EMERGING INFECTIOUS DISEASES®

20 YEARS

Malaria

July 2015

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On the Cover

Marianne North (1830–1890)

Foliage, Flowers, and Seed-vessels of a Peruvian Bark Tree (1870s)

Oil on card
20 x 11.5 in / 50.8 x 29.2 cm

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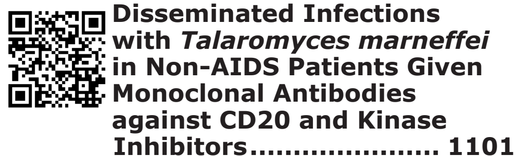

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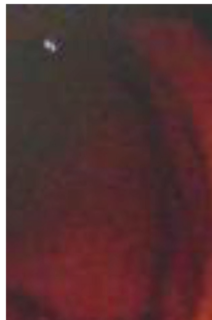

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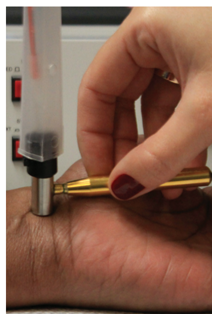

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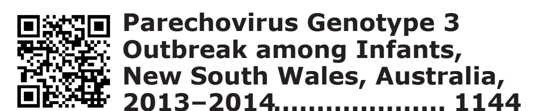

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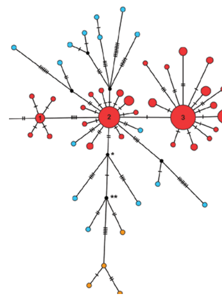

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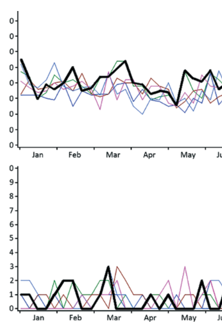

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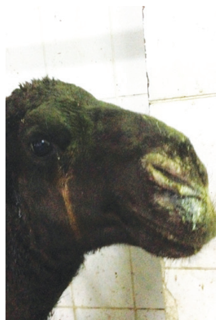

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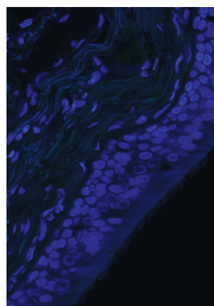

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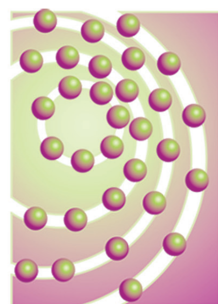

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EMERGING INFECTIOUS DISEASES®

20 YEARS

Presenting the ongoing challenges that emerging microbial threats pose to global health

SYNOPSIS

Disseminated Infections with *Talaromyces marneffei* in Non-AIDS Patients Given Monoclonal Antibodies against CD20 and Kinase Inhibitors

Jasper F.W. Chan, Thomas S.Y. Chan, Harinder Gill, Frank Y.F. Lam, Nigel J. Trendell-Smith, Siddharth Sridhar, Herman Tse, Susanna K.P. Lau, Ivan F.N. Hung, Kwok-Yung Yuen, Patrick C.Y. Woo

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Learning Objectives

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

1. Distinguish the clinical and epidemiologic characteristics of *T. marneffei* infection, based on a case series report
2. Discuss the recent emergence of disseminated *T. marneffei* infection in non-AIDS patients with hematologic malignant neoplasms treated with targeted therapies
3. Identify possible mechanisms of action underlying disseminated *T. marneffei* infection in non-AIDS patients with hematologic malignant neoplasms treated with targeted therapies

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Infections with the fungus Talaromyces (formerly Penicillium) marneffei are rare in patients who do not have AIDS. We report disseminated T. marneffei infection in 4 hematology patients without AIDS who received targeted therapy with monoclonal antibodies against CD20 or kinase inhibitors during the past 2 years. Clinicians should be aware of this emerging complication, especially in patients from disease-endemic regions.

