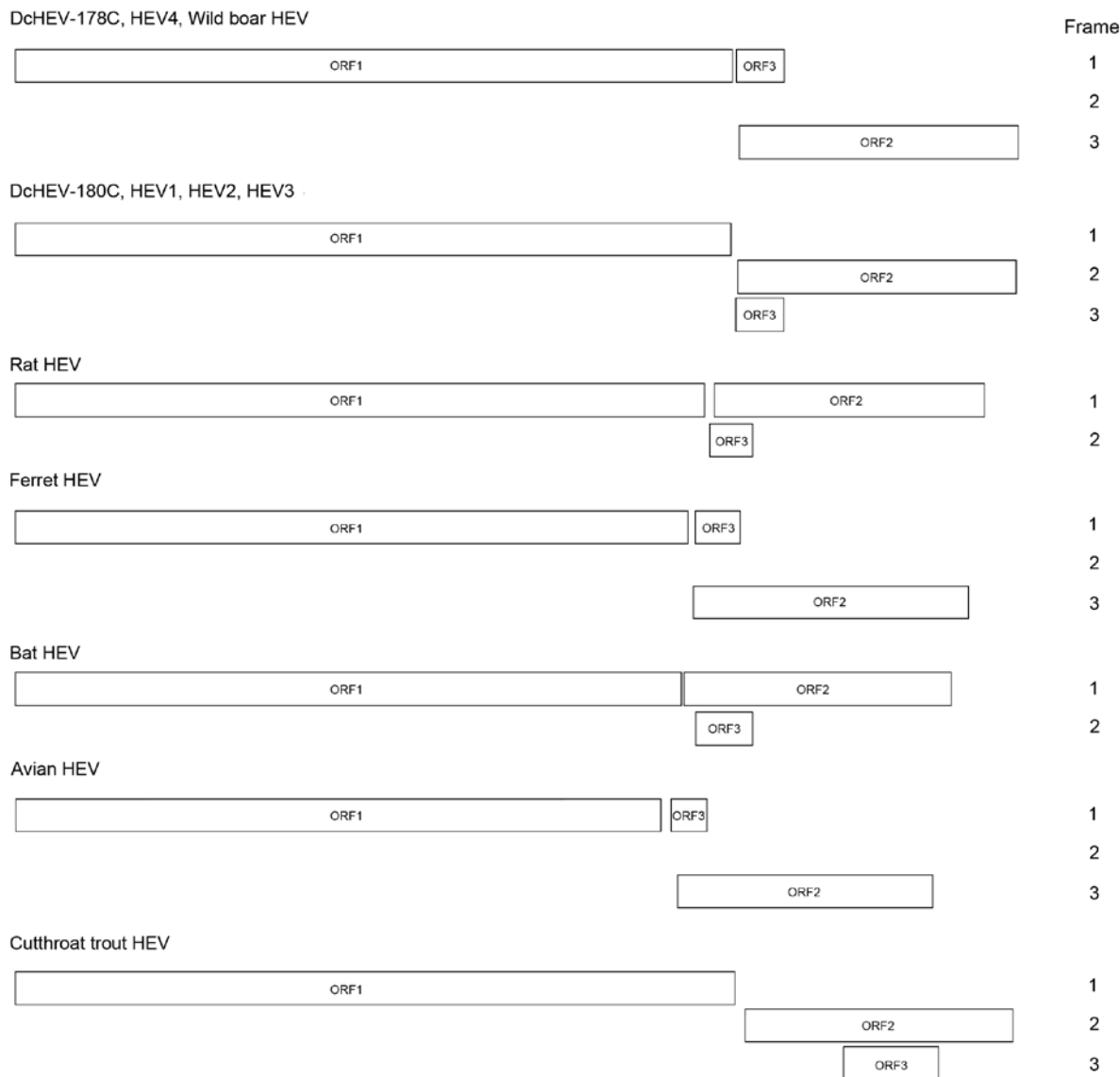


Figure 1. Predicted genomic organization of hepatitis E virus (HEV) from dromedary camel (DcHEV) and other HEVs, considering the reading frame of open reading frame (ORF) 1 as frame 1.

to only 1 possible start codon for its ORF2 but 3 possible start codons for its ORF3. To our knowledge, this presence or absence of such a U insertion in different strains of the same HEV has never been observed in other HEV genotypes and is unique to DcHEV. Although different regions of the DcHEV genome possessed characteristics associated with different kinds of HEV, no significant recombination was detected between DcHEV and the other HEVs. Because different regions of the genomes of DcHEV resembled those of different HEV genotypes, and even the genomes of different strains of DcHEV resembled those of different HEV genotypes, we propose that DcHEV should constitute a new HEV genotype.

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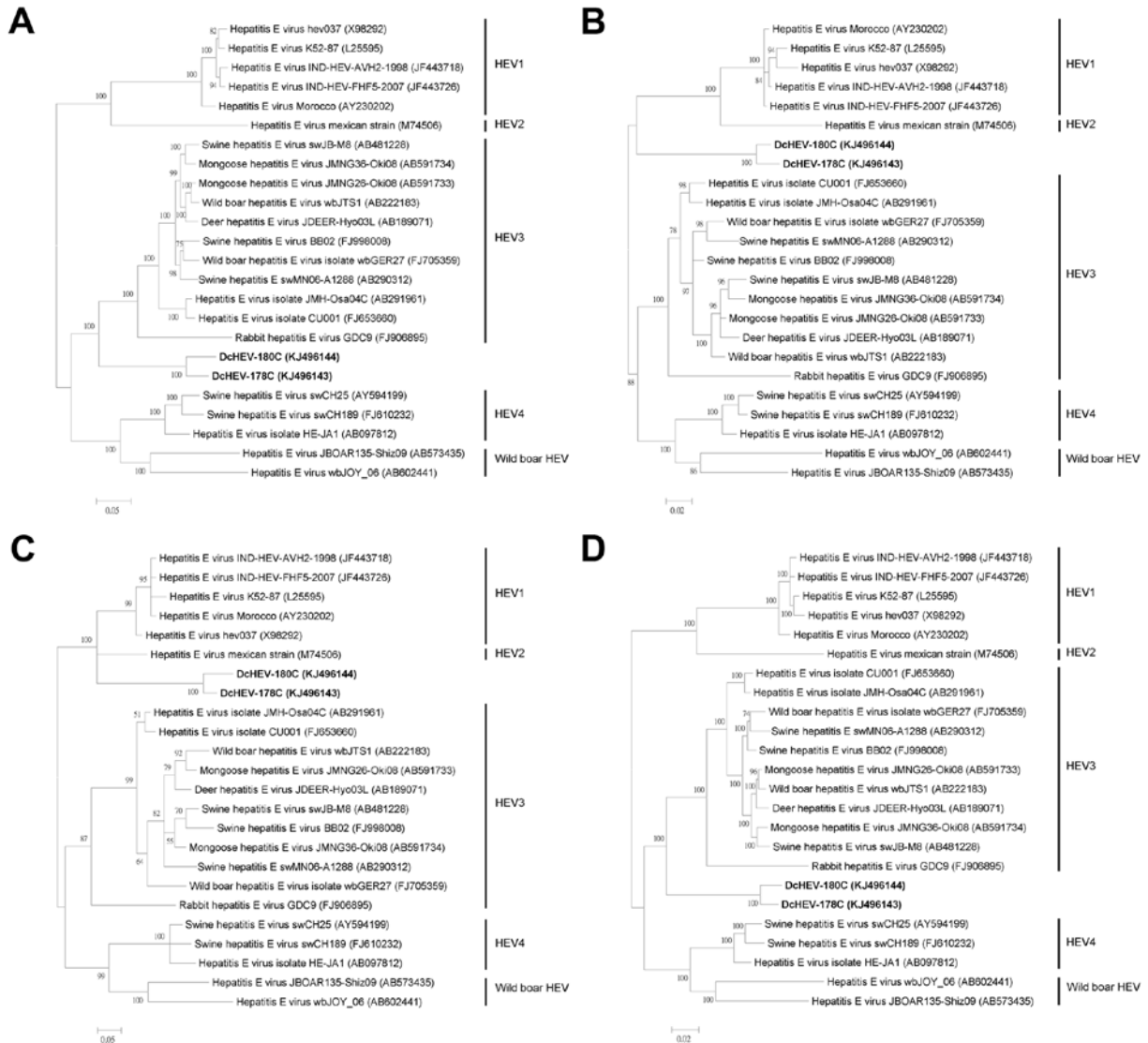


Figure 2. Phylogenetic analyses of open reading frame (ORF) 1 (A), ORF2 (B), ORF3 (C), and ORF1/ORF2 proteins, excluding the hypervariable region (HVR) (D) of hepatitis E virus (HEV) from dromedary camels (DcHEV). The trees were constructed by using Bayesian methods of phylogenetic reconstruction (www.fifthdimension.jp/products/mrbayes5d/), and ProtTest-suggested JTT+I+G+F, MtMam+I+G+F, HIVw+I+G+F, and JTT+I+G+F (<http://darwin.uvigo.es/software/prottest.html>) are the optimal substitution models for ORF1, ORF2, ORF3, and concatenated ORF1/ORF2 excluding HVR, respectively. For this analysis we included amino acid positions 1698, 660, 113, and 2282 in ORF1, ORF2, ORF3 and concatenated ORF1/ORF2 excluding HVR, respectively. For ORF2 and concatenated ORF1/ORF2 excluding HVR, the scale bars indicate the estimated number of substitutions per 50 aa. For ORF1 and ORF3, the scale bars indicate the estimated number of substitutions per 20 aa. Boldface indicates the 2 strains of DcHEV with complete genomes sequenced in this study.

Dr Woo is professor and head of microbiology at The University of Hong Kong. His research focuses on novel microbe discovery and microbial genomics.

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Address for correspondence: Patrick C.Y. Woo, State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong, China; email: pcywoo@hkucc.hku.hk



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MERS Coronaviruses in Dromedary Camels, Egypt

Daniel K.W. Chu,¹ Leo L.M. Poon,¹
Mokhtar M. Gomaa, Mahmoud M. Shehata,
Ranawaka A.P.M. Perera, Dina Abu Zeid,
Amira S. El Rifay, Lewis Y. Siu, Yi Guan,
Richard J. Webby, Mohamed A. Ali,
Malik Peiris, and Ghazi Kayali

We identified the near-full-genome sequence (29,908 nt, >99%) of Middle East respiratory syndrome coronavirus (MERS-CoV) from a nasal swab specimen from a dromedary camel in Egypt. We found that viruses genetically very similar to human MERS-CoV are infecting dromedaries beyond the Arabian Peninsula, where human MERS-CoV infections have not yet been detected.

Middle East respiratory syndrome (MERS) is a pneumonic illness caused by a novel lineage C beta-coronavirus (CoV). During September 2012–January 20, 2014, a total of 178 confirmed cases in humans resulted in 76 deaths (1). Primary infections have originated from countries within the Arabian Peninsula, although travel-associated cases and some secondary transmission have been reported in other countries. Limited human-to-human transmission has resulted in clusters of cases, some associated with multiple rounds of human-to-human transmission (2); the remaining sporadic cases in humans are presumed to be of zoonotic origin.

MERS-CoV genomes are phylogenetically classified into 2 clades, clade A and B clusters (3). The viral genomes detected in the earliest cases in humans (clade A cluster; EMC/2012 and Jordan-N3/2012) are genetically distinct from the others (i.e., clade B). The proximate source of human infection remains unclear. MERS-CoV is related to, but not identical to, viruses detected in bats (4,5). A short RNA fragment of the conserved viral polymerase region identical to MERS-CoV has been identified in *Taphozous perforates* bats, but these findings need to be confirmed (6).

Author affiliations: The University of Hong Kong, Hong Kong, China (D.K.W. Chu, L.L.M. Poon, R.A.P.M. Perera, Y. Guan, M. Peiris); National Research Centre, Giza, Egypt (M.M. Gomaa, M.M. Shehata, D.A. Zeid, A.S. El Rifay, M.A. Ali); HKU-Pasteur Research Pole, Hong Kong (L.Y. Siu); St. Jude Children's Research Hospital, Memphis, Tennessee, USA (R.J. Webby, G. Kayali)

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Serologic studies of domestic livestock in Jordan, Saudi Arabia, Qatar, United Arab Emirates, and Egypt have found high seroprevalance to a MERS-like CoV in dromedary camels but not in other domestic animals (7–11). An investigation of domestic animals in the vicinity of 2 persons with related infections detected fragments of viral RNA in dromedary camels in contact with these persons but whether this represented transmission from a unidentified source to humans and dromedaries, transmission from humans to dromedaries, or vice versa is not clear (12).

The Study

We collected nasal swab specimens from 110 apparently healthy dromedaries (*Camelus dromedarius*) >6 years of age in abattoirs on 12 occasions during June–December 2013 (Table 1). Serum was collected from 52 of these dromedaries. Serum from 179 persons working in the camel abattoirs also was collected. Median age of these workers was 38 years (range 9–67 years), 84% were male, and 25 reported comorbidities (i.e., cardiovascular, renal, diabetes, other). Collection of the human specimens was approved by the ethics committee of the National Research Centre (Giza, Egypt), and Institutional Animal Care and Use Committee approval for collection of animal samples was obtained from St Jude Children's Research Hospital (Memphis, TN, USA).

Real-time reverse transcription PCR (RT-PCR) targeting upstream of E gene of MERS-CoV was used for screening. The open reading frame (ORF) 1a gene was used for confirmation as recommended by the World Health Organization (www.who.int/csr/disease/coronavirus_infections/MERS_Lab_recos_16_Sept_2013.pdf?ua=1). We also used a previously described pan-CoV nested

Table 1. Results of testing nasal swab specimens from dromedary camels by RT-PCR for MERS-CoV and for other CoVs, Egypt, 2013*

| Location of animals sampled | No. samples tested | No. MERS-CoV positive† | No. other CoV positive. (virus identified) |
|------------------------------|--------------------|------------------------|--|
| Alexandria‡ | 17 | 0 | 0 |
| Cairo | | | |
| Abattoir 1§ | 46 | 0 | 3 (BCoV) |
| Abattoir 2§ | 10 | 3 | 0 |
| Nile Delta region, abattoir§ | 37 | 1 | 5 (BCoV) |

*RT-PCR, reverse transcription PCR; MERS, Middle East respiratory syndrome; CoV, coronavirus; BCoV, bovine coronavirus.

Nasal swabs from dromedary camels were placed in phosphate-buffered saline/glycerol transport medium, kept on ice during the field trip and stored at –80°C on arriving at the laboratory. Specimens were shipped in dry ice to the University of Hong Kong laboratory, where they were stored at –80°C until testing.

†Taken as positive only when both upstream of E gene and open reading frame 1a RT-PCR were positive.

‡Locally reared animals.

§Imported from Sudan or Ethiopia.

¹These authors contributed equally to this article.

PCR targeting the viral RNA-dependent RNA polymerase (RdRp) region (13), and PCR products were analyzed by sequencing (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/6/14-0299-Techapp1.pdf).

We detected MERS-CoV RNA in 4 (3.6%) of 110 nasal swab specimens from dromedary camels with the upstream of E gene assay (cycle threshold [C_t] 23.2–36.8), confirmed by the ORF1a assay (C_t 23.2–39.1), fulfilling the World Health Organization criteria for diagnosis of MERS-CoV infection (Table 1). PCR was repeated from a fresh RNA extract to confirm positive results. One positive sample was obtained from a camel in an abattoir in the Nile Delta

region in November 2013 and 3 other samples from an abattoir in Cairo in December 2013. Virus culture attempts in Vero E6 cells (ATCC CRL-1586) so far have been unsuccessful. The pan-CoV nested PCR detected CoV in an additional 8 specimens from dromedary camels. Sequence analyses of these additional positive samples showed that these amplicon sequences were genetically similar to those of bovine coronavirus (BCoV) (>99% nucleotide similarity). No animal was co-infected with MERS-CoV and BCoV-like viruses. The animals positive for either MERS-CoV or BCoV-like virus were all imported from Sudan or Ethiopia for slaughter.

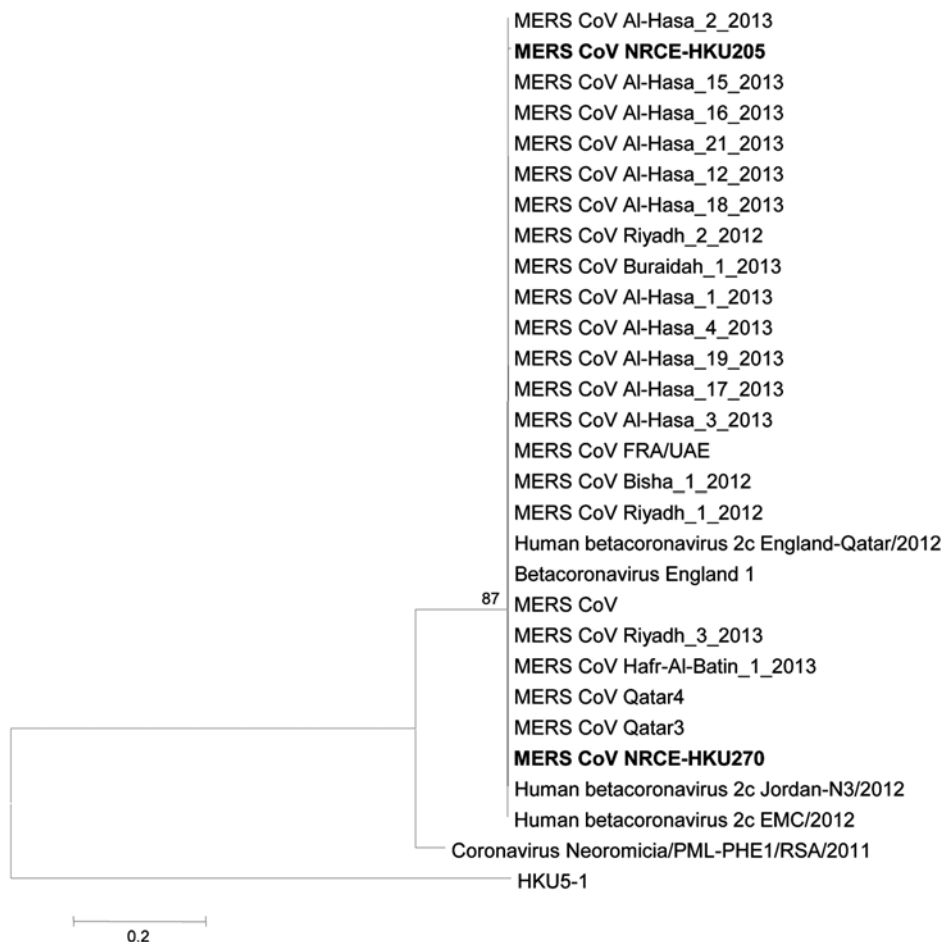


Figure 1. Phylogenetic analyses of a partial RNA-dependent RNA polymerase (RdRp) sequence determined from samples from dromedary camels (*Camelus dromedarius*) NRCE-HKU205 and NRCE-HKU270 that were positive for Middle East respiratory syndrome coronavirus (MERS-CoV). The viral RdRp region analyzed is a highly conserved region of the genome (covering motif B of RdRp) in nonstructural protein 12, at position 15202–15582 of MERS-CoV genome. The partial RdRp sequence of NRCE-HKU205 (GenBank accession no. KJ477102) and NRCE-HKU270 (GenBank accession no. KJ477103) was aligned with human MERS-CoVs (GenBank accession nos. KF600652, KF600630, KF600651, KF186567, KF600627, KF186564, KF600634, KF600632, KF600644, KF600647, KF600645, KF186565, KF186566, KF745068, KF600620, KF600612, KC667074, KC164505, KF192507, KF600613, KF600628, KF961222, KF961221, KC776174, and JX869059) and other representative animal betacoronaviruses (GenBank accession nos. HKU5–1, EF065509; BtCoV/PML/Neo cf. zul/RSA/2011, KC869678). Bat CoV HKU5–1 and bat CoV/PML/Neo cf. zul/RSA/2011 were included in the analysis as outgroups because they are phylogenetically closest to MERS-CoV. Phylogenetic trees were constructed by using MEGA5 (14) with neighbor-joining method. Numbers at nodes indicate bootstrap values determined by 500 replicates. Only bootstrap values >70 are denoted. Bold type indicates MERS-CoV identified in the current study. Scale bars indicate the estimated genetic distance of these viruses.

Table 2. Percentage identity between ORFs of dromedary camel MERS-CoV (NRCE-HKU205) and human MERS-CoV (EMC/2012) at the nucleotide and amino acid levels*

| ORF | % Identity to HCoV-EMC/2012 | |
|-------|-----------------------------|------------|
| | Nucleotide | Amino acid |
| ORF1a | 99.5 | 99.2 |
| ORF1b | 99.5 | 99.7 |
| S | 99.2 | 98.9 |
| ORF3 | 99.0 | 98.0 |
| ORF4a | 99.0 | 100 |
| ORF4b | 99.4 | 99.1 |
| ORF5 | 99.4 | 98.6 |
| E | 100 | 100 |
| M | 100 | 100 |
| N | 99.4 | 99.2 |
| ORF8b | 99.1 | 98.2 |

*ORF, open reading frame; MERS, Middle East respiratory syndrome; CoV, coronavirus; H, human; E, envelope; M, membrane; N, nucleocapsid.

On phylogenetic analysis, the partial RdRp sequences from MERS-CoV-positive samples NRCE-HKU205 and NRCE-HKU270 grouped within the cluster of MERS-CoV (Figure 1). The viral load in the other 2 specimens was too low to provide amplicons suitable for genetic sequencing. Viral RNA from NRCE-HKU205, the first positive specimen detected, was selected for more detailed genomic sequencing. We amplified viral RNA by PCR, using primers specific for overlapping regions of human MERS-CoV genome. PCR products were sequenced and assembled to produce a near full-length genome, lacking only the 3' untranslated region (29908 nt, >99% of the MERS-CoV genome) (online Technical Appendix). The camel MERS-CoV genome has an overall nucleotide similarity of 99.2%–99.5% and deduced amino acid similarity of 98.0%–100% to ORFs of human MERS-CoV EMC/2012 (Table 2). The 5' untranslated region and internal transcriptional regulatory sequences of NRCE-HKU205 were identical to published human MERS-CoV sequences.

Using MERS-CoV EMC/2012 as a reference sequence, we found 12 aa differences (residues 23, 26, 194, 434, 666, 696, 756, 886, 888, 918, 1158, and 1333 of EMC/2012) in the spike protein of dromedary MERS-CoV NRCE-HKU205 and 1 aa deletion (residue 1293 of EMC/2012) in the N terminal of the transmembrane domain. NRCE-HKU270 virus spike does not have a deletion of residue 1293. Of these, only residue 434 falls within the proposed receptor binding domain of spike protein, but it is located at the core (stem) subdomain of the receptor binding domain, suggesting that the camel MERS-CoV is still likely to bind human CD26 (online Technical Appendix Figure). The biological impact of this difference and other amino acid differences needs to be fully explored.

We used phylogenetic analyses of the full genome, the spike gene, and nucleocapsid gene of MERS-CoV NRCE-HKU205 to compare with the same genes of other

human MERS-CoVs (Figure 2). NRCE-HKU205 is within the clade A group but distinct from MERS-CoV EMC/2012, the only other MERS-CoV isolate available in our laboratory, excluding laboratory cross-contamination as an explanation for the detection of MERS-CoV from the dromedary specimens.

Using a previously described pseudoparticle neutralization assay (8), we detected antibodies against MERS-CoV (titer ≥ 20) in 48 (92.3%) of 52 dromedary serum samples with titers ranging from 20 to ≥ 640 . Dromedary NRCE-HKU205 had a serum antibody titer of 640, possibly indicating a developing serologic response, as was noted previously (7), or the possibility of re-infection. Serum was not available from the other 3 animals with MERS-CoV RNA-positive nasal swab specimens.

Serum from 179 persons working in the dromedary abattoirs was negative for antibody to MERS-CoV. This finding includes 114 persons working in the 2 abattoirs from which the MERS-CoV-positive animal swab specimens were obtained.

Conclusions

Our findings confirm that MERS-CoV infects dromedary camels and that this virus is genetically very similar to a MERS-CoV that is infecting humans. The detection of MERS-CoV in nasal swab specimens of camels in 2 of 12 sampling occasions in abattoirs, taken together with the high seropositivity to MERS-CoV in dromedaries previously reported, supports the contention that MERS-CoV infection is common in dromedaries. Studies of dromedaries within camel herds and through the animal marketing system supplying abattoirs are needed to define the epidemiology of the infection. Our findings strengthen the plausibility that dromedaries may be a potential source of human infection and emphasize the need for detailed epidemiologic investigation of the exposure histories of humans with MERS. However, the lack of serologic evidence of infection of humans working in these abattoirs suggests that transmission of this virus to humans is uncommon. The detection of MERS-CoV in dromedaries in Egypt, in animals imported from Sudan and Ethiopia, suggests that cases may occur in humans beyond the Arabian Peninsula. MERS CoV diagnostic tests should be considered for all patients with unexplained severe pneumonia in Egypt, northeastern Africa, and beyond.

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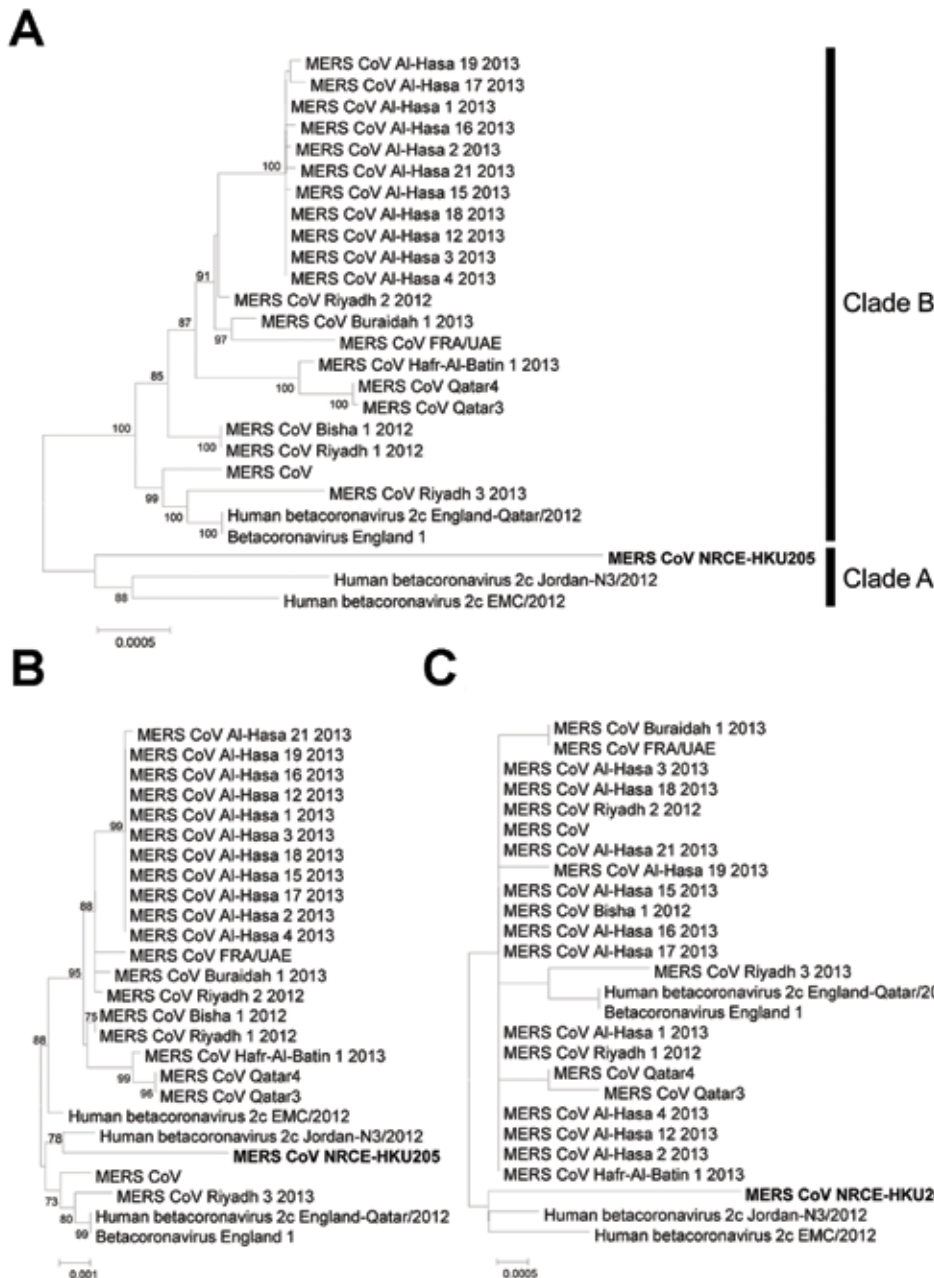


Figure 2. Phylogenetic analyses of Middle East respiratory syndrome coronavirus (MERS-CoV) from dromedary camels. Genomic (A), spike (B), and nucleocapsid (C) sequences of the dromedary camel MERS-CoV NRCE-HKU205 (GenBank accession no. KJ477102) were aligned with the corresponding human MERS-CoV (N = 25) sequences retrieved from GenBank (accession nos. as in Figure 1 legend). Phylogenetic trees were constructed by using MEGA5 (14) with neighbor-joining method. Numbers at nodes indicate bootstrap values determined by 500 replicates. Only bootstrap values >70 are denoted. Bold type indicates MERS-CoV identified in the current study. Scale bars indicate the estimated genetic distance of these viruses.

Dr Chu is a postdoctoral fellow at the Centre of Influenza Research at the University of Hong Kong. His primary research interests are virus ecology, evolution of coronavirus, astroviruses, and zoonotic infections.

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Addresses for correspondence: Malik Peiris, School of Public Health, The University of Hong Kong, 21 Sassoon Rd, Pokfulam, Hong Kong Special Administrative Region, China; email: malik@hku.hk; Ghazi Kayali, Division of Virology, Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, TN, USA; email: ghazi.kayali@stjude.org



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Unraveling the Mysteries of Middle East Respiratory Syndrome Coronavirus

John T. Watson, Aron J. Hall, Dean D. Erdman, David L. Swerdlow, and Susan I. Gerber

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel CoV known to cause severe acute respiratory illness in humans; $\approx 40\%$ of confirmed cases have been fatal. Human-to-human transmission and multiple outbreaks of respiratory illness have been attributed to MERS-CoV, and severe respiratory illness caused by this virus continues to be identified. MERS-CoV was first reported in September 2012, and subsequent investigations documented illness onsets as early as April 2012 (1). As of February 23, 2014, the World Health Organization has reported 182 laboratory-confirmed cases of MERS-CoV infection, including 79 deaths, indicating an ongoing risk for transmission to humans in the Arabian Peninsula (2). The median age of reported case-patients is 52 years (range 2–94 years); most cases are in males (3). Most index case-patients have at least reported 1 chronic comorbid condition (4) and have resided in, or recently traveled to, Jordan, Qatar, United Arab Emirates, Oman, Kuwait, or Saudi Arabia (3). In France, Germany, Italy, United Kingdom, and Tunisia confirmed cases of MERS-CoV have been identified in travelers returning from these countries (3). Although a zoonotic reservoir of MERS-CoV has been speculated, very little is known about the specific exposures that result in primary human cases.

MERS-CoV infection causes severe acute hypoxemic respiratory failure, extrapulmonary organ dysfunction, and high rates of death; however, the spectrum of illness and clinical course are not fully defined (5). Evidence suggests that MERS-CoV is capable of limited human-to-human transmission, which results in outbreaks in family and health care settings (5,6). The 182 reported cases include multiple distinct spatiotemporal clusters and 32 identified infections in health care workers (3). Modeling performed to assess the extent of human infection and the transmission

potential of MERS-CoV (as of August 2013) estimated that most symptomatic case-patients had not been detected but that chains of transmission were not self-sustaining when infection control was implemented (7). Despite evidence of human-to-human transmission, the number of contacts infected by persons with confirmed infections appears to be limited; sustained transmission in the community has not been documented (3). The Hajj, the annual religious pilgrimage to Saudi Arabia, involved 1.37 million pilgrims from 188 countries in 2013 but resulted in no reports of confirmed cases in the weeks after the pilgrimage (3).

Little is known about the pathogenic potential and transmission dynamics of MERS-CoV. Although multiple health care-associated clusters have been identified (4), further investigation is needed of the specific risk factors for transmission within health care facilities. Basic information about the temporal and causal patterns of viral shedding and their relationships to clinical outcomes is critical to further understand the virus and to shape prevention and control measures needed to limit transmission. Standard, contact, and airborne precautions appear to be effective in limiting transmission and are recommended by the Centers for Disease Control and Prevention to manage known or suspected MERS-CoV infection in hospitalized patients as a primary means of preventing and controlling transmission (8).

Potential animal reservoirs and mechanism(s) of transmission of MERS-CoV to humans remain unclear. Of the minority of case-patients for whom information is available about exposure to animals, few have reported owning or visiting a farm with camels, goats, sheep, chickens, ducks, or other animals (4). A zoonotic origin for MERS-CoV was initially suggested by its high genetic similarity to bat CoVs (9) and the identification of closely related viruses in bats (10). Recent reports have described additional data from camels. These include real-time reverse transcription PCR detection and limited sequencing of MERS-CoV from 3 camels from a farm in Qatar linked

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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to 2 infections in humans in October 2013 (11) and, more recently, in camels in Saudi Arabia (12), and antibodies against MERS-CoV in camel serum from the Arabian Peninsula, including serum from the United Arab Emirates drawn in 2003 (13–17). However, more epidemiologic data linking cases to infected animals are needed to determine whether a particular animal species is a host for the virus, a source of human infection, or both.

This month's issue of *Emerging Infectious Diseases* presents results of a study that provides evidence of MERS-CoV in dromedary camels in Egypt (18). Only 3 other reports of MERS-CoV detection in animals have been published: 1 in a bat and 2 in camels (11,12,19). However, these reports were based on limited genetic information. In contrast, on the basis of their sequence analysis of nearly the entire viral genome showing >99% nt sequence identity with human MERS-CoV, Chu et al. provide the most compelling evidence thus far of MERS-CoV infection in dromedary camels (18). Although the authors also found neutralizing antibodies to MERS-CoV (or a MERS-like CoV) in most of the camels, they did not find serologic evidence of infection in the abattoir workers who had contact with the infected animals. This finding leaves key questions about zoonotic transmission unanswered. Most notably, it remains unclear whether zoonotic transmission of MERS-CoV occurs between camels and humans and, if so, what the directionality and risk factors are for such transmission. These lingering gaps in knowledge about MERS-CoV emphasize the need for more epidemiologic study to determine risk factors for human infection, more population-level data on the prevalence of MERS-CoV in camels, risk factors for infection and shedding in camels, and continued vigilance for other possible sources of infection. Also, the camels tested were in Egypt and were locally reared or imported from Sudan or Ethiopia, countries in which no cases have been identified in humans. Thus, the geographic area for surveillance should be widened beyond the Arabian Peninsula and include eastern Africa, which is a source for importation of dromedary camels. This study emphasizes the need to further define exposure information for all MERS-CoV cases regarding camels and other animals, as well as exposure to ill humans who might have undetected MERS-CoV infections. Understanding the role of dromedary camels and possibly other animals in transmission of MERS-CoV to humans remains a priority for future investigation to enable development of targeted control measures and prevent future cases and deaths from this emerging pathogen.

Dr Watson is a medical officer with the Division of Viral Diseases, Centers for Disease Control and Prevention, in Atlanta, Georgia, USA. His research interests include the epidemiology and control of viral respiratory diseases.

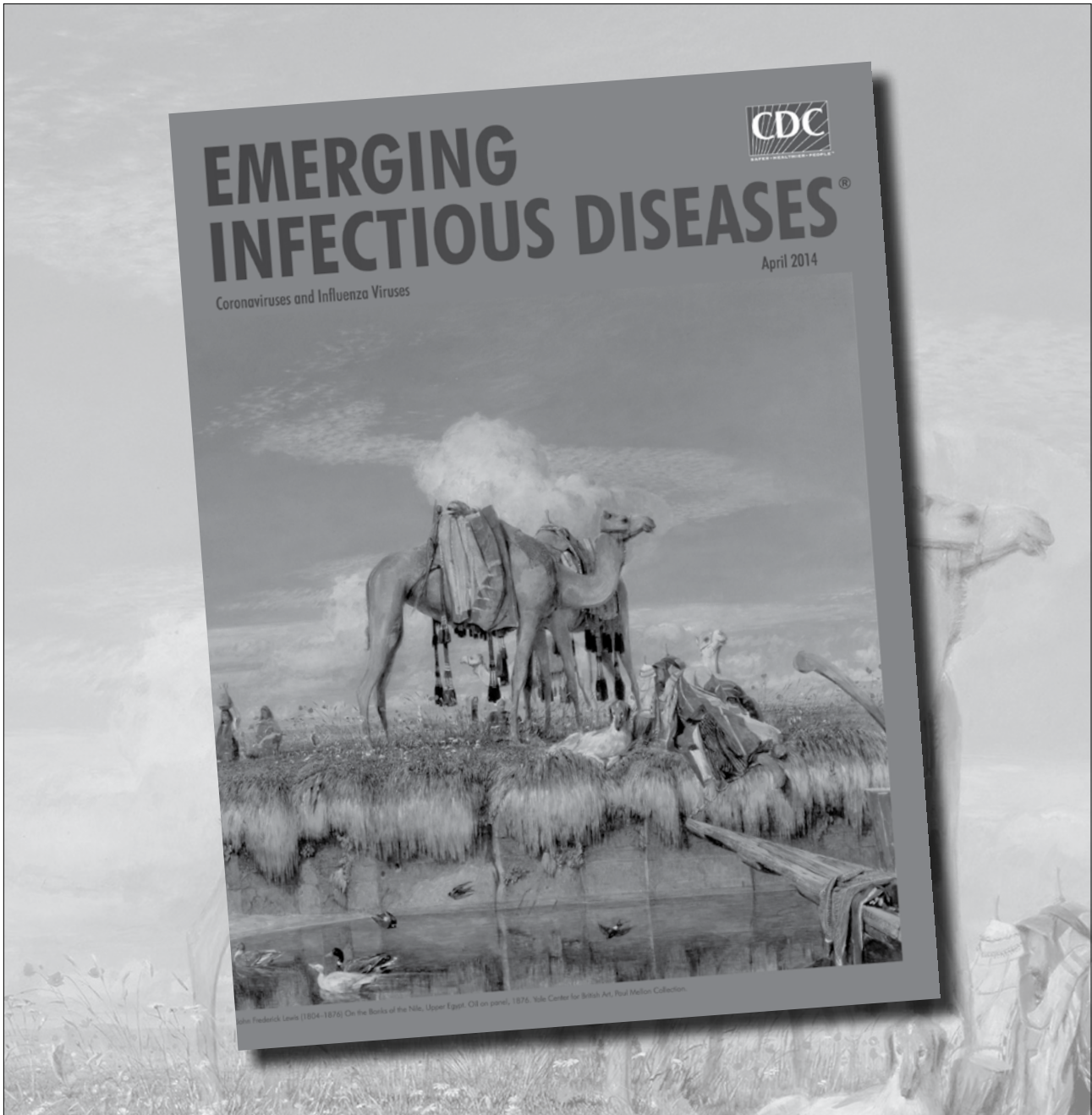
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Address for correspondence: John T. Watson, Division of Viral Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A34, Atlanta, GA 30333, USA; email: acq4@cdc.gov

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Hepatitis E Virus Infection, Papua New Guinea, Fiji, and Kiribati, 2003–2005

To the Editor: We report hepatitis E virus (HEV) infection rates in 3 South Pacific island countries—Papua New Guinea (PNG), Fiji, and Kiribati—determined from results of HEV IgG testing. During 2003–2005, specimens were collected from volunteers as part of a study of the epidemiology of viral hepatitis (1,2). Participants recruited were apparently healthy adults in the community and mother–infant pairs (specifically infants who were receiving, or had recently completed, their vaccinations). No specific inclusion/exclusion criteria were applied. Samples were collected from outpatient clinics and hospitals in PNG from Port Moresby, Goroka, Mt Hagen, Madang, and Daru. Samples from Fiji were collected in Suva from outpatient clinics and hospital wards. A proportion of samples from children were taken from nonjaundiced inpatients in PNG and Fiji. In Kiribati, samples were collected from participants at village preschools,

vaccination clinics, and outpatient clinics on North Tarawa and North Tabiteuea (2). These were convenience samples and therefore might not be nationally representative cohorts. We obtained ethics permission for the study from appropriate national agencies. Signed informed consent was obtained from each participant or, for children, from a parent or guardian.

From this sample pool, in a time sequential manner, the first serum samples were assayed: 545 from PNG (48 Goroka, 99 Mt Hagen, 87 Daru, 47 Madang, 156 Port Moresby), 265 from Fiji, and 238 from Kiribati. We evaluated samples using the Wantai (PE2) HEV IgG ELISA kit (Wantai Pharmaceutical Enterprise, Beijing, China), which detects IgG for all 4 known strains of human HEV. The assays were used according to the manufacturer's instructions, and repeat equivocal results were defined as negative (3,4). HEV IgG positivity was highest in PNG (15.2%), followed by Kiribati (8.8%) and Fiji (2.2%) (Table). IgG positivity did not differ significantly between adults and children (<16 years of age) (PNG: 16.1% vs. 11.4%, $p = 0.23$; Kiribati: 6.0% vs. 13.3%, $p = 0.06$; and Fiji: 1.7% vs. 3.3%, $p = 0.42$ [Fisher exact test]).

To investigate potential parent–child transmission, we tested mother/child (MC); father/child (FC); and when possible, mother/father/child (MFC) sets. We found no transmission association: In PNG, we tested 88 sets (67 MC, 2 MFC, and 19 FC); in Fiji, 29 sets (20 MC and 9 MFC); and in Kiribati, 65 sets (59 MC and 6 MFC); of the 11 PNG, 1 Fijian, and 8 Kiribati HEV IgG–positive children, none had IgG–positive parents. Because these samples were tested retrospectively, ascertaining the HEV IgG status of other family members was not possible.

The high percentage of HEV–seropositive children <5 years of age in PNG and Kiribati implies active viral circulation in these countries. This is an unusual finding, compared with findings from seroprevalence studies in developing countries where IgG prevalence increases with age (3). The reason for this difference remains to be determined. It is unlikely to relate to acute HEV infection in hospitalized children sampled because the Wantai assay measures IgG, not IgM. The finding that these young seropositive children commonly have seronegative parents suggests that parent–child transmission is not the primary

Table. Seroprevalence of HEV, Papua New Guinea, Fiji, and Kiribati, 2003–2005*

| Age group, y | Papua New Guinea† | | Fiji‡ | | Kiribati‡ | |
|--------------|-------------------|----------------------|--------|----------------------|-----------|----------------------|
| | All | HEV IgG+, % (95% CI) | All | HEV IgG+, % (95% CI) | All | HEV IgG+, % (95% CI) |
| Children | 12/105‡ | 11.4 (6.6–18.9) | 3/91‡ | 3.3 (1.1–9.2) | 12/90 | 13.3 (7.8–21.2) |
| <1 | 0/21 | 0 | 1/23 | 4.3 | 1/8 | 12.5 |
| 1–<2 | 3/26 | 11.5 | 1/19 | 5.3 | 2/18 | 11.1 |
| 2–<5 | 6/24 | 25 | 1/35‡ | 2.9 | 6/48 | 12.5 |
| 5–<10 | 1/23‡ | 4.3 | 0/10‡ | 0 | 0/10 | 0 |
| 10–<16 | 1/6 | 16.7 | 0/3‡ | 0 | 0/2 | 0 |
| Unknown | 1/5 | 20 | 0/1 | 0 | 3/4 | 75 |
| Adults | 71/440 | 16.1 (14.2–21.3) | 3/174 | 1.7 (0.6–4.9) | 9/148 | 6.0 (3.2–11.1) |
| 16–19 | 7/24 | 29.2 | 0/8 | 0 | 0/2 | 0 |
| 20–29 | 19/162 | 11.7 | 2/76 | 2.6 | 2/61 | 3.3 |
| 30–39 | 18/143 | 12.6 | 0/41 | 0 | 3/53 | 3.7 |
| 40–49 | 13/70 | 18.6 | 1/28 | 3.6 | 2/19 | 5.3 |
| 50–59 | 5/22 | 22.7 | 0/14 | 0 | 2/9 | 22.2 |
| 60–69 | 3/7 | 42.9 | 0/5 | 0 | 0/2 | 0 |
| 70–79 | 0/4 | 0 | 0/0 | 0 | 0/2 | 0 |
| Unknown | 0/9 | 0 | 0/1 | 0 | 0/0 | 0 |
| Total | 83/545‡ | 15.2 (12.5–18.5) | 6/265‡ | 2.3 (1.0–4.9) | 21/238 | 8.8 (5.8–13.1) |

*An expanded version of this table that includes sex data is available online (wwwnc.cdc.gov/EID/article/20/7/13-0562-T1.htm). HEV, hepatitis E virus; +, positive.

†No. HEV IgG–positive persons/total no. persons in each group.

‡In these groups.

mechanism of infection in the population studied, which is in accord with published data (4).