Talaromyces (formerly *Penicillium*) *marneffei* is a pathogenic, thermal dimorphic fungus that causes systemic mycosis in Southeast Asia. *T. marneffei* infection is characterized by fungal invasion of multiple organ systems, especially blood, bone marrow, skin, lungs, and reticuloendothelial tissues, and is highly fatal, especially when diagnosis and treatment are delayed (*1*,*2*). This disease is found predominantly in AIDS patients and occasionally those with cell-mediated immunodeficiencies involving the interleukin-12/interferon-γ (IFN-γ) signaling pathway, such as congenital STAT1 mutations or acquired autoantibodies against IFN-γ (*1*,*3*–*6*). The infection has rarely been reported among hematology patients, including those from disease-endemic regions (*7*,*8*).

At Queen Mary Hospital in Hong Kong, a 1,600-bed university teaching hospital that has a hematopoietic stem cell transplantation service, where a wide range of invasive fungal infections have been observed (*9*,*10*), only 3 cases of *T. marneffei* infection were encountered in >2,000 hematology patients in the past 20 years, despite the longstanding availability of mycologic culture and serologic testing (*7*,*8*,*11*,*12*). In contrast, the infection was commonly reported among AIDS patients (*13*).

In the past 2 years, we have been alerted by 4 unprecedented cases of disseminated *T. marneffei* infection among non-AIDS hematology patients given targeted therapies, including monoclonal antibodies (mAbs) against CD20 and kinase inhibitors, which are being increasingly used in recent years. We report details for these 4 hematology case-patients. The study was approved by the institutional review board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster in Hong Kong.

Case-Patient 1

Patient 1 was a 56-year-old Filipino man with Waldenström macroglobulinemia, idiopathic thrombocytopenic purpura, and primary biliary cirrhosis. He had fever, night sweating, productive cough, and left facial pain for 1 week and bloody diarrhea for 2 days. He had previously received fludarabine, dexamethasone, and rituximab (mAb against CD20, 18 months earlier) for treatment of Waldenström macroglobulinemia (Table 1). The idiopathic thrombocytopenic purpura was controlled with intravenous immunoglobulin and maintenance prednisolone and mycophenolate sodium. A chest radiograph showed a small cavitary lesion in the right lower lobe. His symptoms and signs did not resolve after he received empirical intravenous imipenem/cilastatin and metronidazole (Table 2).

A colonoscopy showed multiple shallow ulcers at the terminal ileum (Figure 1). Histologic analysis of an ulcer biopsy specimen showed slough of an acutely inflamed ulcer but no microorganisms. However, histologic analysis of a specimen from a nasopharyngeal biopsy performed for persistent left facial pain showed abundant yeast cells engulfed by foamy macrophages (Figure 2). Culture of terminal ileal ulcer biopsy specimens, stool samples, and nasopharyngeal biopsy specimens yielded *T. marneffei*. A contrast-enhanced cranial computed tomography (CT) scan showed 2 lesions (3–4-mm) with rim enhancement and perifocal edema at the right occipital and left parieto-occipital lobes. A thoracic CT scan showed 2 cavitary lesions (4–8 mm) in the right upper and lower lobes.

Immunologic testing showed that the patient was negative for HIV and autoantibodies against IFN-γ. His CD3+ and CD8+ counts were within references ranges, but he had mild CD4+ lymphopenia (Table 2). His fever and symptoms resolved with after 2 weeks of treatment with intravenous liposomal amphotericin B, followed by oral voriconazole. Reassessment colonoscopy (at 2 months) and CT scan (at 6 months) showed complete resolution of all lesions.

Case-Patient 2

Patient 2 was a 44-year-old Chinese man who had fever for 2 days. He had previously received chemotherapy and mAbs against CD20 (rituximab, 14 months earlier; obinutuzumab, concomitant) for refractory chronic lymphocytic leukemia (CLL) involving bone marrow (Table 1). He was empirically given intravenous piperacillin/tazobactam and anidulafungin (Table 2). Histologic analysis of a trephine biopsy specimen showed persistent CLL with plasmacytic differentiation, and Grocott staining showed yeasts with central septa in small clusters. Culture of peripheral blood and bone marrow aspirate yielded *T. marneffei*. A change in antifungal treatment to intravenous amphotericin B led to defervescence and clearance of fungemia. He was given oral itraconazole as maintenance therapy. He remained well until 2 months later when he was hospitalized for deteriorating CLL complicated by neutropenic fever with multiorgan failure caused by other opportunistic infections (Table 1). He died 5 months after the episode of disseminated *T. marneffei* infection.