To investigate whether HEV seropositivity was higher in certain areas, we partitioned the data into regions defined by participant's place of birth and tribal ethnicity. In Fiji and Kiribati, no significant region association was detected. However, in PNG, the proportion of HEV antibody-positive specimens was greater among participants from highland communities (altitude >1,500 m) than from lowland communities (20.4% vs. 9.7%, $p = 0.01$). (Port Moresby has a mixed immigrant population and was excluded from this analysis).

The reason for the higher proportion of HEV IgG-positive specimens among participants in highland than lowland communities is unclear but might be explained by increased zoonotic transmission. In highland regions, pigs are more frequently kept, and the animals are kept closer to home (5). HEV genotypes 1 and 2 are hyperendemic to many developing countries and typically cause waterborne outbreaks of acute hepatitis in humans (6). Genotypes 3 and 4 are endemic to industrialized countries and are known to be a porcine zoonosis (7). Genotype 3 has been found in pigs in New Caledonia (8).

In our investigation of 3 developing nations in Oceania, we found that HEV IgG positivity varies substantially between, and within, countries; it is high in PNG (15.2%) and low in Fiji (2.2%). By using the same sensitive, diagnostic assay, the seroprevalence of HEV in blood donors in New Zealand was reportedly 4% (9). In New Caledonia, 1.7% of 351 military recruits tested positive (10). In our study, the sampling method limits the applicability of the data to the general population. Nevertheless, our findings suggest HEV infection should be considered in cases of unexplained hepatitis.

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**John S. Halliday,¹
G.L. Abby Harrison,¹
Anthony Brown,
Jeremy G. Hunter,
Richard Bendall, David Penny,
Tebuka Toatu,
Mohammad Y. Abdad,
Paul Klenerman,
Eleanor Barnes,
and Harry R. Dalton**

Author affiliations: University of Oxford, Oxford, UK (J.S. Halliday, A. Brown, P. Klenerman, E. Barnes); Royal Melbourne Hospital, Melbourne, Victoria, Australia (J.S. Halliday); Walter and Eliza Hall Institute of Medical Research, Melbourne (G.L.A. Harrison); University of Exeter, Truro, UK (J.G. Hunter, R. Bendall, H.R. Dalton); Massey University, Palmerston North, New Zealand (D. Penny); Disease Surveillance, Control and Research Unit, Secretariat of the Pacific, Suva, Republic of the Fiji (T. Toatu); and Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea (M.Y. Abdad)

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¹These authors contributed equally to this article.

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Address for correspondence: John S. Halliday, Department of Gastroenterology, Royal Melbourne Hospital, 300 Grattan St, Parkville 3050, Victoria, Australia; email: johnshalliday@gmail.com

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Diagnosis of Trombiculosis by Videodermoscopy

To the Editor: Dermoscopy (also known as dermatoscopy, epiluminescence microscopy, and surface microscopy) is a noninvasive technique that enables rapid and magnified ($\times 10$) in vivo observation of the skin and detection of morphologic details often not visible to the naked eye. Videodermoscopy, which is performed with a probe equipped with lenses providing higher magnification (up to $\times 1,000$) and connected to a personal computer, enables more detailed inspection of the skin than does manual dermoscopy and enables storage of digital images. Both techniques have been widely used for the differential diagnosis and monitoring of pigmented lesions; however, a role for these techniques in the diagnosis and follow-up of other skin disorders has recently emerged (1,2). Their usefulness for diagnosing several parasitic disorders of the skin (e.g., scabies, pediculosis, phthiriasis, larva migrans, tungiasis, myiasis, and tick infestations) has led to introduction of the term entodermoscopy. In the hands of trained physicians, these techniques are more effective than traditional methods (e.g., parasite identification by microscopic examination of samples obtained by skin scraping); they are well accepted by patients and particularly suitable for mass screening and posttreatment follow-up examinations (1–8).

We describe a puzzling case in which videodermoscopy enabled a definitive diagnosis of trombiculosis. Trombiculosis is a common but underreported ectoparasitosis that is probably often misdiagnosed.

In January 2013, a 66-year-old man from eastern Sicily, Italy, reported diffuse intense pruritus that persisted despite various treatments administered in the previous months for a well-documented diagnosis of

scabies. The condition had considerably impaired his quality of life, causing family concerns and missed workdays. Physical examination revealed multiple excoriations and pinpoint erythematous macules scattered throughout the trunk and lower legs (Figure, panel A), but no burrows or other findings suggestive of scabies were detectable with use of a common magnification lens. An accurate and thorough examination by videodermoscopy (at $\times 150$ magnification) revealed a reddish mite strongly attached to the skin on the patient's right shin. In the stored images, a larval *Neotrombicula autumnalis* mite was subsequently identified (Figure, panel B). A diagnosis of cutaneous trombiculosis was made, and the patient was instructed to avoid further environmental exposure; his symptoms were consequently relieved.

Trombiculosis is an infestation of the skin by the larval stage of various species of mites belonging to the phylum Arthropoda, class Arachnida, subclass Acarina. *N. autumnalis* mites are more diffuse in the temperate and humid European environment, where adult individuals live and reproduce on the soil, especially during warmer and wet late summer months. Eggs usually hatch at the end of autumn, and new mites, which at their larval stage are obligate parasites of warm-blooded hosts, usually feed and grow on the skin of small rodents and dogs, injecting lytic enzymes to digest cutaneous cells. Humans engaged in

outdoor activities or staying in the countryside for professional or recreational purposes can become occasional hosts of this ectoparasite. Infection is more common in autumn and should be suspected for persons at risk (e.g., farmers, hunters, children) who have an itchy eruption with a likely environmental cause (9).

No specific medications are required to treat trombiculosis in humans. Usually effective measures are use of repellents, avoidance of exposure by wearing adequate clothing when in mite-infested areas, and washing of body and clothes with soap and hot water immediately after exposure. Itch can sometimes be relieved by supportive care with oral antihistamines or topical corticosteroids (9). Antimicrobial drugs might be needed to cure bacterial superinfection resulting from repeated scratching.

Trombiculosis is not considered rare, but it is underreported and, probably, often misdiagnosed. Cutaneous findings are nonspecific, and an accurate anamnesis is essential for making this challenging diagnosis. Because the patient reported here denied any professional or recreational outdoor activities, a single clinical examination would probably have led to a wrong diagnosis of a nonspecific itchy dermatitis, leading to use of inadequate or needless medications. Also, our experience confirms that common magnification lenses and even dermoscopy at $\times 10$ magnification have some limitations;

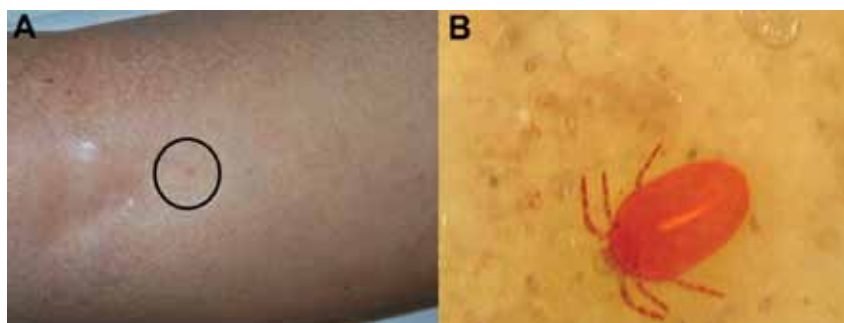


Figure. Clinical features of a nonspecific lesion (circle) (A) and its corresponding, unequivocal dermoscopy findings (B), showing a *Neotrombicula autumnalis* mite attached to the skin (original magnification $\times 150$).

parasites can easily be missed or barely noticeable so that their identification can be quite difficult. In such instances, videodermatoscopy might lead to the diagnosis and should be considered as a useful diagnostic aid. Image storage and sharing can also facilitate collaboration with experts and can enable timely recognition of unusual parasitic disorders imported from different geographic areas or tropical countries.

The cost of the equipment varies according to resolution quality, magnification capability, and image storage facility; costs range from 500 (for simple systems) to 10,000 (for sophisticated systems) euros. The expense is greatly outweighed by the advantages of avoiding the high cost of managing outbreaks of epidemic parasitoses resulting from misdiagnosis, treatment failures, and incomplete posttreatment monitoring (10).

Videodermatoscopy is a noninvasive way to diagnose some pruritic disorders while avoiding unnecessary, uncomfortable, and sometimes expensive investigations and treatments. Physicians without access to such equipment should consider promptly referring patients to the nearest available videodermatoscopy service for effective management.

**Maria R. Nasca,
Francesco Lacarrubba,
and Giuseppe Micali**

Author affiliation: University of Catania, Catania, Italy

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Address for correspondence: Maria Rita Nasca, Dermatology Clinic, University of Catania, A.O.U. Policlinico Vittorio Emanuele, P.O. Gaspare Rodolico, Via S. Sofia 78, 95123 Catania, Italy; email: nasca@policlinico.unict.it

Distinguishing Nontuberculous Mycobacteria from Multidrug-Resistant *Mycobacterium tuberculosis*, China

To the Editor: Mycobacteria are commonly characterized by positive acid-fast staining. Most mycobacterial species belong to the nontuberculous mycobacteria (NTM), excluding species in the *Mycobacterium tuberculosis* complex and *M. leprae*. Both *M. tuberculosis* and NTM can induce pulmonary infection with similar symptoms and pulmonary radiographic findings (1). These similarities have led to difficulty in distinguishing these infections clinically.

As in many developing countries, the acid-fast stain is the only bacteriologic basis for diagnosing tuberculosis (TB) in primary health care institutions in China, where facilities are limited for *M. tuberculosis* culture, strain identification, and drug resistance detection. Thus, NTM is easily misdiagnosed as *M. tuberculosis*, and multidrug-resistant (MDR) TB is unable to be accurately identified. Patients with misdiagnosed TB usually are treated with the standard anti-TB regimens recommended by the Chinese government (i.e., 2HRZE/4HR [2 months of isoniazid (INH), rifampin (RIF), pyrazinmid, and ethambutol, followed by 4 months of INH and RIF 1 time daily] and 2H₃R₃Z₃E₃/4H₃R₃ [2 months of INH, RIF, pyrazinamide, and ethambutol followed by 4 months of INH and RIF 3 times weekly]) (2), which often results in treatment failures. Misdiagnosis is a key hurdle for effective prevention and treatment of TB (3–5). To evaluate the effect of misdiagnosis on TB prevention, we determined the proportion of patients with MDR TB and NTM infection in primary health care institutions in Zhejiang Province, China. Our

findings would be useful for improving TB prevention and treatment.

During 2011–2012, sputum samples from 13,882 patients suspected of having TB in 8 counties in Zhejiang Province were used to culture mycobacteria. Each sample was seeded onto 2 pieces of Löwenstein-Jensen medium. A total of 1,410 samples grew mycobacteria confirmed as acid-fast bacilli by using Ziehl-Neelsen staining. The 1,410 samples were further identified by using the Mycobacteria Identification Array Kit (CapitalBio, Beijing, China). The kit contains 17 types of bacilli-specific 16S rRNA probes (i.e., *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. gilvum*, *M. xenopi*, *M. smegmatis*, *M. aurum*, *M. terrae*, *M. goodii*, *M. chelonae/abscessus*, *M. phlei*, *M. scrofulaceum*, *M. fortuitum*, *M. szulgai*, *M. ulcerans*, *M. marinum*, and *M. kansasii*). With this method, *M. tuberculosis* and NTM can be distinguished, and the species of NTM can be identified (6–8). Of 1,410 positive strains, we identified 1,332 (94.5%) as *M. tuberculosis* and 78 (5.5%) as NTM. NTM strains were further identified as follows: *M. intracellulare*, 39 isolates; *M. chelonae/abscessus*, 12 isolates; *M. kansasii*, 13 isolates; *M. avium*, 3 isolates; *M. fortuitum*, 4 isolates; and *M. scrofulaceum* and *M. szulgai*, 1 isolate each. For 5 isolates, strain could not be classified.

We detected drug resistance of 1,332 *M. tuberculosis* strains using a

Tuberculosis Drug Resistance Detection Array Kit (CapitalBio) (9). The mutant points for RIF resistance were identified as follows: *rpoB/C531G*, *C531T*, *CG531AC*, *A526C*, *A526G*, *A526T*, *C526A*, *C526G*, *C526T*, *T533C*, *A516G*, *A516T*, *G516T*, *T511C*, *T511G*, *C513A*, *A513T*, and *C522T* (Table). Moreover, the kit contained 5 mutant points for INH resistance, including *katG* (*G315A*, *G315C*, *G315T*, and *C315*) and *inhA* (*C-15T*) (Table). Of 1,332 *M. tuberculosis* strains, we identified 1,115 (83.7%) RIF/INH-sensitive strains, 88 (6.6%) MDR TB strains, 83 (6.2%) INH-resistant strains, and 47 (3.5%) RIF-resistant strains. Of the 1,410 positive strains, 88 (6.2%) were MDR *M. tuberculosis* strains.

The epidemiology of TB in Zhejiang Province reflects the situation in China and some developing countries (10). The clinical diagnosis and treatment of >80% TB cases in China is performed mainly by primary health care institutions. However, almost 80% of these medical institutions do not have the capability to culture *M. tuberculosis*, detect drug resistance, and identify strains (7). Of 1,410 strains obtained from the patients in 8 counties of Zhejiang Province, 218 (15.5%) were MDR TB, INH resistant, and RIF resistant. These affected patients could not be effectively treated with the national standard regimens. Specifically, 88 patients with MDR TB would be at risk for extensively drug-resistant TB, and 83

patients with INH-resistant TB and 47 with RIF-resistant TB would be at risk for MDR TB. In addition, we identified 78 (5.5%) NTM strains. With the acid-fast stain, these illnesses would be misidentified as TB and, in most instances, also would be reported as treatment failures. Clearly, accurate diagnosis provided by the technologies used in this study for distinguishing NTM and *M. tuberculosis*, *Mycobacterium* strain identification, and drug-resistance detection would increase the cure rate and effectively prevent TB epidemics.

For INH resistance, *katG315* was a main mutant point of the *M. tuberculosis* strain; 140 (81.4%) of the 172 INH-resistant mutations were related to *katG315*. For RIF resistance, *rpoB531* was a main mutant point; 84 (60.0%) of 140 RIF-resistant mutations were associated with *rpoB531*. Therefore, in future studies, more attention should be paid to the molecular epidemiology of *katG315* and *rpoB531*.

In conclusion, using the techniques for *M. tuberculosis* culture, *Mycobacterium* strain identification, and drug-resistance detection is necessary. It should be urgently pursued for accurate TB diagnosis in primary health care institutions in China to improve the prevention, treatment, and control of TB.

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Table. Gene mutations of 214 drug-resistant tubercle bacilli, Zhejiang Province, China, 2011–2012

| Drug | Mutant points | Mutant times | |
|-----------|---|--|--|
| | | Mutant times for single site, no. (%)* | Total no. mutant times of sites related to drug resistance |
| Isoniazid | <i>inhA-15</i> (C→T) | 32 (18.6) | 172† |
| | <i>katG315</i> (G→C), (G→A) | 140 (81.4) | |
| Rifampin | <i>rpoB511</i> (T→C) | 10 (7.1) | 140‡ |
| | <i>rpoB513</i> (A→C) | 2 (1.4) | |
| | <i>rpoB516</i> (A→T), (A→G), (G→T) | 19 (13.6) | |
| | <i>rpoB526</i> (A→G), (A→T), (C→G), (C→T) | 21 (15) | |
| | <i>rpoB531</i> (C→G), (C→T) | 84 (60.0) | |
| | <i>rpoB533</i> (T→C) | 4 (2.9) | |

*No. mutant times for single site/total no. mutant times of sites related to drug resistance.

†A strain simultaneously had *katG315* (G→C) and *inhA-15* (C→T).

‡Five strains had the double mutation of *rpoB*.

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**Kaijin Xu, Sheng Bi,
Zhongkang Ji, Haiyang Hu,
Feisu Hu, Beiwen Zheng,
Bing Wang, Jingjing Ren,
Shigui Yang, Min Deng,
Ping Chen, Bing Ruan,
Jifang Sheng, and Lanjuan Li.**

Author affiliations: State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China (K. Xu, S. Bi, Z. Ji, H. Hu, F. Hu, B. Zheng, B. Wang, J. Ren, S. Yang, M. Deng, P. Chen, B. Ruan, J. Sheng, L. Li); and Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou (K. Xu, L. Li)

DOI: <http://dx.doi.org/10.3201/eid2006.130700>

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Address for correspondence: Lanjuan Li, 79 Qingchun Rd, Hangzhou 310003, P.R. China; email: ljli@zju.edu.cn

Schmallenberg Virus Circulation in High Mountain Ecosystem, Spain

To the Editor: Schmallenberg virus (SBV) is an emerging vector-borne virus mainly associated with *Culicoides* spp. midges (1,2). Factors affecting the density and distribution of vectors may help determine the prevalence of SBV infection in particular areas. Altitude could be one limiting factor for virus transmission; however, little information is available regarding SBV in high-altitude regions.

During December 29, 2012–February 21, 2013, morphologic anomalies were identified in 4 stillborn calves from different farms in northeastern Spain, and infection with SBV was suspected. The cases were clustered in the Ripollès and Garrotxa regions of Catalonia and appeared in beef cattle herds that spent the grazing season (May–November) in the alpine meadows (>2,000 m altitude) of the National Game Reserve of Freser-Setcases in the Eastern Pyrenees Mountains. The calves had severe arthrogryposis, ankylosis of several joints, abnormal curvature of the vertebral column, and severe muscle atrophy. Malformations of the central nervous system included bilateral hydrocephalus, cerebellar hypoplasia, and micromyelia, characterized by the presence of few neurons in the ventral horns and moderate to severe bilateral reduction of white matter in the ventral and lateral funiculi.

SBV infection was confirmed by real-time reverse transcription qualitative PCR (RT-qPCR) (1,3) or serologic testing in 3 of the 4 calves and all 4 of the mothers (Table). Serum samples were tested by using a commercial indirect ELISA (ID.vet; Innovative Diagnostics, Montpellier, France) and a virus neutralization test using the BH80/11–4 isolate (provided by the Friedrich-Loeffler-Institut, Isle of Riems, Germany) (4). Consistent results were obtained from both of these techniques, and the proportions of calves positive by ELISA and RT-qPCR were similar to those found in previous studies (5).

The neurologic and musculoskeletal lesions found in the calves indicated that fetal infection probably occurred at 5–6 months' gestation (6). Gestation started in mid-April to mid-May; therefore, maternal infection most probably occurred in late summer 2012 (September–October), when cows were grazing in the alpine meadows.

We then performed a serologic study in domestic and sympatric wild ruminants from the National Game

Table. Results of serologic and molecular analyses of SBV in sympatric wild and domestic ruminants, Eastern Pyrenees, Spain, 2010–2013*

| Ruminants and SBV cases | No. animals | SBV serologic testing results | | | | | | | RT-qPCR | |
|-------------------------|-------------|-------------------------------|------------|---------------|----------|------------------|------------|-----------------------|---------|--|
| | | 2010 Ratio | 2011 Ratio | 2012 | | | 2013 Ratio | 2013 Prev, % (95% CI) | | |
| | | | | Mar–Aug Ratio | Sep–Dec† | | | | | |
| | | | | Ratio | Ratio | Prev, % (95% CI) | | | | |
| Wild | | | | | | | | | | |
| Chamois‡ | 260 | 0/45 | 0/89 | 0/21 | 5/81 | 6.2 (0.9–11.4)§ | 3/24 | 12.5 (0–25.7)§ | – | |
| Roe deer¶ | 20 | – | 0/6 | 0/9 | – | – | 4/5 | 80.0 (44.9–100) | – | |
| Mouflon# | 75 | – | 0/29 | 0/21 | – | – | 0/25 | – | – | |
| Fetuses | | | | | | | | | | |
| Chamois | 7 | – | – | – | – | – | 0/7 | – | – | |
| Mouflon | 1 | – | – | – | – | – | 0/1 | – | – | |
| Roe deer | 1 | – | – | – | – | – | 0/1 | – | – | |
| Domestic** | | | | | | | | | | |
| Cattle | 130 | – | 0/9 | – | 26/30 | 86.7 (74.5–98.8) | 79/91 | 86.8 (79.9–93.8) | – | |
| Sheep | 60 | – | 0/30 | – | 14/30 | 46.7 (28.8–65.5) | – | – | – | |
| Goat | 13 | – | 0/4 | – | 2/9 | 22.2 (0–49.4) | – | – | – | |
| SBV cases | | | | | | | | | | |
| Stillborn calves | 4 | – | – | – | 0/1 | – | 2/3 | – | 1/4†† | |
| Mothers of calves | 4 | – | – | – | – | – | 4/4 | – | – | |

*SBV, Schmallenberg virus; ratio, no. positive/no. tested; prev, prevalence; RT-qPCR, real-time qualitative reverse transcription PCR; –, no data or not applicable.

†First evidence of SBV circulation in the study area was a seropositive chamois on September 3, 2012.

‡Two sampling periods, March–May and August–December. Only March–May in 2013.

§Differences not statistically significant.

¶Sampling period April–May.

#Sampling period April–June.

**Two sampling periods, October–November 2011 and November 2012–April 2013.

††Brain, thymus, and abomasum fluid samples positive; liver and kidney samples negative.

Reserve of Freser-Setcases, which comprises 20,200 ha of alpine and subalpine ecosystems. We analyzed serum samples from 355 wild ruminants hunted during August 2010–May 2013; species sampled included Pyrenean chamois (*Rupicapra pyrenaica*), European mouflon (*Ovis aries musimon*), and roe deer (*Capreolus capreolus*). We also analyzed samples from fetuses of these species obtained in April 2013 (Table), as well as animals from 8 cow herds and 4 sheep–goat mixed herds; a mean of 14 samples were collected per herd during 2 sampling periods (Table). Two of the mixed sheep–goat herds were sampled during both sampling periods. All serum samples underwent ELISA testing; positive results were confirmed by virus neutralization (4).

Domestic ruminants sampled during October–November 2011 were seronegative, whereas all farms sampled during November 2012–April 2013 had infected animals (Table). High mean seroprevalence was found in cow herds; 105 (86.8% [95% CI

80.7%–92.8%]) of 121 animals tested were infected. Seroprevalence was lower but still high for mixed sheep–goat herds; 16 (41% [95% CI 25.6%–56.5%]) of 39 animals were infected. The earliest evidence of SBV in the study area came from a seropositive Pyrenean chamois hunted on September 3, 2012; this date coincides with the estimated months when cows that delivered stillborn calves were infected. For wild ungulates tested from September 2012 onwards, overall SBV seroprevalence was statistically higher (χ^2 33.47, 2 d.f., $p < 0.0001$) in roe deer (4/5, 80% [95% CI 44.9%–100%]) than in Pyrenean chamois (8/105, 7.6% [95% CI 2.5%–12.7%]) and mouflon (0/23). Differences in seroprevalence for summer through autumn 2012 compared with spring 2013 in Pyrenean chamois were not significant (Table).

Roe deer seroprevalence was similar to the 88.9% reported in Belgium in December 2011, which contrasted with the lower seroprevalence observed in red deer, 54.6%, for the same month in the same study (7).

Differences in seroprevalence between wild host species might be related to differences in exposure to SBV vectors depending on habitat selection, vector feeding habits, or host-specific factors; altitude might be an additional factor affecting exposure (8). Thus, the lower altitude habitat selection of roe deer and the housing of domestic ruminants in valley areas could explain the higher seroprevalence observed in these species compared with that in Pyrenean chamois and mouflon.

All fetuses of wild ruminants had negative serologic test results for SBV, and no gross lesions indicating infection were observed (Table). However, the potential reproductive disorders that SBV infection can cause in these species are unknown.

Our findings support the hypothesis that SBV can circulate in alpine meadows at >2,000 m altitude and confirm the appearance of SBV in late summer and autumn 2012 in the high mountain ecosystem of the Eastern Pyrenees in Spain. A variety of domestic and wild ruminants showed

susceptibility to SBV infection, but differences in seroprevalence suggest different roles for sympatric ruminants in SBV epidemiology. The role of vector species in the transmission of SBV in alpine ecosystems should be analyzed.

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**Xavier Fernández-Aguilar,
Joan Pujols,
Roser Velarde, Rosa Rosell,
Jorge R. López-Olvera,
Ignasi Marco, Marti Pumarola,
Joaquim Segalés,
Santiago Lavín,
and Oscar Cabezón**

Author affiliations: Centre de Recerca en Sanitat Animal, Barcelona, Spain (X. Fernández-Aguilar, J. Pujols, R. Rosell, J. Segalés, O. Cabezón); Universitat Autònoma de Barcelona, Barcelona (X. Fernández-Aguilar, R. Velarde, J.R. López-Olvera, I. Marco, M. Pumarola, J. Segalés, S. Lavín, O. Cabezón); Institut de Recerca i Tecnologia Agroalimentàries, Barcelona (J. Pujols); and Generalitat de Catalunya Departament d'Agricultura, Barcelona (R. Rosell)

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Address for correspondence: Oscar Cabezón, Servei d'Ecopatologia de Fauna Salvatge, Wildlife Diseases Research Group, Departament de Medicina i Cirurgia Animals, Edifici V. Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain; email: oscar.cabezón@cresa.uab.cat

Novel Henipa-like Virus, Mojiang Paramyxovirus, in Rats, China, 2012

To the Editor: The genus *Henipavirus* (family *Paramyxoviridae*) contains 3 established species (*Hendra virus*, *Nipah virus*, and *Cedar virus*) and 19 newly identified species, including 1 full-length sequenced virus, Bat Paramyxovirus Eidhel/GH-M74a/GHA/2009 (1,2). The zoonotic pathogens *Hendra virus* and *Nipah virus* have been associated with lethal neurologic and respiratory diseases in humans, horses, and pigs (3–5). The known natural reservoirs of henipaviruses are fruit bats (1,3); these viruses have not been reported in other wild animals. We report on a novel henipa-like virus, Mojiang paramyxovirus (MojV), in rats (*Rattus flavipectus*) in China.

In June 2012, in Mojiang Hani Autonomous County, Yunnan Province, China, severe pneumonia without a known cause was diagnosed in 3 persons who had been working in an abandoned mine; all 3 patients died. Half a year later, we investigated the presence of novel zoonotic pathogens in natural hosts in this cave. For the investigation, we collected anal swab samples from 20 bats (*Rhinolophus ferrumequinum*), 9 rats (*R. flavipectus*), and 5 musk shrews (*Crocidura dracula*) from the mine for virome analysis.

All samples were processed by using a virus particle-protected nucleic acid purification method, followed by sequence-independent PCR amplification of extracted RNA and DNA (6). The amplified viral nucleic acid libraries were then sequenced by using an Illumina Genome Analyzer II (Illumina Trading, Beijing, China) for a single read of 81 bp. All raw reads were then aligned to the nonredundant protein database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/RefSeq/) by using BLASTx (<http://blast.ncbi.nlm.nih.gov/>).

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nih.gov/Blast.cgi) after filtering reads as described (6). The taxonomy of the aligned reads was parsed by using the MEGAN4 MetaGenome Analyzer (7).

On the basis of the nonredundant protein alignment results, we identified 38 sequence reads that were classified as *Henipavirus* spp. However, the sequences shared low nucleotide and amino acid identities with known henipaviruses. The reads were then used for reads-based PCR to identify the partial genome of this virus. The remaining genomic sequences were determined by using genome walking. The 5' and

3' untranslated regions were obtained by nested PCR with combined specific primers and henipavirus-specific degenerate primers as described (8), and the exact sequences of the 5' and 3' genome termini were determined by rapid amplification of cDNA ends.

MojV shares similar features with known henipaviruses. The virus has a genome length of 18,404 nt (submitted to GenBank under accession no. KF278639), and has the characteristic henipavirus gene order: 3'-nucleocapsid (N) protein (539 aa); P/V/W/C proteins (phosphoprotein; 694 aa, 464

aa, 434 aa, 177 aa); matrix protein (340 aa); fusion protein (545 aa); attachment glycoprotein (625 aa); and large (L) protein (2,277 aa)-5' (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/6/13-1022-Techapp1.pdf). The predicted conserved sequences between genes showed features characteristic of henipaviruses (online Technical Appendix Table). The central domain of the N protein contains 3 conserved motifs common in all paramyxoviruses: QXW [I/V] X₃K [A/C] XT, FX₂T[I/L][R/K]Φ[G/A][L/I/V] XT, and FX₄YPX₂ΦSΦAMG, where Φ

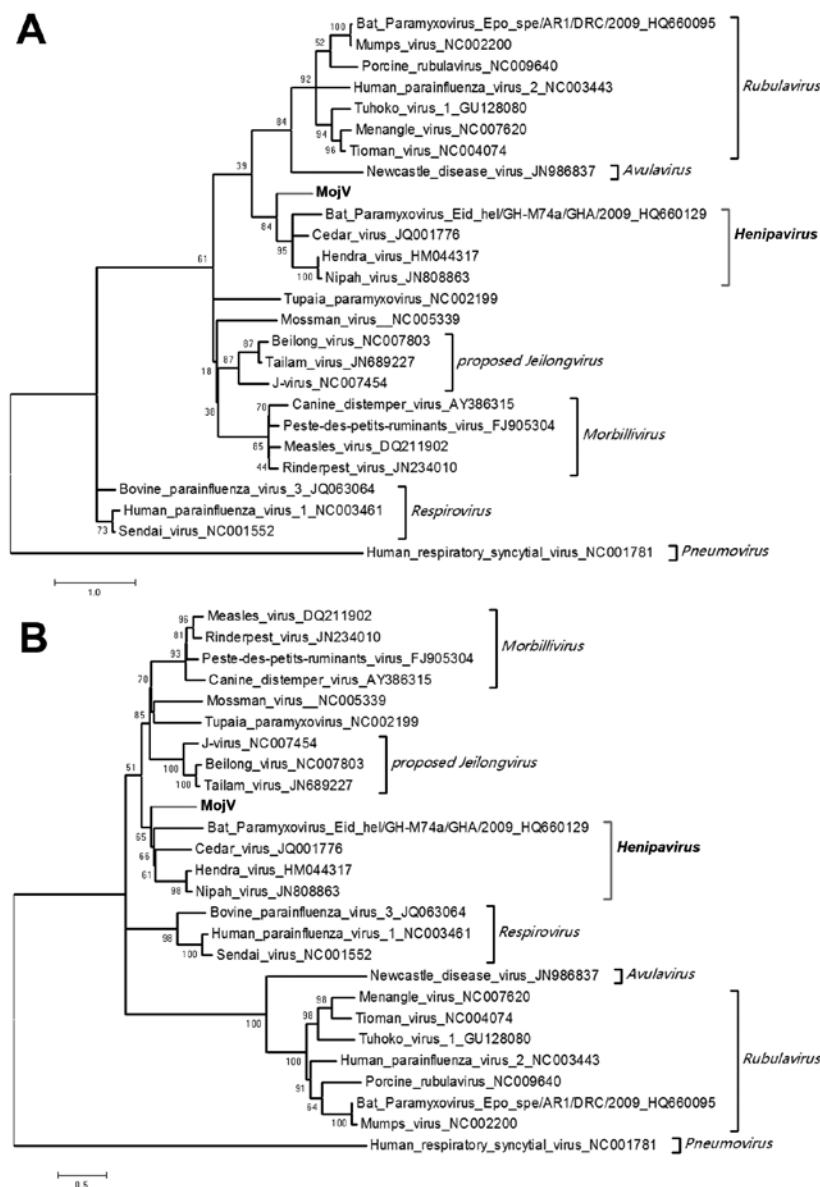


Figure. Phylogenetic trees based on the nucleocapsid proteins (A) and large proteins (B) of Mojiang paramyxovirus (MojV) and other previously reported paramyxoviruses. Bold font indicates MojV and *Henipavirus* spp. Scale bars indicate nucleotide substitutions per site.

is an aromatic amino acid (9). In addition, the RNA editing site (AAAAGG) for the processing of V and W proteins conserved in the phosphoprotein gene sequences of Hendra virus and Nipah virus was found, and 6 conserved domains within the L proteins of the order *Mononegavirales* (8) were found in the MojV L protein.

The nucleotide identities of predicted MojV genes exhibited similarity with genes of known henipaviruses: N (53.0%–57.0% identity), phosphoprotein (37.8%–43.0% identity), matrix (59.5%–63.4% identity), fusion (47.5%–51.4% identity), attachment glycoprotein (36.6%–41.8% identity), and L (55.9%–58.6% identity) genes. Using MEGA5 (10), we used the phylogenetic trees based on N and L proteins to describe the evolutionary relationships between MojV and members of the family *Paramyxoviridae* (Figure). MojV clustered with the 4 members of the genus *Henipavirus* and was distant from other clusters. Thus, considering the similar genome features between MojV and other henipaviruses, we confirmed that MojV could be classified as a new species closely related to *Henipavirus* spp.

Specific nested primer sets targeting the L gene of MojV were designed to separately re-evaluate the 34 anal swab samples and some tissue samples. Of 9 anal swab samples from the *R. flavipectus* rats, 3 were positive for MojV, and a tissue sample from 1 of the 3 MojV-positive rats was also MojV positive (tissue was not collected from the other 2 rats). All 20 samples from *R. ferrumequinum* bats and all 5 samples from *C. dracula* musk shrews were MojV negative. The 3 MojV-positive anal swab samples were cultured in Vero E6, Hep2, and BHK21 cells for virus isolation; no cytopathic effects or viral replication was detected after 2 blind subculture passages.

Our study showed the presence of a rodent-origin, henipa-like virus, MojV, in China. *R. flavipectus* rats are the natural reservoir of MojV. This finding and its context indicate that

Henipavirus spp. viruses might infect more mammalian hosts than previously thought and that bats may not be the only hosts of henipaviruses.

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**Zhiqiang Wu,¹ Li Yang,¹
Fan Yang,¹ Xianwen Ren,¹
Jinyong Jiang, Jie Dong,
Lilian Sun, Yafang Zhu,
Hongning Zhou, and Qi Jin**

Author affiliations: Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China (Z. Wu, L. Yang, F. Yang, X. Ren, J. Dong, L. Sun, Y. Zhu, Q. Jin); Institute of Pathogen Biology, Beijing (Z. Wu, L. Yang, F. Yang, X. Ren, J. Dong, L. Sun, Y. Zhu, Q. Jin); and Yunnan Institute of Parasitic Diseases, Puer, China (J. Jiang, H. Zhou)

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Address for correspondence: Qi Jin, No. 6 Rongjing East St, Yizhuang, Beijing, 100176, China; email: zdsys@vip.sina.com

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¹These authors contributed equally to this article.

***Streptococcus suis* Infection and Malignancy in Man, Spain**

To the Editor: *Streptococcus suis* is an emerging zoonotic agent. Human infection is associated with occupational exposure to swine. Affected persons are usually, but not always, healthy (1,2). Immunosuppressive conditions can predispose persons to *S. suis* infection, and cancer has classically been associated as a risk factor for *S. suis* infection (1,2). Nevertheless, the actual number of reported cases is low (2–7). We describe a severe case of *S. suis* infection in a man who had not been exposed to swine but for whom disseminated cancer was diagnosed 5 months after the infection.

In 2012, a 57-year-old alcoholic man from Spain, who had no other medical conditions and no contact with animals sought care for headache and vomiting for 24 hours. He reported a 4-day history of fever and a painful right shoulder. At admission, temperature was 38.9°C, blood pressure 180/100 mm Hg, heart rate 68 beats/min, and respiratory rate 24 breaths/min. Neck stiffness and lethargic mental status were noted.

Laboratory tests revealed the following values: leukocytosis of 14×10^9 (reference range $3.9\text{--}10 \times 10^9$) cells/L with 90.4% neutrophils, platelets 100×10^9 (reference $135\text{--}333 \times 10^9$) cells/L, hemoglobin 16 (reference $12.6\text{--}16.6$) g/dL, creatinine 131 (reference $0\text{--}111$) $\mu\text{mol/L}$, and C-reactive protein 243 (reference $0\text{--}5$) mg/L. Lumbar puncture yielded turbid cerebrospinal fluid (CSF), with high opening pressure (>32 cm H_2O), pleocytosis (0.4×10^9 leukocytes/L; 88% neutrophils), high protein level (70 [reference range $15\text{--}45$] mg/dL) and a low glucose level (<0.3 [reference $2.2\text{--}4.1$] mmol/L). CSF showed gram-positive cocci in chains. Cefotaxime,

dexamethasone, and mannitol were administered. After septic shock and respiratory insufficiency developed, the patient was transferred to the intensive care unit.

Streptococcus spp. grew in blood and CSF cultures. Although initially misidentified as *S. bovis*, the pathogen was confirmed as *S. suis* by sequence analysis of the 16S rRNA gene. Multilocus sequence typing (<http://ssuis.mlst.net>) identified this isolate as sequence type (ST) 3.

The patient was transferred to the medical ward 18 days after admission. Neurologic examination demonstrated vestibular ataxia, hearing loss, and diplopia resulting from cranial nerve VI palsy. Furthermore, a diagnosis of subacromial/subdeltoid bursitis led to arthroscopic debridement. Ceftriaxone was administered for 4 weeks. Results of abdominal computed tomography and echocardiogram were within normal limits. Because the *Streptococcus* organism was initially identified as *S. bovis*, colonoscopy and assessment of tumor markers were also requested; results were within normal limits.

After the patient was discharged (4 weeks after admission), diplopia and the shoulder mobility limitation completely resolved, but bilateral deafness and ataxia persisted. Five months later, the patient was readmitted for severe hypercalcemia. Positron-emission and computed tomography revealed liver, lung, and bone metastases. Tumor markers were elevated (carcinoembryonic antigen 4,152 [reference range $0\text{--}4.3$] $\mu\text{g/L}$; monoclonal antibody CA-19-1 9,233 [0–39] U/mL). The patient died of multiorgan failure 21 days after admission. Necropsy revealed a disseminated esophageal adenocarcinoma.

S. suis is an encapsulated gram-positive, catalase-negative facultative anaerobe coccus, positive for Lancefield group antigens R-S or T. This pathogen of swine is infrequently transmitted to humans (1–3,6);

recently, however, the number of *S. suis* cases in humans has increased substantially. Most cases have been reported in Europe and Southeast Asia, where pig farming is intensive (1). Although cases are usually sporadic, 2 outbreaks in China (1998 and 2005) caused a substantial number of deaths. Exposure to infected pigs was demonstrated for almost all patients. However, some patients had not been exposed to animals (1,2,4). *S. suis* can be an opportunistic pathogen in immunocompromised persons (1,2). Splenectomy is a well-established risk factor. Other predisposing factors are alcoholism, heart disease, and diabetes (1,4,5).

Although cancer is accepted as a risk factor (1,2), the reported number of cases with associated malignancy is quite low (Table). For all cases except one, cancer was diagnosed before or during the episode of infection. A primary adrenal lymphoma was diagnosed 1 year after *S. suis* meningitis (6), but probably an underlying defect in humoral immunity was already present. The patient reported here probably had subclinical malignancy at the time of infection. Although we cannot rule out a spurious relationship between cancer and infection, we believe that malignancy, in combination with the patient's alcoholism, led to an immunosuppressed condition that facilitated the development of infection.

S. suis leads to a wide spectrum of clinical manifestations, meningitis being the most common (1–3,6–8). A higher frequency of sensorineural hearing loss is characteristic of *S. suis* meningitis (1). In the patient reported here, meningitis was complicated by permanent deafness, ataxia, and transient diplopia; to our knowledge, only 2 other cases complicated by diplopia have been reported (8,9).

S. suis ST3 belongs to ST clonal complex 1 and is associated with serotype 2 (<http://ssuis.mlst.net>). Although clonal complex 1 accounts for most *S.*

Table. Cases of *Streptococcus suis* infection and cancer reported in the literature*

| Year | Country of origin | Patient age, y/sex | Clinical presentation | Malignancy | Time of malignancy diagnosis† | Animal contact | Infection outcome | Ref. |
|------|-------------------|--------------------|-----------------------------------|--|--------------------------------------|------------------------|-------------------|------------|
| 2012 | Spain | 57/M | Meningitis, arthritis, bacteremia | Disseminated esophageal adenocarcinoma | 5 mo after <i>S. suis</i> infection. | No | Survived | This study |
| 2007 | Italy | 68/M | Meningitis | Lung squamous cell carcinoma | At admission | No | Survived | (2) |
| 2006 | Greece | 59/M | Endocarditis | Colon carcinoma | At admission | Yes (farmer) | Survived | (3) |
| 2004 | Hong Kong | 81/F | Cellulitis, bacteremia | Breast malignancy | Before <i>S. suis</i> infection | Yes | Survived | (4) |
| 2001 | Thailand | NA | NA | Stomach cancer | Before <i>S. suis</i> infection | NA | NA | (5) |
| 1994 | Taiwan | 61/M | Meningitis | Primary adrenal B-cell lymphoma | 1 y after <i>S. suis</i> infection. | Yes (pig farmer) | Survived | (6) |
| 1983 | The Netherlands | 76/M | Meningitis | Pancreatic carcinoma | Before <i>S. suis</i> infection | Yes (pig keeper) | Survived | (7) |
| 1981 | The Netherlands | 52/M | Meningitis | Stomach carcinoma | Before <i>S. suis</i> infection | Yes (cut meat for dog) | Survived | (7) |

*Ref., reference; NA, not available.

†With regard to the episode of *S. suis* infection.

suis infections in humans (1,10), genotype ST3 is extremely rare. To our knowledge, only 1 other human case of *S. suis* ST3 infection has been reported, also in Spain (10).

The patient reported here had severe *S. suis* infection with no prior exposure to swine but with undiagnosed neoplasia. In patients with no exposure to swine, we recommend searching for other predisposing factors, such as malignancy or other immunodeficiencies.

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**Silvia Gómez-Zorrilla,
Carmen Ardanuy,
Jaime Lora-Tamayo,
Jordi Cámara,
Dolors García-Somoza,
Carmen Peña, and Javier Ariza**

Author affiliation: University of Barcelona, Barcelona, Spain

DOI: <http://dx.doi.org/10.3201/eid2006.131167>

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Address for correspondence: Silvia Gómez-Zorrilla, Infectious Diseases Service, Hospital Universitario de Bellvitge, Bellvitge Biomedical Research Institute, Feixa Llargà s/n 08907, Hospitalet de Llobregat, Barcelona, Spain; email: sgomez@bellvitgehospital.cat

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.

***Bartonella* spp. and *Yersinia pestis* Reservoirs, Cusco, Peru**

To the Editor: *Bartonella* spp. are gram-negative alphaproteobacteria that are transmitted between the reservoir and mammal host by hematophagous insects (1). The genus *Yersinia* comprises 11 species, of which *Y. pestis* is the causative agent of plague, a deadly rodent-associated, fleaborne zoonosis (2). Despite the large number of plague cases reported in humans and the large amount of data about human-infecting *Bartonella* spp. in Peru (3), no data have been published about which rodent species are reservoirs of these pathogens in this country.

La Convención Province, where cases of bartonellosis occurred during 1998 (4), is located in the northeastern part of Cusco, Peru. Although, to our knowledge, no human cases of plague have been reported in this province, a plague outbreak was recently detected in Junin Province (M.A. Quispe-Riclude, pers. comm.), which is located northwest of La Convención Province.

A total of 28 rodents were captured during 2010–2011 in 3 villages (Alto Ivochote, Aguas Calientes, and Yomentoni) in the Echarate District, La Convención Province. Traps had been set in intradomiciliary, peridomiciliary, and extradomiciliary settings. Spleens of animals were obtained, and DNA was isolated by using the Illustra Tissue and Cells Genomic Prep Mini Spin Kit (GE Healthcare, Little Chalfont, UK).

Rodents were examined for *Bartonella* spp. DNA by using a PCR and primers CS443f and CS1210r specific for a 767-bp fragment of the citrate synthase gene (5). Screening for plague was performed by using PCR primers Yp1 and Yp2 specific for a plasminogen activator protein (*pla*) encoded by the *Y. pestis*-specific pPLA plasmid (6).

New and published *Bartonella* spp. and *Y. pestis* sequences were obtained from GenBank and compared by using the nucleotide-nucleotide basic local sequence alignment tool (BLAST) (blastn) program (www.ncbi.nlm.nih.gov/Class/MLACourse/Modules/BLAST/nucleotide_blast.html). The χ^2 test was used to determine statistical differences in the prevalence of both pathogens among host species and villages.

Overall prevalences for *Y. pestis* and *Bartonella* spp. were 17.9% and 21.4%, respectively (Table). Co-infections with both bacteria were found in 3 (10.7%) rodents: 2 *Hylaeamys perenensis* rodents and 1 *Oecomys* spp. rodent. *Bartonella* prevalence was higher in *H. perenensis* rats than in *Rattus rattus* rodents ($p < 0.001$). Rodents positive for *Bartonella* spp. were found in the 3 study villages, and prevalence for Aguas Calientes was higher than that for Alto Ivochote ($p < 0.001$). One of 8 rodents trapped inside houses and 1 of 2 rodents trapped at peridomestic sites were positive for *Bartonella* spp.

Sequence analysis identified 3 citrate synthase gene sequences (GenBank accession nos. KF021602–KF021604) that had 98% and 99% sequence similarity to genotypic variant A3 of the undescribed *Bartonella*

genogroup A, which was obtained from *Oryzomys palustris* rats in the southeastern United States (7). One genotype (isolate B259) was identified in *H. perenensis* rats, and 2 other genotypes were identified in 1 *H. perenensis* rodent and 1 *Oecomys* spp. rodent (isolates B273 and B280, respectively). We propose that the genotype of isolates B273 and B280 is variant A6 and the genotype of isolate B259 is variant A7. A previous study reported that the A, B, and C genogroups contain independent species (8).

The *pla* amplicons (GenBank accession nos. KF214264–KF214266) had 98% sequence identity with *Y. pestis* reference sequences. Plague prevalence was higher in *H. perenensis* rats than in *R. rattus* rats ($p < 0.05$). Infected rodents were found in all villages studied except Yomentoni, and prevalence in Aguas Calientes was higher than in Alto Ivochote ($p < 0.01$). Two (25%) of 8 rodents trapped inside houses were infected with *Y. pestis*.

This study suggests that infections of rodents with *Bartonella* spp. and *Y. pestis* are common and widespread throughout the Echarate District. It also shows the role of *H. perenensis* and *Oecomys* spp. rodents as reservoirs of both pathogens. This role was confirmed by amplifying the chromosomal ferric iron uptake regulation gene by using PCR primers Yp1 and Yp2, as described by Hinnebusch et al. (9). The epidemiologic role of rodent-borne *Bartonella* spp. as a cause of disease in humans is emerging in the Americas. This role has been suggested by identification of a novel rodent-associated *Bartonella* strain causing febrile illness in the

Table. Prevalence of *Bartonella* spp. and *Yersinia pestis* in rodents from Echarate District, Cusco, Peru

| Study area | Rodent host species (no.) | No. (%) positive for <i>Yersinia pestis</i> | No. (%) positive for <i>Bartonella</i> spp. |
|-----------------|---------------------------------|---|---|
| Alto Ivochote | <i>Rattus rattus</i> (20) | 3 (15) | 0 (0) |
| Alto Ivochote | <i>Hylaeamys perenensis</i> (1) | 0 | 1 (100) |
| Aguas Calientes | <i>Hylaeamys perenensis</i> (2) | 2 (100) | 2 (100) |
| Aguas Calientes | <i>Oecomys</i> spp. (1) | 1 (100) | 1 (100) |
| Yomentoni | <i>Rattus rattus</i> (4) | 0 | 1 (25) |
| Total | (28) | 6 (21.4) | 5 (17.9) |

rural southwestern United States (10) and a strain of pathogenic *B. elizabethae*, a bacteria that can cause human endocarditis, in the Huayllacallán Valley in Peru (3).

Because most identified *Bartonella* spp. have been reported as infectious agents for humans, our results should prompt public health concern. However, our findings require further investigation about the pathogenicity of these *Bartonella* genotypes. The detection of both pathogens in intradomestic and peridomestic areas where humans are in close contact with rodents could indicate that the incidence of both diseases in humans from Echarate District might be underestimated.

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**Aarón Martín-Alonso,
Mayday Soto, Pilar Foronda,
Elsa Aguilar,
Guillermo Bonnet,
Rosa Pacheco,
Basilio Valladares,
and María A. Quispe-Ricalde**

Author affiliations: University of La Laguna, Canary Islands, Spain (A. Martín-Alonso, P. Foronda, G. Bonnet, B. Valladares, M. A. Quispe-Ricalde); and National University of San Antonio Abad, Cusco, Peru (M. Soto, E. Aguilar, R. Pacheco)

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Address for correspondence: Pilar Foronda, Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Avda. Fco. Sanchez s/n, 38203, Tenerife, Canary Islands, Spain; email: pforonda@ull.edu.es

Buruli Ulcer Disease in Republic of the Congo

To the Editor: Buruli ulcer, which is caused by the *Mycobacterium ulcerans* bacterium, is a severe disabling necrotic disease of the skin, occurring mainly in swampy rural areas of western and central Africa. This tropical disease is neglected, despite being the third most common mycobacterial disease of humans, after tuberculosis and leprosy. The disease has become substantially more frequent over the past decade, particularly around the Gulf of Guinea, and has been detected or suspected in at least 31 countries (1). Clinical diagnosis of Buruli ulcer disease should be confirmed by PCR, as recommended by the World Health Organization (WHO); and case-patients should be treated with rifampin/streptomycin daily for 8 weeks (therapy available since 2004), combined, if necessary, with surgery.

Although confirmed cases of Buruli ulcer disease have been reported in all countries neighboring the Republic of the Congo (hereafter called Congo) (2–4), only 1 report of a confirmed case in Congo has been published (5) (Figure, panel A). During 2007–2012, a total of 573 clinical cases of Buruli ulcer disease were reported to WHO by the National Leprosy, Buruli Ulcer and Yaws Control Program in Congo. We report 108 cases (19% of all cases reported) that

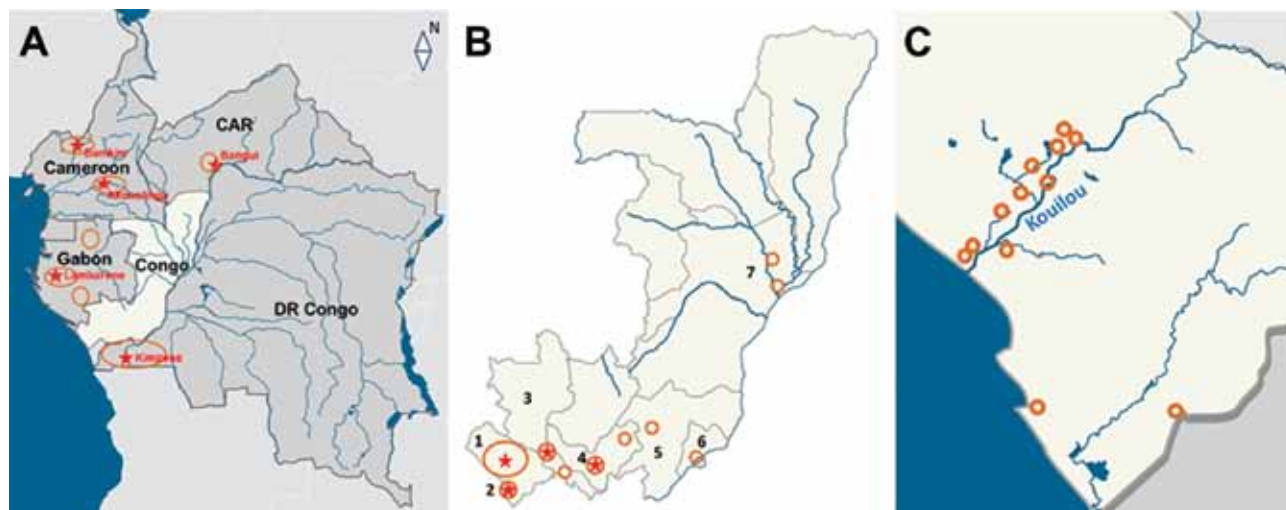


Figure. Buruli ulcer–endemic areas in the Republic of the Congo (RC) and neighboring countries. A) Buruli ulcer cases have been reported in all countries neighboring RC. CAR, Central African Republic; DR Congo, Democratic Republic of the Congo. B) RC (representing the white area in panel A). The numbers indicate the 7 departments or communes (of 12 total) where PCR-positive cases of Buruli ulcer disease were diagnosed. 1, Kouilou Department; 2 Pointe Noire Commune; 3, Niari Department; 4, Bouenza Department; 5, Pool Department; 6, Brazzaville Commune; 7, Cuvette Department. C) Kouilou Department (department 1 in panel B). Most Buruli ulcer case-patients from Kouilou Department were living close to the Kouilou River. Stars indicate locations of health centers that treat Buruli ulcer disease; circles indicate areas where persons with Buruli ulcer disease were identified. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/20/6/13-1498-F1.htm)

were confirmed, in accordance with WHO recommendations, by quantitative PCR, the most sensitive and specific testing method available (6).

The National Leprosy, Buruli Ulcer, and Yaws Control Program, with the support of the Raoul Follereau Foundation (Paris, France), performed passive and active surveillance of Buruli ulcer in Congo during 2007–2012. Fine-needle aspirate or swab samples were obtained from patients with suspected Buruli ulcer and sent to Angers University Hospital (Angers, France) for confirmation by quantitative PCR as described (6,7). Of the 283 samples analyzed, 114 (40%) from 108 different patients were PCR positive. Of the 114 PCR-positive samples, 20 (18%) were fine-needle aspirate samples and 94 (82%) were swab samples (at least 2 swabs/lesion). The 108 case-patients included 60 (56%) female and 48 (44%) male patients; 56% of the case-patients were <15 years of age. The most common clinical form of the disease (86% of cases) was the ulcerative stage with edema or plaque. All confirmed Buruli ulcer case-patients

were treated in accordance with WHO recommendations (8): antibiotic treatment (rifampin/streptomycin) plus surgery if necessary. All patients with nonconfirmed cases were treated according to the alternative diagnosis reached by the clinician.

Our findings show that Buruli ulcer disease affects persons in several of Congo's administrative divisions (Figure, panel B); of the 108 patients, 77 (71%) were from Kouilou Department (Figure, panel C). The village of residence was recorded for 55 of these 77 patients, 46 (84%) of whom lived in 9 villages along the Kouilou River, encompassing an area of $\approx 50 \text{ km} \times 20 \text{ km}$: Madingo-Kayes, Kanga, Loukouala, Mfilou, Koubotchi, Mboukoumassi, Tchisseka, Magne, and Loaka villages. This disease-endemic area includes 2 lakes, Dinga and Nanga, both of which are fed by the Kouilou River. The remaining 31 (29%) confirmed patients (i.e., those not living in Kouilou Department) lived in Niari Department (9%), Bouenza Department (6.5%), Pool Department (3%), or Cuvette Department (5.5%) or in Pointe

Noire Commune (2%) or Brazzaville Commune (3%) (Figure, panel B).

The distribution of Buruli ulcer cases in Congo is unusual. The Kouilou River region was most affected, but several other areas, all in southern Congo, have confirmed Buruli ulcer patients. Cuvette Department is the 1 exception; although it is in northeastern Congo, this department did have a cluster of cases. The cases in Cuvette were identified (and the infections were diagnosed and treated) during active research into Buruli ulcer during 2009–2010. (Note that there has been no survey in this region since 2010.)

Buruli ulcer is also endemic in some areas of the countries neighboring Congo. In the Democratic Republic of the Congo, the disease is highly endemic in the Bas Congo region, which shares a border with departments in southern Congo where the disease is endemic (9). By contrast, the small cluster of cases diagnosed in Cuvette Department in northeastern Congo seems to be isolated from other areas where the disease is known to be endemic.

M. ulcerans is known to be associated with wetlands, and the Kouilou River environment is certainly suitable for its spread (10). Identification of this zone as a high-risk area for Buruli ulcer disease will help the Ministry of Health improve early detection, biological confirmation, and treatment programs. In the other regions, active and continuous surveillance is necessary to establish a detailed map of the villages and areas where Buruli ulcer disease is endemic; such information would enable the implementation of targeted control activities. However, active surveillance in Congo has substantially declined since 2011. Our findings support the reactivation of such surveillance campaigns to ensure the early identification and confirmation of Buruli ulcer cases and to improve patient management.

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**Estelle Marion, Damas Obvala,
Jeremie Babonneau,
Marie Kempf,
Kingsley B. Asiedu,
and Laurent Marsollier**

Author affiliations: Fondation Raoul Follereau, Pobè, Bénin (E. Marion); Centre Hospitalier Universitaire d'Angers, Angers, France (E. Marion, J. Babonneau, M. Kempf, L. Marsollier); INSERM, Angers (E. Marion, J. Babonneau, L. Marsollier); Ministère de la Santé, Brazzaville République du Congo (D. Obvala); and World Health Organization, Geneva, Switzerland (K.B. Asiedu)

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Address for correspondence: Estelle Marion, ATOMycA, INSERM Avenir Team, U892, CHU Angers, France; email: stel.marion@yahoo.fr

Rapid Metagenomic Diagnostics for Suspected Outbreak of Severe Pneumonia

To the Editor: Recent outbreaks of severe pneumonia or acute respiratory distress syndrome (ARDS) have attracted much public interest. Given current awareness levels, clinical personnel and health officials must rapidly and adequately respond to suspected outbreaks to prevent public disturbances. We report a case that highlights the potential of next-generation sequencing (NGS) to complement conventional diagnostics in such scenarios.

On March 29, 2013, a police officer (patient 1) was admitted to the emergency department of the University Medical Centre Hamburg-Eppendorf in Hamburg, Germany, because of ARDS. The patient was given mechanical ventilation; all diagnostic test results for pathogens commonly known to cause pneumonia were negative (www.virus-genomics.org/supplementaries/EID1406.pdf). Although treatment with antimicrobial drugs was immediately initiated, the patient died 6 days later of multiple organ failure.

On April 5, a second police officer (patient 2) from the same county was admitted to the same emergency department because of ARDS. He was transferred to the intensive care unit and given mechanical ventilation. Similar to the situation for patient 1, diagnostic test results were negative, prompting the news media to suspect an outbreak of a novel or mutated virus (1,2). Especially because of simultaneous outbreaks of avian influenza and infections with Middle East respiratory syndrome coronavirus in other parts of the world, these reports caused serious concern among the public and health officials.

After the death of patient 1 and hospitalization of patient 2, we subjected

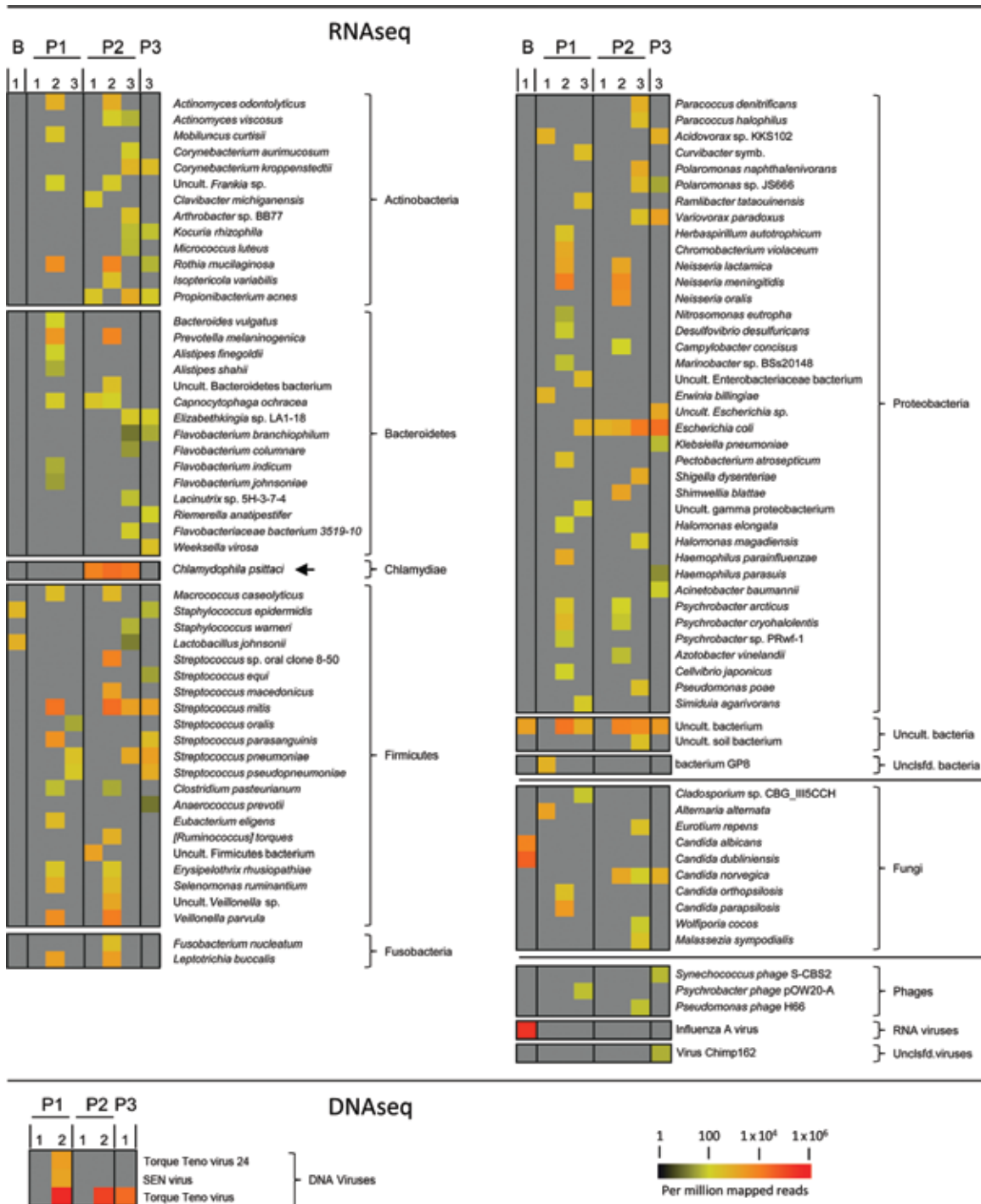


Figure. Next-generation sequencing of RNA (RNaseq) and DNA (DNaseq) isolated from bronchoalveolar lavage (BAL) samples from 3 patients with severe pneumonia, northern Germany. Shown are data from BLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis of de novo assembled sequence contigs (www.virus-genomics.org/supplementals/EID1406.pdf). Relative abundance of contig reads mapping bacterial, fungal, or viral species is indicated by a heat map (scale bar). White areas indicate that no reads were detected. Diagnostic samples were obtained from 3 patients (lanes P1, P2, and P3). Lane B, control BAL sample (analyzed by using RNaseq only) from an influenza patient; lane 1, MS: analysis on the Illumina MiSeq platform (www.illumina.com/systems.ilmn); lane 2, HS: analysis on the Illumina HiSeq platform (www.illumina.com/systems.ilmn); lane 3, HS dpl., RNA samples depleted of human rRNA before analysis on an HiSeq instrument. *Chlamydia psittaci*, which was unequivocally detected in all samples from patient 2 but not in samples from the other patients, is indicated by an arrow. Symb., symbiont; Uncult., uncultured; Unclsfd., unclassified; SEN virus, strain of Torque teno virus.

nucleic acids extracted from bronchoalveolar lavage (BAL) specimens from both patients to NGS by using a MiSeq sequencer ([www.illumina.com/systems.illumina](http://www.illumina.com/systems/illumina)). To enable rapid and unbiased detection of bacterial and viral agents, we did not enrich specific sequences. The entire workflow (www.virus-genomics.org/supplementaries/EID1406.pdf) was completed within 50 hours.

First-line analysis clearly identified *Chlamydophila psittaci* in the RNA sample from patient 2, but no sequences of obvious pathogenic origin were detected in samples from patient 1 (Figure). *C. psittaci*, an intracellular bacterium, can be transmitted by inhaling aerosolized secretions or feces from infected birds (3). Person-to-person transmission of this bacterium is rare (4,5). Ornithosis, a disease characterized by severe pneumonia and influenza-like symptoms, might develop in persons infected with this bacterium. Because of the rarity of the disease, standard diagnostic panels usually do not include *C. psittaci*. After 11 days of antimicrobial drug treatment, the condition of patient 2 improved, and the patient was transferred to a general hospital ward.

On April 29, a third police officer (patient 3) who had been sharing office space with patient 2, came to the hospital with symptoms of pneumonia. After antimicrobial drug treatment, his condition rapidly improved and the patient was discharged on day 7.

At admission of patient 3, samples from all 3 patients were analyzed at greater read depth by using a HiSeq system. RNA sequencing identified commensal bacteria in all samples, but *C. psittaci* was present only in samples from patient 2 (Figure). Likewise, subsequent high-throughput 16S rRNA sequencing (6), PCR, and serologic analysis unequivocally identified a *C. psittaci* infection in patient 2, but not in patients 1 or 3 (www.virus-genomics.org/supplementaries/EID1406.pdf).

We did not detect viral pathogens in any samples. At the DNA level, most nonhost reads originated from nonpathogenic single-stranded DNA anelloviruses (7). No RNA viruses were found, although influenza A(H3N2) virus was readily identified in a MiSeq analysis of a control BAL sample from a patient with a diagnosis of influenza A (PCR cycle threshold 32) (Figure). Furthermore, pairwise BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) did not reveal the presence of unknown sequence contigs that were shared among the patients, as would be expected in case of infection with a novel viral agent. Together with the confirmed *C. psittaci* infection in patient 2, the absence of a common pathogen signature strongly suggests that the cases were unrelated.

We used a comprehensive metagenomic approach to resolve cases suspected of representing an ongoing outbreak. The method used enabled diagnosis of a *C. psittaci* infection within a reasonable timeframe to allow for timely clinical intervention. These findings strongly suggest that NGS methods can complement conventional diagnostics (8–10) and also highlight their potential to aid clinical personnel and health agencies in making appropriate decisions during suspected outbreaks. Clearly, however, NGS-based methods will have to be further standardized and validated before their full potential in diagnostic settings can be realized.

The absence of pathogenic sequences in patients 1 and 3 might suggest that their clinical symptoms had noninfectious causes. However, although samples were collected during the acute phase of clinical symptoms, and despite our ability to detect an influenza A infection in controls, we cannot fully exclude the possibility that a potentially causative pathogen present at low levels might have evaded detection. Thus, systematic and correlative studies evaluating the sensitivity of NGS-based detection

methods in different diagnostic entities are urgently needed.

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**Nicole Fischer,¹ Holger Rohde,¹
Daniela Indenbirken,
Thomas Günther,
Kerstin Reumann,
Marc Lütgehetmann,
Thomas Meyer, Stefan Kluge,
Martin Aepfelbacher,
Malik Alawi,
and Adam Grundhoff**

Author affiliations: University Medical Centre Hamburg–Eppendorf, Hamburg, Germany (N. Fischer, H. Rohde, M. Lütgehetmann, T. Meyer, S. Kluge, M. Aepfelbacher, M. Alawi); German Center for Infection Research, Hamburg (N. Fischer, A. Grundhoff); and Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg (D. Indenbirken, T. Günther, K. Reumann, M. Alawi, A. Grundhoff)

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¹These authors contributed equally to this article.

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Address for correspondence: Adam Grundhoff, Research Group Virus Genomics, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Martinistrasse 52, Hamburg D-20251, Germany; email: adam.grundhoff@hpi.uni-hamburg.de

Respiratory Infection with Enterovirus Genotype C117, China and Mongolia

To the Editor: Enteroviruses (EVs) are small, nonenveloped viruses of the family *Picornaviridae* (1). EVs are classified into 12 species according to the molecular and antigenic properties of their viral capsid protein (VP1). To date, 7 species are known

to infect humans, including EV-A to EV-D and rhinovirus A, B, and C (www.picornastudygroup.com/taxa/species/species.htm)

EV-C117 was a newly found EV-C genotype. It was identified in a nasopharyngeal sample from a hospitalized child, 3 years and 9 months of age, with community-acquired pneumonia in Lithuania in 2012 (2,3). However, aside from this case, little is known about the prevalence and clinical significance of EV-C117. Here, we report the detection of EV-C117 in children in China and Mongolia with respiratory tract infections (RTIs).

During March 2007–March 2013, we screened for EV-C117 in respiratory samples from patients with RTIs in China and Mongolia, including nasopharyngeal aspirates collected from 3,108 children in China who had lower respiratory tract infections when they were admitted to Beijing Children's Hospital (4) and swab samples from 2,516 patients in Mongolia with influenza-like illness (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/6/13-1596-Techapp1.pdf). Respiratory viruses in samples from China were screened by using multiplex PCR and single PCR assays as described (4). Samples from Mongolia were screened by using the FTD Respiratory Pathogens Multiplex Assay Kit (Fast-track Diagnostics, Luxembourg City, Luxembourg). EV-positive samples were further genotyped by using reverse transcription PCR (RT-PCR) and primers sequentially targeting the VP1 region (5), the 5'-untranslated region (5'-UTR)/VP4/VP2 region (6) and the 5'-UTR (7). A 394-nt amplicon corresponding to the 5'-UTR of EVs was obtained from 10 children in China; a 598-nt amplicon corresponding to the 5'-UTR/VP4/VP2 region was obtained by RT-PCR from 5 children in Mongolia. Blastn analysis (www.blast.ncbi.nlm.nih.gov/Blast.cgi) of PCR amplicons showed that only amplicons detected in 2 children from China

(patients BCH096A and BCH104A) and 2 children from Mongolia (patients MGL126 and MGL208) had the highest similarity (95%–98%) to the EV-C117 prototype strain LIT22.

To further confirm that these 4 strains belong to EV-C117, we attempted to amplify the full-length viral genome sequences. However, we only obtained full-length viral genome sequences from the 2 strains found in patients from China (GenBank accession nos. JX560527 [patient BCH096A], and JX560528 [patient BCH104A], respectively). For the remaining 2 strains from Mongolia, MGL126 (5'UTR/VP4/VP2: KF726102; VP1: KF726100) and MGL208 (5'UTR/VP4/VP2: KF726103; VP1: KF726101), we obtained the sequence of the 5'-UTR/VP4/VP2 region and VP1 gene. Phylogenetic analysis of these sequences showed that they all belonged to genotype EV-C117 (Figure, panels A and B).

Virus isolation for EV-C117 by using Vero and H1-HeLa cells was unsuccessful. Through blastn and phylogenetic analyses, we also found that the previously identified EV-C strain HC90835 (EU697831, from Nepal) (8), EV-C104 strain CL-C22 (EU840734, EU840744, and EU840749, from Switzerland) (9) and a rhinovirus strain SE-10-028 (JQ417886, from South Korea), also belong to EV-C117 (Figure, panel A), indicating that EV-C117 is widely distributed geographically. Because a large proportion of EV infections are subclinical or mild (1), the prevalence of EV-C117 should be further estimated by using serologic investigations in general populations.

The VP1 sequences of the EV-C117 strains isolated in China and Mongolia were 89.9%–95.6% (nt) and 95.2%–98.3% (aa) identical to the EV-C117 prototype strain LIT22 (patient JX262382). Alignment analysis of amino acid sequences showed differences between strains isolated in this study and LIT22, i.e., Ser¹⁵ (strains

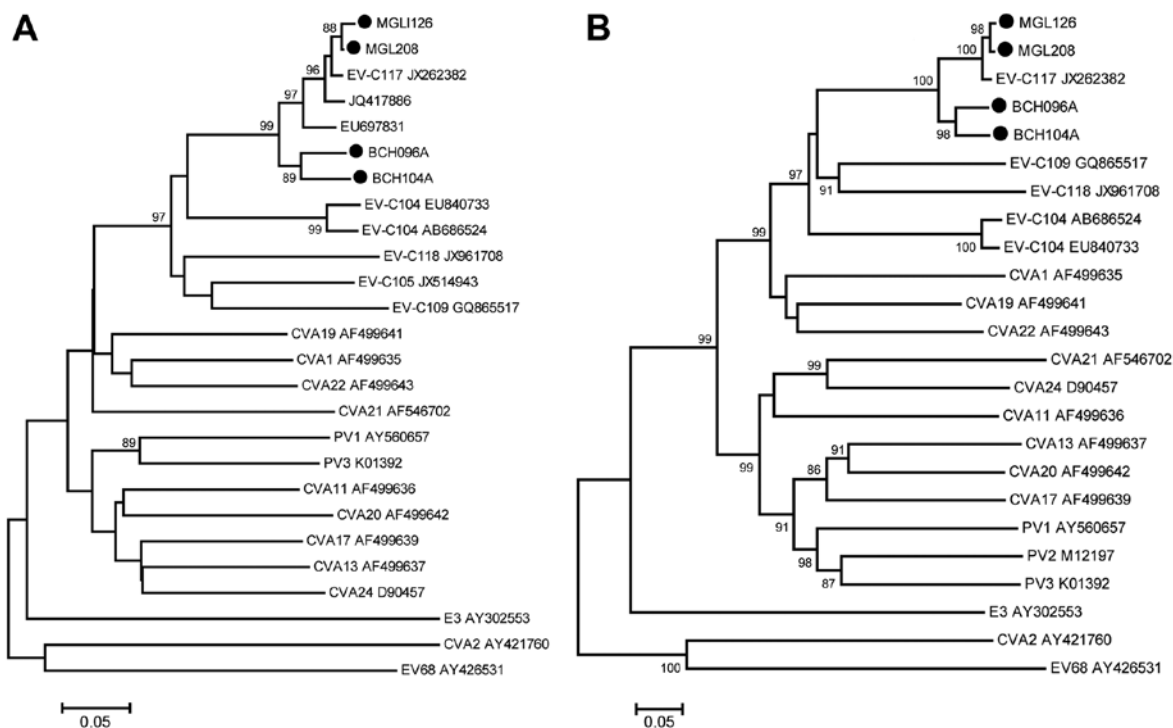


Figure. Phylogenetic analysis of enterovirus genotype C117 (EV-C117) based on nucleotide sequences. Phylogenetic trees were generated with 1,000 bootstrap replicates. Neighbor-joining analysis of the targeted nucleotide sequence was performed by using the Kimura 2-parameter model with Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (www.megasoftware.net). The EV-C117 strains identified in this study are indicated by black circles. Enterovirus 68, cocksackievirus (CV) A2, and echovirus (E) 3 (GenBank accession nos. AY426531, AY421760, and AY302553) were used as outgroups. PV, poliovirus. A) Phylogenetic analysis of the VP 4/VP2 region (399 nt, corresponding to nt 673–1,071 of EV-C117 prototype strain LIT22 [JX262382]). B) Phylogenetic analysis of the viral protein 1 region (888 nt, corresponding to nt 2416–3303, numbered according to the sequence of LIT22). Scale bars represent nucleotide substitutions per site.

in this study) versus Asn¹⁵ (LIT22). In addition, we found that the strains from patients in China contain Lys⁶³ and Ala⁹⁰, and those from Mongolia have Thr⁹³, Asn⁹⁷, and Ser²⁷⁶. The biological significance of these mutations is unknown.

Of these 4 EV-C117-positive children, 3 were hospitalized with respiratory disease (online Technical Appendix Table 2); the nonhospitalized child (MGL208) had a sore throat, but no other signs or symptoms. The viral loads of EV-C117 and co-detected viruses were quantified by using real-time PCR (methods available upon request), with a median EV-C117 load of 2.9×10^5 RNA copies/mL (range $1.1\text{--}4.8 \times 10^5$ RNA copies/mL [Technical Appendix Table 2]). EV-C117 was the only virus detected in patients

BCH104A and MGL126. Respiratory syncytial virus (5.0×10^6 copies/mL) and rhinovirus (1.5×10^5 copies/mL) were detected in patient BCH096A, and influenza virus A (IFVA, H3N2; 5.1×10^{10} copies/mL) and human bocavirus (3.7×10^2 copies/mL) were detected in patient MGL208.

The co-detection of viruses in 2 of the EV-C117-positive patients raises the question of what role EV-C117 plays in RTIs. However, it is notable that EV-C117 was the only virus detected in the other 2 patients. This finding indicates that, at least in patients with low resistance (patient BCH104A had severe bacterial infection before EV-C117 was detected and patient MGL126 had congenital heart disease), EV-C117 might be associated with RTIs. In addition, the strain

isolated in Nepal and the strain isolated in Switzerland, EV-C117, were both detected in specimens collected from patients with RTIs (8,9). Collectively, these data indicate the respiratory tropism of EV-C117. Additional epidemiologic and virologic studies on EV-C117 may be warranted to establish its role in RTIs.

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**Zichun Xiang,¹
Sosorbarmyn Tsatsral,¹
Chunyan Liu,¹ Linlin Li, Lili Ren,
Yan Xiao, Zhengde Xie,
Hongli Zhou, Guy Vernet,
Pagbajabyn Nymadawa,
Kunling Shen,
and Jianwei Wang**

Author affiliations: MOH Key Laboratory of Systems Biology of Pathogens, Beijing, China; (Z. Xiang, L. Li, L. Ren, J. Wang); Institute of Pathogen Biology, Beijing (Z. Xiang, L. Li, L. Ren, Y. Xiao, H. Zhou, J. Wang); National Center of Communicable Diseases, Ulaanbaatar, Mongolia (S. Tsatsral, P. Nymadawa); Beijing Children's Hospital Affiliated to Capital Medical University, Beijing (C. Liu, Z. Xie, K. Shen); Fondation Mérieux, 69365 Lyon, France (G. Vernet); and Mongolian Academy of Medical Sciences, Ulaanbaatar (P. Nymadawa)

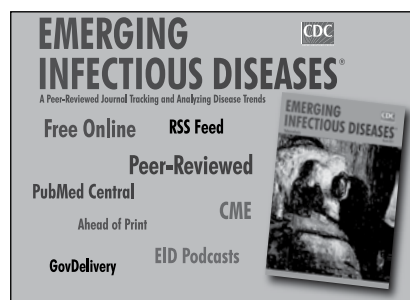
Address for correspondence: Jianwei Wang, 9# Dong Dan San Tiao, Dongcheng District, Beijing 100730, China; email: wangjw28@163.com

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Address for correspondence: Jianwei Wang, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, MOH Key Laboratory of Systems Biology of Pathogens and Christophe Mérieux Laboratory, IPB, CAMS-Fondation Mérieux, Institute of Pathogen Biology (IPB), Chinese Academy of Medical Sciences (CAMS), 9# Dong Dan San Tiao, Beijing 100730, China; email: wangjw28@163.com



Bufavirus in Feces of Patients with Gastroenteritis, Finland

To the Editor: For nearly 3 decades, human parvovirus B19 (B19V) was considered to be the only pathogenic parvovirus found in humans. Since 2005, several new human parvoviruses have been found, including human bocaviruses 1–4 and human parvovirus 4 (PARV4) (1–5), and during 2012, metagenomic analysis of fecal samples from children in Burkina Faso with acute diarrhea showed a highly divergent parvovirus, which was named bufavirus (BuV) (6). Its sequence in the coding region showed <31% similarity with known parvoviruses, the closest genera being *Protoparvovirus* and *Amdoparvovirus*. Subsequent studies, on the basis of PCR results, showed that 4% of fecal samples from Burkina Faso (n = 98) and 1.6% from Tunisia (n = 63) harbored either of 2 genotypes of this new virus, which belongs to the species *Primate protoparvovirus 1* of the genus *Protoparvovirus* (6,7; <http://ictvonline.org>).

To assess the occurrence of BuV in northern Europe, we analyzed 629 fecal samples from patients of all ages (median 51.5 years, range 0–99) in Finland who had gastroenteritis. To gain a more complete representation of BuV occurrence, we obtained samples retrospectively from routine diagnostics for bacterial and viral gastroenteritis-inducing pathogens (HUSLAB, Helsinki University Central Hospital Laboratory Division, Helsinki, Finland) and analyzed all samples available during the collection periods.

The samples originally sent to HUSLAB for bacterial diagnosis (bacterial cohort, n = 243) had been analyzed during October 2012–March 2013 for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia*

¹These authors contributed equally to this work.

spp., *Vibrio cholerae*, and *Escherichia coli* (subtypes enterohemorrhagica, enteropatogena, enterotoxigenic and enteroagregativa) by using culture or PCR (8). In 81 (33.3%) of the samples, ≥ 1 bacterial pathogen was found.

The samples originally sent for viral diagnosis (viral cohort, n = 386) had been tested in HUSLAB for norovirus during April–May, 2013 by using reverse transcription quantitative PCR (RT-qPCR)(HUSLAB in-house). Further diagnosis for rotavirus and adenovirus had been requested by physicians from 105 (27.2%) of 386 samples (Diarlex MB antigen detection assay, Orion Diagnostica, Espoo, Finland), and for astrovirus from 33 (8.6%) samples (RT-PCR, HUSLAB in-house). A viral pathogen was discovered in 141 (36.5%) samples; in 139, the pathogen was norovirus.

The samples had been sent from diverse locations within Finland, and thus were not from a few isolated outbreaks. No further information on patients and samples was available for either cohort, and not enough samples were left for retrospective analysis of additional pathogens. The Ethics Committee of the Hospital District of Helsinki and Uusimaa approved the study.

BuV DNA was detected by using a new real-time qPCR with the

following primers and probe: BuV forward, 5'-ACAGTGTAGACAGTG-GATCAAACCTT-3'; BuV reverse, 5'-GTTGTGGTTGGATTGTGGT-TAGTTC-3'; BuV qPCR probe, 5'-FAM-CGGAAGAGATTTTGA-CAGTGCYTAGCAA-BHQ1 3'. The detailed qPCR protocol is shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/6/13-1674-Techapp1.pdf). The analytical sensitivity of the RT-qPCR assay was 5–10 copies per reaction.

Of the 629 fecal samples, 7 (1.1%) were positive for BuV DNA, of which 4 were from the bacterial cohort and 3 from the viral cohort. BuV DNA quantity was low in all samples, ranging from 1.9×10^3 to 3.2×10^4 copies per milliliter of fecal supernatant (Table). In contrast to the original discovery of the virus in children with diarrhea (6), all positive samples were from adults (median age 53 years, range 21–89 years). All BuV DNA–positive results were confirmed by repeated BuV qPCR, by amplifying and sequencing another area of the virus, or by both methods (Table): all sequenced amplicons were more similar to the BuV genotype 1 (online Technical Appendix Figure) (6). Two of the BuV-positive samples were from the same patient, taken 4 days apart, and the latter sample also harbored norovirus. The additional 6 BuV-positive samples

were negative for the other viral or bacterial pathogens tested.

Seven fecal samples collected from adults in Finland contained BuV DNA, indicating that circulation of the virus is restricted neither to children nor to Africa. However, the low DNA loads in all the positive samples suggest that BuV might not be the primary cause of these cases of gastroenteritis. A known gastroenteritis-inducing pathogen (norovirus) was found in 1 of the 7 BuV-positive samples. We did not observe any clustering of the 7 positive samples into a specific period (Table).

Although the association with gastroenteritis seems weak, BuV might cause symptoms of other types. We did not include feces from healthy subjects for comparison. The identified BuV DNA in our samples could originate from previous or current infections unrelated to gastroenteritis, or be associated with prolonged virus secretion in the respiratory or digestive tracts, a phenomenon shown, e.g., for human bocavirus1 (9,10). Acquisition of the virus from a food source cannot be ruled out, although 1 patient harbored the DNA for at least 4 days, during which a 10-fold increase in viral load was observed.

Overall, this study shows that BuV circulates in northern Europe and can be found in the feces of patients with

Table. Samples collected for bacterial and viral testing that were subsequently positive for bufavirus DNA*

| Sample no. | Sample cohort | Quantity, copies/mL supernatant | Age, y/sex | Pathogens tested for by HUSLAB† | Other pathogens found | Sampling date | Sequenced region, nt, divergence (%) from JX027295‡ |
|------------|---------------|---------------------------------|------------|---------------------------------|-----------------------|---------------|---|
| 1 | Bacterial | 5.2×10^3 | 21/M | Bacteria | 0 | 2012 Dec 4 | VP2, 2786–4495, 0.88 |
| 2 | Bacterial | 1.9×10^4 | 38/M | Bacteria | 0 | 2013 Jan 6 | VP2, 2786–4495, 0.71 |
| 3§ | Bacterial | 1.9×10^3 | 53/M | Bacteria | 0 | 2013 Jan 11 | § |
| 4 | Bacterial | 3.7×10^3 | 46/M | Bacteria | 0 | 2013 Apr 27 | VP2, 2786–4495, 0.76 |
| 5 | Viral | 3.4×10^3 | 77/M | Norovirus | 0 | 2013 Apr 19 | VP2, 2786–4495, 1.60 |
| 6¶ | Viral | 3.6×10^3 | 89/F | Norovirus | 0 | 2013 Apr 20 | Partial NS, 16–1080, 1.13 |
| 7¶ | Viral | 3.2×10^4 | 89/F | Norovirus | Norovirus | 2013 Apr 23 | VP2, 2786–4495, 1.36 |

*VP2, viral protein 2; NS, nonstructural.

†Samples originally sent to HUSLAB (Helsinki, Finland) for bacterial diagnosis were analyzed for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Vibrio cholerae*, and *Escherichia coli* (subtypes enterohemorrhagic, enteropatogena, enterotoxigenic, and enteroagregativa) by using culture or PCR. Bufavirus-positive samples could not be analyzed for the presence of pathogens other than those originally tested for because the samples had been discarded.

‡Sequence divergence analyzed by using the DNA distance matrix in BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). The bufavirus sequences were submitted to GenBank (accession nos. KJ461874–KJ461879).

§This sample was positive for bufavirus by quantitative PCR. However, we were not able to amplify another region of the virus from this sample, likely caused by a low amount of the virus in the sample, which had the lowest copy number among the positive samples.

¶Samples from the same patient, collected 4 days apart.

gastroenteritis. Despite the absence of known pathogens among 6 of 7 BuV-shedding patients, the causative role of BuV in gastroenteritis remains uncertain. Serologic studies will help clarify a possible association between BuVs and diarrhea or other diseases.

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**Elina Väisänen, Inka Kuisma,
Tung G. Phan, Eric Delwart,
Maija Lappalainen,
Eveliina Tarkka, Klaus Hedman,
and Maria Söderlund-Venermo**

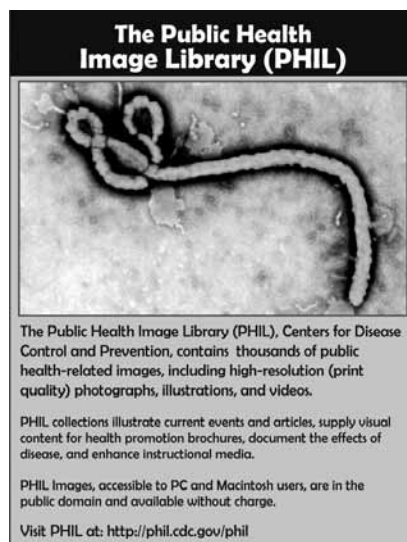
Author affiliations: Faculty of Medicine, University of Helsinki, Helsinki, Finland (E. Väisänen, I. Kuisma, K. Hedman, M. Söderlund-Venermo); Blood Systems Research Institute, San Francisco, California, USA (T.G. Phan, E. Delwart); University of California, San Francisco (T.G. Phan, E. Delwart); and Helsinki University Central Hospital, Helsinki (M. Lappalainen, E. Tarkka, K. Hedman)

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Address for correspondence: Elina Väisänen, University of Helsinki, Haartman Institute: Virology; Haartmaninkatu 3, Helsinki 00290, Finland; email: elina.vaisanen@helsinki.fi



Human Granulocytic Anaplasmosis Acquired in Scotland, 2013

To the Editor: Human granulocytic anaplasmosis is a tick-borne disease caused by *Anaplasma phagocytophilum*, an obligate intracellular gram-negative bacterium that infects granulocytes. The usual clinical signs and symptoms include nonspecific fever, chills, headache, and myalgia. Infection is usually mild or asymptomatic, but severe systemic complications can occur, leading to a need for intensive care and estimated fatality rates of 0.5%–1.0% (1,2).

A. phagocytophilum was first described in 1932 in Scotland as the causative agent of tick-borne fever in sheep (3). Although some clinical cases of human granulocytic anaplasmosis have been reported in Europe, mostly from Slovenia, Sweden, and Poland (4), most cases have occurred in the United States. This difference cannot be explained by the prevalence of the pathogen in ticks or human exposure to the pathogen because the 3% prevalence of *A. phagocytophilum* among *Ixodes ricinus* ticks in Europe seems to be nearly as high as that among ticks in the United States (2). The median seroprevalence rate for *A. phagocytophilum* infection among humans in Europe is 6.2%, reaching up to 21% (2). This incongruence between seroprevalence rate and number of human cases might be associated with underdiagnosis of cases (2), a high rate of asymptomatic disease (5), or cross-reactivities in serologic tests that might lead to overestimation of seroprevalence rate (5).

In August 2013, an immunocompetent 40-year-old man sought treatment for fever ($\approx 39^{\circ}\text{C}$) and other nonspecific symptoms such as malaise, myalgia, and severe headache 3 days after becoming aware of several tick bites received while on a hiking

vacation in Scotland. The man had removed the 3 tick nymphs from his legs immediately after their discovery and stored them in a plastic container; they were later sent to the Consultant Laboratory for Tick-borne Encephalitis in Berlin, Germany, for analysis. When the patient returned to Germany, 5 days after the onset of symptoms and 8 days after tick removal, a blood sample was collected (sample 1) and the bite sites were swabbed with a sterile cotton bud. By that time, the fever was gone, but malaise and other symptoms persisted. The patient began taking doxycycline, and within 2 days all symptoms subsided and the patient recovered completely.

A second blood sample was collected 28 days after tick removal (sample 2). Complete blood counts and chemistry panels were performed for both samples. All values were within the reference range except that for lactate dehydrogenase (248 U/L), which was moderately increased over the baseline value of <245 U/L in sample 1. Values did not differ substantially between the 2 samples.

DNA from whole-blood samples and swabs was extracted (QIAamp DNA Blood Mini Kit; QIAGEN, Hilden, Germany) and tested for *A. phagocytophilum*, *Babesia* spp., *Borrelia* spp., and *Rickettsia* spp. by using commercially available rapidSTRIFE assays for *Anaplasma*, *Babesia*, *Borrelia*, *Rickettsia* (all Analytik Jena AG; Jena, Germany). DNA extracted from blood and swab samples was negative for all tested pathogens.

After the tick specimens were taxonomically identified as *I. ricinus*, DNA/RNA was extracted (blackPREP Tick DNA/RNA Kit; Analytik Jena AG) and tested for the same pathogens. All 3 ticks were negative for *Babesia* spp., *Borrelia* spp., and *Rickettsia* spp., but 2 were positive for *A. phagocytophilum*.

Indirect immunofluorescence assays (Focus Diagnostics, Cypress, CA, USA) performed on the paired

serum samples revealed an increased *A. phagocytophilum*-specific IgM titer, from 20 at 5 days after symptom onset to 80 at 20 days later; the *A. phagocytophilum*-specific IgG titer rose from a high titer of 800 to >3,200 over this period.

The presence and 4-fold increase of *A. phagocytophilum*-specific IgM and IgG in paired serum samples confirmed the diagnosis of human granulocytic anaplasmosis in accordance with Centers for Disease Control and Prevention criteria (6). As described previously for several cases of human granulocytic anaplasmosis, patient blood counts were within reference limits but serum lactate dehydrogenase level was elevated (7). The diagnosis was further corroborated by detection of *A. phagocytophilum* DNA in 2 of the 3 ticks removed from the patient's skin. PCR amplification failed to detect *A. phagocytophilum* DNA in the patient's blood, consistent with previous studies documenting frequent lack of *A. phagocytophilum* DNA detection in whole blood and a substantial drop in PCR positivity after the acute phase of illness (8).

Human granulocytic anaplasmosis is not usually reported in Scotland like it is in the rest of Europe. The case originated from an area with long-established disease occurrence in ruminants, but the literature reports only 1 case of human infection in southwestern Scotland (9), ≈500 km from where this infection was probably acquired. Correct diagnosis would have been difficult had the patient not conserved the ticks and contacted the Consultant Laboratory for Tick-borne Encephalitis immediately after returning to Germany. A large number of human granulocytic anaplasmosis cases might be missed because general practitioners may not be aware of the pathogen's existence or its distribution.

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**Peter Hagedorn, Maren Imhoff,
Christian Fischer,
Cristina Domingo,
and Matthias Niedrig**

Author affiliations: Robert Koch-Institut, Berlin, Germany (P. Hagedorn, M. Imhoff, C. Domingo, M. Niedrig); and Charité-Universitätsmedizin Berlin, Berlin (C. Fischer)

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Address for correspondence: Peter Hagedorn, Robert Koch Institute, Nordufer 20, 13353, Berlin, Germany; email: hagedornP@rki.de

Genetic Relatedness of Dolphin Rhabdovirus with Fish Rhabdoviruses

To the Editor: Rhabdoviruses are enveloped, single-stranded, negative-sense RNA viruses that comprise a large and diverse family in the order *Mononegavirales* and infect arthropods, plants, fish, and mammals. There are 9 genera of rhabdoviruses: *Cytorhabdovirus*, *Ephemerovirus*, *Lysavirus*, *Novirhabdovirus*, *Nucleorhabdovirus*, *Perhabdovirus*, *Sigmavirus*, *Tibrovirus*, and *Vesiculovirus*. In addition, a substantial number of plant, vertebrate, and invertebrate rhabdoviruses have not been classified (1). Three genera (*Novirhabdovirus*, *Perhabdovirus*, and *Vesiculovirus*) comprise members that infect fresh water and marine fish (2). Fish rhabdoviruses pose a serious problem for aquaculture because of worldwide outbreaks of disease caused by novirhabdoviruses, perhabdoviruses, and vesiculoviruses (3,4).

In 1992, a rhabdovirus-like virus was isolated from lung and kidney of a white-beaked dolphin (*Lagenorhynchus albirostris*) that had beached along the coast of the Netherlands (5). Although no macroscopic or microscopic lesions were observed at necropsy, negative contrast electron microscopy showed typical rhabdovirus-like, bullet-shaped particles in

Vero cell cultures that showed a focal cytopathic effect (5). After this rhabdovirus-like virus was injected intracerebrally into brains of 1-day-old suckling mice, they died within 5 days (5). We report genetic and phylogenetic characterization of a dolphin rhabdovirus (DRV) and evaluated the seroprevalence of DRV-neutralizing antibodies by using serum samples from various marine mammals collected during a 10-year period (2003–2012).

To characterize DRV, we performed random sequence amplification and deep sequencing with the 454 GS Junior Instrument (Roche, Basel, Switzerland) with DRV-infected Vero cell supernatants as described (6). From this analysis, we determined the complete coding sequence of DRV covered by 42,080 of 49,292 reads (minimum coverage 4 reads, average coverage 872 reads).

Genomic termini of DRV were determined by using a 3' and 5' rapid amplification of cDNA ends PCR and Sanger sequencing of obtained PCR amplicons. The complete genome of DRV (GenBank accession no. KF958252) consists of 11,141 nt and has a typical rhabdovirus gene arrangement of 5 major open reading frames (ORFs) in the order 3'-nucleoprotein (N), phosphoprotein (P), matrix (M) protein, glycoprotein (G), and large (L) protein-5' (Figure, panel A, Appendix, wwwnc.cdc.gov/EID/article/20/6/13-1880-F1.htm). No additional ORFs ≥ 300 nt were detected. Between the major ORFs of DRV, intergenic sequences were present that ranged in size from 34 (P–M) to 83 (G–L) nucleotides. Putative transcription initiation and transcription termination polyadenylation sequences were AAC(A/G/U) and AUGA_n, respectively.

The deduced amino acid sequence of genes of DRV and several other rhabdoviruses were aligned by using MUSCLE in MEGA5 version 5.2 (7). Ambiguous aligned regions

were removed by using the Gblocks program (8). Phylogenetic analysis of the L and G genes was performed by using the neighbor-joining method in MEGA5 (7). This analysis showed that DRV is most closely related to fish rhabdoviruses of the genera *Perhabdovirus* and *Vesiculovirus* and unassigned fish rhabdoviruses with strong bootstrap support (Figure, panels B–D, Appendix).

Deduced amino acid sequences of the 5 major genes had the highest, although weak, homology with those of various fish rhabdoviruses by pairwise identity analyses: N (48%) with hybrid snakehead virus (HSHV), *Monopterus albus* rhabdovirus (MARV), and *Siniperca chautsi rhabdovirus* (SCRV); P (18%–20%) with eel virus European X (EVEX), HSHV, MARV, and SCRV; M (27%–33%) with lake trout rhabdovirus, Swedish sea trout rhabdovirus, and EVEX; G (30%–32%) with perch rhabdovirus, lake trout rhabdovirus, Swedish sea trout rhabdovirus, HSHV, MARV, SCRV, and EVEX; and L (54%–56%) with perch rhabdovirus, HSHV, and EVEX. This close relationship with fish rhabdoviruses is surprising because DRV was isolated from tissues of a mammal and propagated in mammalian cell lines at 37°C, which does not occur with related viruses isolated from fish.

To evaluate whether DRV or related viruses circulate among species of cetaceans, we performed serologic screening by using a virus neutralization assay as described (5). The specificity of this assay was tested by using a panel of rhabdovirus-specific antisera obtained from cetaceans of various species (5). The serum samples had been collected for diagnostic purposes from mainly juvenile cetaceans stranded along the coast of the Netherlands during 2003–2012. These species included 2 Atlantic white-sided dolphins (*Lagenorhynchus acutus*), 79 harbor porpoises (*Phocoena phocoena*), 9 striped dolphins (*Stenella*

coeruleoabla), and 6 white-beaked dolphins (*Lagenorhynchus albirostris*). Serum samples from 145 bottlenose dolphins (*Tursiops truncatus*) from the collection of the Dolphinarium Harderwijk (Harderwijk, the Netherlands) were also tested. DRV-neutralizing antibodies were detected in serum samples from 1 bottlenose dolphin (7%), 5 striped dolphins (55%), 1 white-beaked dolphin (17%), and 3 harbor porpoises (4%). These results suggested that DRV or closely related viruses continue to infect members of cetacean species (6).

Although rhabdovirus evolutionary pathways are complicated (9), our analysis suggests that DRV is a possible derivative of fish rhabdoviruses. DRV might have originated from an unidentified fish rhabdovirus and might cycle between fish and marine mammals, similar to that suggested for cycling of vesicular stomatitis virus between arthropods and terrestrial mammals (10). Future analyses of sequences from other marine mammal rhabdovirus sequences might support the validity of our phylogenetic analysis and result in creation of a new group containing marine mammal rhabdoviruses.

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**Jurre Y. Siegers,
Marco W.G. van de Bildt,
Cornelis E. van Elk,
Anita C. Schürch, Noël Tordo,
Thijs Kuiken, Rogier Bodewes,
and Albert D.M.E. Osterhaus**

Author affiliations: Erasmus Medical Centre, Rotterdam, the Netherlands (J.Y. Siegers, M.W.G. van de Bildt, C.E. van Elk, A.C. Schürch, T. Kuiken, R. Bodewes, A.D.M.E. Osterhaus); Dolphinarium Harderwijk, Harderwijk, the Netherlands (C.E. van Elk);

SOS Dolphin Foundation, Harderwijk, the Netherlands (C.E. van Elk); Institut Pasteur, Paris, France (N. Tordo); and Viroclinics Biosciences, Rotterdam, the Netherlands (A.D.M.E. Osterhaus)

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Address for correspondence: Rogier Bodewes, Department of Viroscience, Erasmus Medical Centre, Dr. Molewaterplein 50, 3015GE Rotterdam, the Netherlands; email: r.bodewes@erasmusmc.nl

Genetic and Ecologic Variability among *Anaplasma phagocytophilum* Strains, Northern Italy

To the Editor: The tick-borne pathogen *Anaplasma phagocytophilum* is an increasing potential public health threat across Europe. Its intraspecific genetic variability is associated with different reservoir host and vector tick species (1–4); however, the roles of various vertebrates as competent reservoirs of *A. phagocytophilum* in Europe need clarification (1). During March 2011–June 2013, we studied the prevalence and genetic variability of *A. phagocytophilum* in 821 questing *Ixodes ricinus* ticks (155 adults [A], 666 nymphs [N] collected by standard blanket dragging) and 284 engorged ixodid ticks (61A, 191N, 21 larvae [L]) collected from humans, dogs, sheep, hunted wild ungulates, live-trapped birds, and rodents. Blood samples from 1,295 rodents (yellow-necked mice [*Apodemus flavicollis*]), bank voles [*Myodes glareolus*], and harvest mice [*Moscardinus avellanarius*]) were also analyzed. All animal-handling procedures and ethical issues

The phylogenetic trees for *groEL* (Figure, panel A) and *msp4* (Figure, panel B) loci have similar topologies with strong support for 2 main clades (Figure, panels A and B), each with different host and vector association. The first clade (clade 1) contained sequences from questing *I. ricinus* ticks and engorged ticks collected from humans, dogs, wild ungulates, rodents, sheep, and birds. Our findings suggest that humans are exposed to several *A. phagocytophilum* genotypes exclusively from clade 1 (Figure, panels A and B). Our 3 unique *A. phagocytophilum* sequences were from 3 *I. ricinus* nymphs that fed on the same human clustered within this clade, but no clinical symptoms were observed.

The second clade (clade 2) includes sequences from rodents, specifically, bank voles (*M. glareolus*), other voles and shrews. Among tick species we found *I. persulcatus* to belong to this clade (Figure, panels A and B). We have found no evidence of circulation of this genotype in other hosts or in questing or engorged *I. ricinus* ticks in previously published data or in this study (Figure, panels A and B, clade 2). This finding suggests that the *A. phagocytophilum* genotype associated with mice, voles, and shrews in Europe may be maintained in enzootic cycles by another tick vector, such as *I. trianguliceps*, as observed in the UK for the field vole (*Microtus agrestis*) (8). This so-called ecologic strain probably does not represent an immediate threat to humans in northern Italy, unlike the rodent strain reported in the USA, since it occurs in very low prevalence, and because *I. trianguliceps* is an endophilic tick species that is unlikely to come into contact with humans.

In 1 questing *I. ricinus* tick at the nymphal stage, we detected a *groEL* sequence (KF031399) identical to a sequence isolated from humans with human granulocytic anaplasmosis in Europe (AF033101). The *msp4* sequence for the same sample (KF031406) belonged to clade 1, and

contained sequences of a strain found in 96 infected persons in the United States. This suggests that ≥ 1 human pathogenic strain now circulates in the investigated area. However, we did not find this strain in any of the host-fed ticks analyzed, so the host responsible for maintaining the circulation of this pathogenic strain must be identified before any recommendation for preventive measures can be provided.

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**Ivana Baráková,
Markéta Derdáková,
Giovanna Carpi, Fausta Rosso,
Margherita Collini,
Valentina Tagliapietra,
Claudio Ramponi,
Heidi C. Hauffe,
and Annapaola Rizzoli**

Author affiliations: Fondazione Edmund Mach, Trento, Italy (I. Baráková, Giovanna Carpi, F. Rosso, M. Collini, V. Tagliapietra,

H.C. Hauffe, A. Rizzoli); Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovak Republic (I. Baráková, M. Derdáková); Institute of Parasitology SAS, Košice, Slovak Republic (M. Derdáková); Yale School of Public Health, New Haven, CT, USA (G. Carpi); and Ospedale Santa Chiara, Trento (C. Ramponi)

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Address for correspondence: Anna Paola Rizzoli, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Trento, Italy; email: anna.paola.rizzoli@fmach.it

Zika Virus, French Polynesia, South Pacific, 2013

To the Editor: Isolated in 1947 from a rhesus monkey in Zika forest, Uganda, Zika virus (ZIKV) is a mosquito-borne flavivirus (1). For half a century, ZIKV was described only as causing sporadic human infections in Africa and Asia, which was mostly confirmed by serologic methods (2). In 2007, the first ZIKV outbreak reported outside Africa and Asia was retrospectively documented from biological samples of patients on Yap Island, Federated States of Micronesia, North Pacific, who had received an incorrect diagnosis of dengue virus (DENV) (3,4). We report here the early investigations that led to identification of ZIKV as the causative agent of an outbreak that started in October 2013 in French Polynesia.

French Polynesia is a French overseas territory located in the South Pacific. The ≈270,000 inhabitants live on 67 islands distributed into 5 archipelagoes (Society, Marquesas, Tuamotu, Gambier, and Austral Islands). Surveillance for acute febrile illnesses is coordinated by the Department of Health with the contribution of a sentinel network of public and private practitioners, the main public hospital (Centre Hospitalier

du Taaone), and the public health and research institute (Institut Louis Malardé [ILM]). As part of this syndromic surveillance system, ILM has implemented protocols for detecting arboviruses that are known to cause outbreaks in French Polynesia, such as DENV, or that pose a risk for causing epidemics because of the presence of potential mosquito vectors. In addition, ILM provides DENV serotype identification for other Pacific island countries, including Yap State, as part of the regional surveillance of dengue (5). For that reason, a ZIKV reverse transcription PCR (RT-PCR) protocol by Lanciotti et al. (3) was implemented at ILM.

In October 2013 (week 41), a 53-year-old woman (patient 1) and 2 other members of the household—her 52-year-old husband (patient 2) and

her 42-year-old son-in-law (patient 3)—experienced a mild dengue-like illness consisting of low fever (<38°C), asthenia, wrist and fingers arthralgia, headache, and rash. Patients 2 and 3 also had conjunctivitis. Patient 1 had swollen ankles and aphthous ulcers. For all 3 patients, results were negative for DENV by RT-PCR and nonstructural protein 1 (NS1) antigen tests (5), for West-Nile virus by RT-PCR, and for chikungunya virus by RT-PCR; results of RT-PCR for ZIKV were equivocal for patients 1 and 2. During week 43, a 57-year-old patient (patient 4) reported similar symptoms; results of RT-PCR for DENV were negative, but results of RT-PCR for ZIKV were positive. ZIKV infection was then confirmed by sequencing of the genomic position 858–1138 encompassing the prM/E protein coding regions of ZIKV

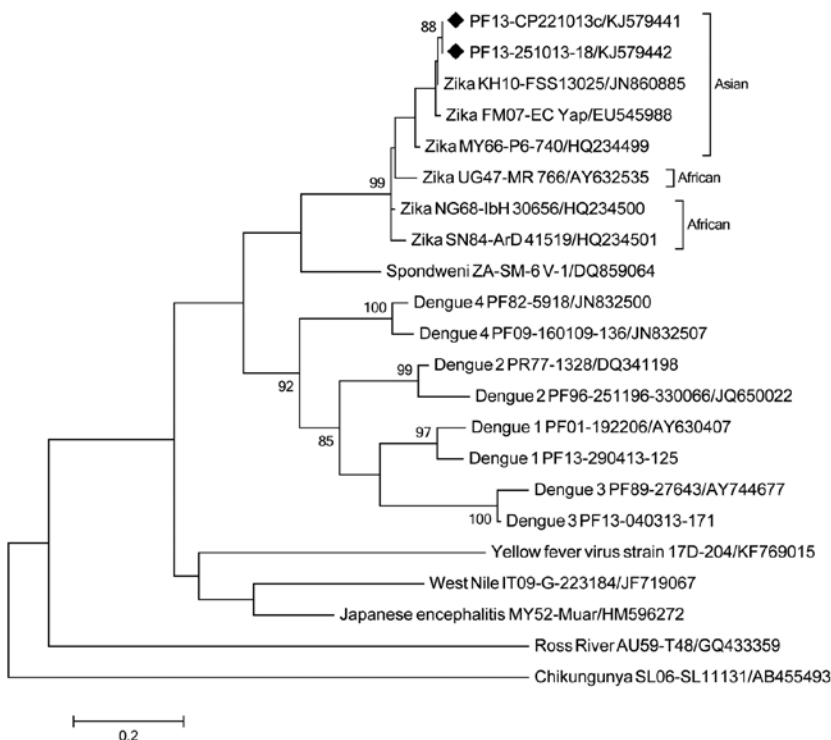


Figure. Phylogenetic analysis of partial M/E genes of 2 ZIKV strains, French Polynesia, 2013. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered is shown for values >85 next to the branches (1,000 replicates). Evolutionary analyses were conducted in MEGA5 (<http://megasoftware.net/>). Strains are labeled by country of origin and date-strain name/GenBank accession number. The 2 ZIKV strains collected in French Polynesia are marked with a black diamond. ZIKV, Zika virus. Scale bar indicates nucleotide substitutions per site.

(GenBank accession no. KJ579441). The protocol was approved by the Ethics Committee of French Polynesia (reference no. 66/CEPF). Phylogenetic analysis of the sequence (Figure) showed ZIKV strain Cambodia 2010-FSS13025 (GenBank accession no. JN860885) as the closest strain (6).

Concomitant with these investigations, the Department of Health recorded an increased number of patients with a mild dengue-like syndrome and rash who were visiting primary care physicians. Given this information, we performed RT-PCR for ZIKV on 10 samples collected during weeks 43 and 44 from patients living in different archipelagoes that had tested negative for DENV NS1 antigen. Four samples gave negative results; 4, positive; and 2, equivocal. Sequencing of 1 ZIKV-positive sample from a patient in Nuku Hiva, Marquesas Islands (GenBank accession no. KJ579442) showed that it had 100% homology with the fragment sequenced from patient 4 who lived in Tahiti, Society Islands. The phylogenetic tree shows that the ZIKV that recently emerged in French Polynesia is similar to Cambodia 2010 and Yap State 2007 strains, which corroborates previous findings of an expansion of ZIKV Asian lineage (7,8). ZIKV was then isolated by inoculating Vero cells with RT-PCR samples positive for ZIKV. After 6 days of propagation, ZIKV-infected cells were detected by indirect immunofluorescence assay using specific hyperimmune mouse ascitic fluids provided by the Institut Pasteur (Dakar, Senegal) (9).

By week 51, the practitioners' network recorded 5,895 patients with suspected ZIKV infections, leading to an estimate of 19,000 suspected cases when extrapolated to other care centers (adjusted to the mean consultation visits). Serum from 584 patients was tested by RT-PCR for ZIKV; 294 samples were positive.

This ZIKV outbreak is the largest documented and the first known to be

caused by an arbovirus other than DENV in French Polynesia. To assess when ZIKV circulation in French Polynesia might have started, we will be conducting a retrospective study on DENV NS1 antigen-negative samples collected before the first ZIKV cases were detected. Investigations of the clinical features of ZIKV infections are ongoing. Particularly, because French Polynesia is experiencing concomitant ZIKV, DENV-1, and DENV-3 outbreaks, attention will be paid to whether sequential infections may affect disease outcome. Otherwise, because French Polynesia hosts several mosquito species, notably *Aedes aegypti*, already known to transmit ZIKV (10), but also other potential vectors, such as *Ae. polynesiensis*, human and entomologic surveillances have been reinforced to clarify the emergence factors of this outbreak.

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**Van-Mai Cao-Lormeau,
Claudine Roche, Anita Teissier,
Emilie Robin, Anne-Laure Berry,
Henri-Pierre Mallet,
Amadou Alpha Sall,
and Didier Musso**

Author affiliations: Institut Louis Malardé, Papeete, Tahiti, French Polynesia (V.-M. Cao-Lormeau, C. Roche, A. Teissier, E. Robin, D. Musso); Direction de la Santé (A.-L. Berry, H.-P. Mallet); and Institut Pasteur, Dakar, Senegal (A.A. Sall)

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Address for correspondence: Van-Mai Cao-Lormeau, Institut Louis Malardé, PO Box 30, 98713 Papeete, Tahiti, French Polynesia; email: mlormeau@ilm.pf



Novel Reassortant Influenza A(H5N8) Viruses, South Korea, 2014

To the Editor: Highly pathogenic avian influenza (HPAI) viruses have caused considerable economic losses to the poultry industry and poses potential threats to animal and human health (www.oie.int/en/ and www.who.int/en/). Since 2003, influenza A(H5N1) viruses with a hemagglutinin (HA) gene derived from A/goose/Guangdong/1/96-like viruses have become endemic to 6 countries (Bangladesh, China, Egypt, India, Indonesia, and Vietnam) (1) (www.cdc.gov/). Furthermore, HPAI viruses with an H5 subtype continue to undergo substantial evolution because of extensive genetic divergence and reassortment between other subtypes of influenza viruses. Especially in China, novel subtypes of H5 HPAI virus, such as influenza A(H5N2), influenza A(H5N5), and influenza A(H5N8) viruses, were reported during 2009–2011 (2,3).

On January 16, 2014, clinical signs of HPAI, such as decreased egg production (60%) and slightly increased mortality rates, were detected in ducks on a breeder duck farm near the Donglim Reservoir in Jeonbuk Province, South Korea. On January 17, a farmer (5 km from the Donglim Reservoir) also reported clinical signs of HPAI in broiler ducks. In addition, 100 carcasses of Baikal teals were found in the Donglim Reservoir.

RNAs extracted from organs (liver, pancreas, and trachea) of 3 dead birds (1 breeder duck, 1 broiler duck, and 1 Baikal teal) were positive for H5 subtype virus by reverse transcription PCR (4). We isolated viruses from suspected specimens by inoculation into embryonated specific pathogen-free chicken eggs. The H5N8 subtype was identified by using HA and neuraminidase (NA) inhibition assays.

Three viruses isolated from domestic ducks and wild birds were

designated A/breeder duck/Korea/Gochang1/2014 (H5N8) (Gochang1), A/duck/Korea/Buan2/2014 (H5N8) (Buan2), and A/Baikal Teal/Korea/Donglim3/2014 (H5N8) (Donglim3). All 8 RNA genome segments of these viruses were amplified by using segment-specific primers and directly sequenced (5). Sequences of the 8 RNA segments of each virus were submitted to GenBank under accession nos. KJ413831–KJ413854.

Gochang1 virus has been shown to be highly pathogenic for chickens (intravenous pathogenicity index 3.0) (6). This finding was consistent with analysis of the HA gene, as shown by a series of deduced basic amino acid sequences (Gochang1, LREKRRKR/GLF, Buan2 and Donglim3, LREKRRKR/GLF) at cleavage sites of HA (6). This outbreak of influenza A(H5N8) infection in South Korea was reported to the World Organisation for Animal Health (7).

Nucleotide identity analysis with BioEdit version 7.2.5 (<http://bioedit.software.informer.com/>) and ClustalW (www.ebi.ac.uk/Tolls/clustalw2) showed that 3 distinct novel influenza A(H5N8) viruses emerged in South Korea. Gochang1 virus had 87%–97% sequence identities in the 8 genome segments with sequences for Buan2 and Donglim3 viruses, which had high sequence identities (>99.5%) with each other. Conservative amino acid residues within receptor binding pockets of HA (including E190, R220, G225, Q226, and G228; H3 numbering) were present in all 3 viruses, which indicated that these viruses retained affinity for the avian (sialic acid-2,3-NeuAcGal) cell surface (8). Although there was an I314V mutation in the NA of the 3 viruses, other mutations that encode oseltamivir and zanamivir resistance were not detected (9).

A BLAST (www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) search and phylogenetic analysis showed that these novel H5N8 subtype viruses likely originated from reassortment

between A/duck/Jiangsu/k1203/2010 (H5N8) virus and other subtypes of avian influenza virus, all of which co-circulated in birds in eastern China during 2009–2012 (10). A phylogenetic tree of partial HA gene sequences for the 3 virus isolates from South Korea and other H5 subtype viruses (n = 72), showed that Gochang1, Buan2, and Donglim3 belong to the proposed H5 clade 2.3.4.6 (Figure) (10).

The H5 and N8 genes of the 3 viruses had high nucleotide identities with A/duck/Jiangsu/k1203/2010 (H5N8) (JQ97369691–98) (H5: Gochang1, 98.9%, Buan2 and Donglim3, 97.2%; N8: Gochang1, 98.5%, Buan2 and Donglim3, 98.1%). For Gochang1 virus, polymerase basic protein 2 (PB2) and nonstructural (NS) protein had the highest identities with A/environment/Jiangxi/28/2009 (H11N9) (PB2 98.6%, NS 97.7%). The other segments showed high genetic identities with A/duck/Jiangsu/k1203/2010 (H5N8) (>98.7%), which suggested that Gochang1 virus was generated by reassortment in which the PB2 and NS genes of A/duck/Jiangsu/k1203/2010 (H5N8) were replaced with those of influenza A(H11N9) viruses.

For Buan2 and Donglim3 viruses, the PB2, HA, nucleoprotein, and NA genes were highly similar to those of A/duck/Jiangsu/k1203/2010 (H5N8) (>97.2%). However, the PB1, polymerase acidic protein, matrix protein, and NS genes of this virus had the highest genetic identities with A/duck/Eastern China/1111/2011 (H5N2) (>98.2%). Therefore, Buan2 and Donglim3 viruses might be reassortants that contain PB2, HA, nucleoprotein, and NA genes from A/duck/Jiangsu/k1203/2010 (H5N8) and PB1, polymerase acidic protein, NS, and matrix genes from A/duck/Eastern China/1111/2011 (H5N2) co-circulating in the same region of China (2,10).

We characterized 3 distinct novel reassortant influenza A(H5N8) HPAI viruses during an influenza outbreak in South Korea. Buan2 and Donglim3

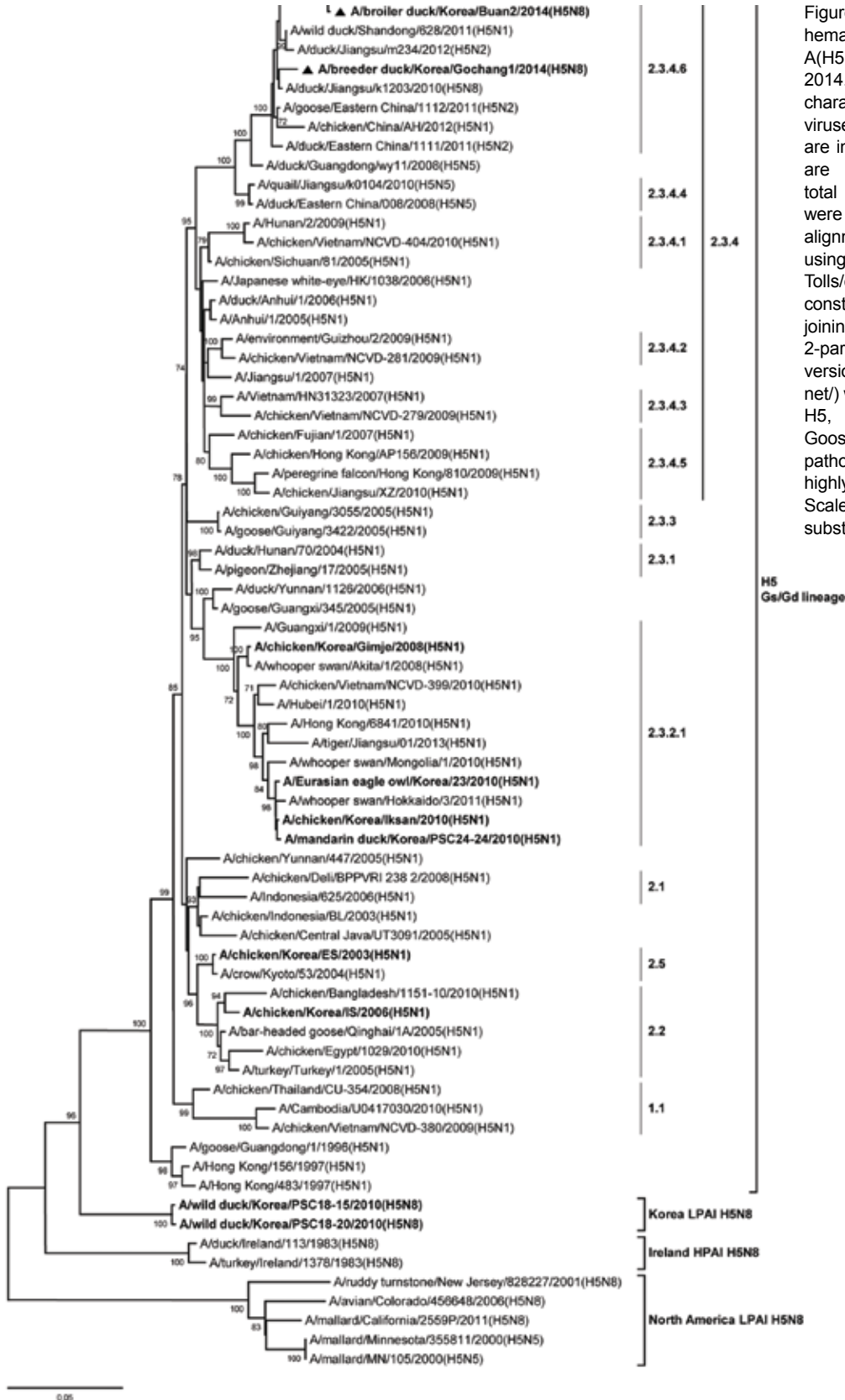


Figure. Phylogenetic tree of hemagglutinin (HA) genes of influenza A(H5N8) viruses, South Korea, 2014. Triangles indicate viruses characterized in this study. Other viruses detected in South Korea are indicated in boldface. Subtypes are indicated in parentheses. A total of 72 HA gene sequences were $\geq 1,600$ nt. Multiple sequence alignment was performed by using ClustalW (www.ebi.ac.uk/Tools/clustalw2). The tree was constructed by using the neighbor-joining method with the Kimura 2-parameter model and MEGA version 5.2 (www.megasoftware.net/) with 1,000 bootstrap replicates. H5, hemagglutinin 5; Gs/Gd, Goose/Guangdong; LP AI, low pathogenic avian influenza; HP AI, highly pathogenic avian influenza. Scale bar indicates nucleotide substitutions per site.

viruses showed high nucleotide identities, which suggested that the outbreak viruses in domestic ducks and Baikal teals might have an identical origin. Although research on the epidemiologic features of this outbreak is currently underway, it seems likely that on the basis of reassortant sequence features of the 8 genome segments, these 3 distinct viruses originated in eastern China. These influenza viruses are a potential threat to the poultry population in South Korea, including gallinaceous birds during movement of domestic ducks through the distribution network of live bird markets.

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**Youn-Jeong Lee, Hyun-Mi Kang,
Eun-Kyoung Lee,
Byung-Min Song,
Jipseol Jeong, Yong-Kuk Kwon,
Hye-Ryoung Kim, Kyu-Jun Lee,
Mi-Seon Hong, Il Jang,
Kang-Seuk Choi, Ji-Ye Kim,
Hyun-Jeong Lee, Min-Su Kang,
Ok-Mi Jeong, Jong-Ho Baek,
Yi-Seok Joo, Yong Ho Park,
and Hee-Soo Lee**

Author affiliation: Animal and Plant Quarantine Agency, Anyang, Gyeonggi, South Korea

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Address for correspondence: Youn-Jeong Lee, Avian Disease Division, Animal and Plant Quarantine Agency, 175 Anyang-ro, Anyang-si, Gyeonggi-do 430-757, South Korea; email: leeyj700@korea.kr

Possible Misidentification of *Mycobacterium yongonense*

To the Editor: Tortoli et al. (1) reported pulmonary disease caused by *M. yongonense* strains isolated from patients in Italy; these strains were identified by sequencing the 16S rRNA, *hsp65*, *rpoB*, and *sodA* genes and the internal transcribed spacer 1 (ITS1) region. The 16S rRNA gene sequence of these isolates showed 100% similarity with those of *M. yongonense* and *M. marseillense*. The isolates were more closely related to *M. yongonense* than to *M. marseillense* in terms of the *hsp65* gene and ITS1 region; however, the *rpoB* gene sequence showed a higher degree of similarity to that of *M. intracellulare* (99.4%) than to that of *M. marseillense* (97.4%). The authors did not mention the similarity of the isolates with *M. intracellulare* in these sequences except for the *rpoB* gene. However, because these sequences showed high similarity to *M. yongonense*, a high degree of similarity to *M. intracellulare* could be inferred.

The initial description of *M. yongonense* highlighted its unique molecular character (2). The 16S rRNA and *hsp65* genes and ITS1 region are closely related to those of *M. intracellulare* ATCC 13950^T; however, the *rpoB* gene is closely related to that of *M. parascrofulaceum* ATCC BAA-614^T (99.4%). No consensus guidelines are available for mycobacterial identification, but the *rpoB* gene has been used widely as a target gene; multilocus sequence analysis also has been used recently (3,4). Although the authors suggest that a variant of *M. yongonense* preceded the acquisition of the *rpoB* gene from *M. parascrofulaceum* by a lateral gene transfer event (3), the isolates described are more similar to *M. intracellulare* than to *M. yongonense* on the basis of the *rpoB*

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gene sequence and multilocus sequence analysis. It is also possible that the isolates are a *M. yongonense* strain that preceded the acquisition of the *rpoB* gene but that are not the same as the initially described *M. yongonense*.

**Sung Kuk Hong
and Eui-Chong Kim**

Author affiliation: Seoul National University Hospital, Seoul, South Korea

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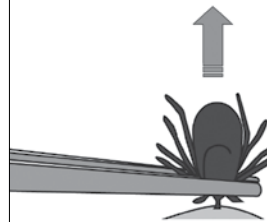
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Address for correspondence: Sung Kuk Hong, Department of Laboratory Medicine, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea; email: trust99@snu.ac.kr

**How to Correctly
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Grasp the tick firmly and as closely to the skin as possible. With a steady motion, pull the tick's body away from the skin. Do not be alarmed if the tick's mouthparts remain in the skin. Cleanse the area with an antiseptic.



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etymologia

Zika Virus

Zika [zēk' ə] Virus

Zika virus is a mosquito-borne positive-sense, single-stranded RNA virus in the family *Flaviviridae*, genus *Flavivirus* that causes a mild, acute febrile illness similar to dengue. In 1947, scientists researching yellow fever placed a rhesus macaque in a cage in the Zika Forest (*zika* meaning “overgrown” in the Luganda language), near the East African Virus Research Institute in Entebbe, Uganda. A fever developed in the monkey, and researchers

isolated from its serum a transmissible agent that was first described as Zika virus in 1952. It was subsequently isolated from a human in Nigeria in 1954. From its discovery until 2007, confirmed cases of Zika virus infection from Africa and Southeast Asia were rare. In 2007, however, a major epidemic occurred in Yap Island, Micronesia. More recently, epidemics have occurred in Polynesia, Easter Island, the Cook Islands, and New Caledonia.

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Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

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Quiet Moment around the Campfire

Byron Breedlove

Frederic Remington was an American painter, sculptor, illustrator, and writer whose works frequently featured cowboys, Native Americans, soldiers, horses, bison, and other iconic features of the rapidly vanishing American West. During his 25-year career, Remington produced approximately 3,000 paintings and drawings, 22 bronze sculptures, a novel, a Broadway play, and more than 100 articles and stories. Remington was 48 years old when he died of peritonitis, a complication of an emergency appendectomy.

In approximately 1900, Remington began working on a series of paintings—now known collectively as the nocturnes—that depict color and light unique to night. Nancy Anderson, curator of American and British Paintings at the National Gallery of Art, Washington, DC, wrote that “In these experimental, complex, and deeply personal paintings, Remington explored the technical and aesthetic difficulties of painting darkness. Surprisingly, his images are

filled with color and light—moonlight, firelight, candlelight.” Many of those paintings also depict danger from humans, animals, or nature, as implied and real threats, sometimes revealed to the viewer and sometimes suggested by the posture or action of Remington’s subjects.

This month’s cover painting, commonly known as *The Cigarette*, was discovered in Remington’s studio after his death. In this painting, four cowboys relax around a small outside a cabin. A plume of smoke rises toward the clear blue-green night sky flecked with a few stars, past a large skin hanging on the side of the cabin. The cabin does not overwhelm the painting but details such as the shadow under the roofline, the seams between logs, the softened edges of the structure, and the tautly stretched skin reveal Remington’s deftness at rendering textures. His use of subdued colors punctuated by the reflected firelight underscores the quiet of the evening’s respite following a long day’s work.

The cowboy in the foreground blocks the campfire so that its muted glow washes out into the middle of the painting. Despite a rifle standing within reach just to the

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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left of the bunkhouse door, this painting does not suggest the foreboding sense of danger characteristic of many of Remington's other nocturnes. As the evening's ritual of cigarettes, coffee, and conversation plays out, the cowboy to the left squats and smokes, perhaps telling a story or swapping tales about days gone by.

The lonely vistas and wilderness settings for many of Remington's works offer, according to Thayer Tolles of the Metropolitan Museum of Art, "a nostalgic, even mythic, look at a rapidly disappearing western frontier, which underwent dramatic transformation in the face of transcontinental transportation, Native American confinement to reservation land, immigration, and industrialization." The once remote places and outposts yielded to encroaching ranches, farms, and towns, as more people pushed westward each year.

The vast migration and increasing population across the vanishing frontier would also bring the potential for transmission of the pathogens that cause such diseases as tuberculosis, measles, pneumonia, and typhoid. Crossing the plains and deserts by horseback, wagon, or train may have exposed settlers to airborne fungal spores that can cause coccidioidomycosis, commonly called "cocci" or "valley fever." As cabins, then towns, then cities were built, crowded conditions afforded more opportunities for exposures to pathogens from animal hosts, such as rodents and bats.

Many of Remington's works portray the Old West as a vast mythical landscape of danger, silence, and beauty.

"*The Cigarette*," however, captures a quiet moment around a campfire in the dwindling evening light, serving as an elegiac farewell to that time and place.

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Address for correspondence: Byron Breedlove, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C14, Atlanta, GA 30329-4027, USA; email: wbb1@cdc.gov

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Zoonotic Filariasis Caused by Novel *Brugia* Nematode, United States

Rotavirus Epidemiology in Bangui, Central African Republic, 2008

MERS-Related Betacoronavirus in *Vespertilio superans* Bats, China

Complete list of articles in the July issue at
<http://www.cdc.gov/eid/upcoming.htm>

Upcoming Infectious Disease Activities

June 24–27, 2014

EMBO Conference on
Microbiology After the Genomics
Revolution—Genomes 2014
Institut Pasteur, Paris
<http://www.genomes-2014.org>

July 27–August 1, 2014

IUMS 2014
International Union of
Microbiological Societies
Montréal, Canada
<http://www.montrealiums2014.org>

September 5–9, 2014

ICAAC 2014
Interscience Conference on
Antimicrobial Agents and Chemotherapy
Washington, DC
<http://www.icaac.org>

October 8–12, 2014

ID Week 2014
Philadelphia, PA
<http://www.idweek.org/>

October 31–November 3, 2014

IMED 2014
Vienna, Austria
<http://imed.isid.org>

November 15–19, 2014

APHA 142nd Annual Meeting & Expo
New Orleans, LA
[http://www.apha.org/meetings/
AnnualMeeting](http://www.apha.org/meetings/AnnualMeeting)

November 30–December 4, 2014

ASLM2014 International Conference
Cape Town International Convention
Centre, South Africa
<http://www.aslm2014.org>

2015

March 8–11, 2015

ICEID
International Conference
on Emerging Infectious Diseases
Atlanta, GA

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to <http://www.ama-assn.org/ama/pub/category/2922.html>. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Adverse Pregnancy Outcomes and *Coxiella burnetii* Antibodies in Pregnant Women, Denmark

CME Questions

- 1. Your patient is a 28-year-old pregnant Danish woman with positive titers against *Coxiella burnetii*. According to the case series by Dr. Nielsen and colleagues, which of the following statements about adverse pregnancy outcomes is correct?**
 - A. Approximately three fourths of women with positive titers against *C. burnetii* had obstetric complications
 - B. No deaths occurred in this case series
 - C. Oligohydramnios was not reported
 - D. Adverse pregnancy outcomes included miscarriage, extrauterine pregnancy, preterm delivery, and delivery of an infant small for gestational age
- 2. According to the case series by Dr. Nielsen and colleagues, which of the following statements about the causal association between adverse pregnancy outcomes and *C. burnetii* infection is correct?**
 - A. Findings from this case series prove that *C. burnetii* infection causes adverse pregnancy outcomes
 - B. Other infections were conclusively ruled out in all the women
 - C. Adverse pregnancy outcome occurred in 8 of 12 pregnancies in which the women were not treated for *C. burnetii* infection, supporting a possible causal association
 - D. All placentae tested by polymerase chain reaction tested positive for *C. burnetii* infection
- 3. According to the case series by Dr. Nielsen and colleagues, which of the following statements about the overall risk for a Q fever associated adverse pregnancy outcome would most likely be correct?**
 - A. The investigators found 9 cases of Q fever–associated adverse pregnancy outcomes in Denmark during a period of 5 years
 - B. Lack of awareness of Q fever–associated adverse pregnancy outcomes in the relevant risk groups means that the true risk is much higher than found in this study
 - C. Cases in this series came from a wide variety of occupations and communities
 - D. Community studies in Denmark and the Netherlands have confirmed the association between Q fever and adverse pregnancy outcomes

Activity Evaluation

| | | | | | |
|---|---|---|---|---|----------------|
| 1. The activity supported the learning objectives. | | | | | |
| Strongly Disagree | | | | | Strongly Agree |
| 1 | 2 | 3 | 4 | 5 | |
| 2. The material was organized clearly for learning to occur. | | | | | |
| Strongly Disagree | | | | | Strongly Agree |
| 1 | 2 | 3 | 4 | 5 | |
| 3. The content learned from this activity will impact my practice. | | | | | |
| Strongly Disagree | | | | | Strongly Agree |
| 1 | 2 | 3 | 4 | 5 | |
| 4. The activity was presented objectively and free of commercial bias. | | | | | |
| Strongly Disagree | | | | | Strongly Agree |
| 1 | 2 | 3 | 4 | 5 | |

CDC Health Information for International Travel 2014

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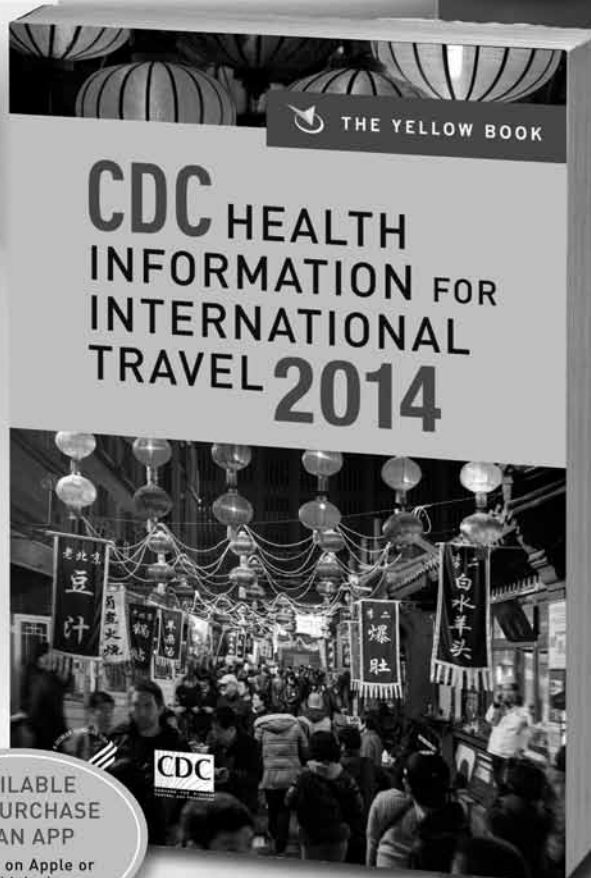
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Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of 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 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymology. 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.