Case-Patient 3

Patient 3 was a 63-year-old Chinese man with myelofibrosis and well-controlled diabetes mellitus. He had intermittent fever, right cervical lymphadenopathy, and productive cough for 4 months. He was given ruxolitinib (kinase

Table 1. Characteristics of 4 case-patients with disseminated *Talaromyces marneffei* infection after targeted therapies*

Characteristic	Case-patient 1	Case-patient 2	Case-patient 3	Case-patient 4
Age, y/sex	56/M	44/M	63/M	67/M
Concurrent conditions	Waldenström macroglobulinemia, idiopathic thrombocytopenic purpura, primary biliary cirrhosis	Chronic lymphocytic leukemia	Myelofibrosis with splenectomy, diabetes mellitus	Acute myeloid leukemia, hypertension
Targeted therapy	Rituximab	Rituximab and obinutuzumab	Ruxolitinib	Sorafenib
Action of therapy	mAb against CD20	mAb against CD20	JAK-1/2 inhibitor	Multikinase inhibitor
Time interval, mo†	18	14 (rituximab) and concomitant (obinutuzumab)	Concomitant	Concomitant
Cumulative dose before *T. marneffei* infection	700 mg/dose iv x 4 doses	700 mg/dose IV x 13 doses (rituximab) and 1,000 mg IV x 3 doses (obinutuzumab)	10–20 mg 2×/d oral x 6.5 mo	400 mg 2×/d oral x 8 mo
Other immunosuppressants (time interval, mo)†	Fludarabine and dexamethasone (39), prednisolone 10 mg/d and mycophenolate sodium 360 mg 2×/d (concomitant)	Fludarabine and cyclophosphamide (48), CHOP (36), bendamustine (13)	None	Mitoxantrone and etoposide (21), daunarubicin (20), clofarabine (18), azacitidine (15), decitabine (15), cytarabine (14)
Clinical manifestations	Terminal ileitis, cerebral abscesses, nasopharyngitis, and multiple cavitary lung lesions	Marrow infiltration and fungemia	Right cervical lymphadenitis and multiple cavitary lung lesions	Fungemia
Specimens positive for *T. marneffei*	Feces, and terminal ileal and nasopharyngeal biopsy specimens	Blood and bone marrow aspirate	Right cervical lymph node	Blood
Highest serum antibody titer against *T. marneffei*	1:320	<1:40	1:320	<1:40
Antifungal treatment (duration, mo)	Amphotericin B (2 weeks) and voriconazole (>21)	Amphotericin B (2 weeks) and itraconaozle (5)	Amphotericin B (2 weeks) and voriconazole (>6)	Amphotericin B (2 weeks) and voriconazole (>5)
Other opportunistic infections	None	Bacteremia (*Mycobacterium chelonae*, *Enterococcus faecium*, and MRCNS), fungemia (*Candida glabrata*), HSV oral mucositis, PJP	Bacteremia (*Klebsiella pneumoniae*)	Herpes zoster at right occiput
Clinical outcome	Responded to antifungal treatment	Clearance of *T. marneffei* fungemia but died of MODS and multiple infections 5 mo after *T. marneffei* infection	Responded to antifungal treatment	Responded to antifungal treatment

*mAb, monoclonal antibody; JAK, Janus kinase; IV, intravenous; CHOP, cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisolone; MRCNS, methicillin-resistant coagulase-negative *Staphylococcus*; HSV, herpes simplex virus; PJP, *Pneumocystis jiroveci* pneumonia; MODS, multiple organ dysfunction syndrome.
†Time interval between end of therapy and onset of symptoms for *T. marneffei* infection.

inhibitor) 6 months before symptom onset because of transfusion-dependent myelofibrosis despite splenectomy 4 years earlier (Table 1). A chest radiograph and thoracic CT scan showed multiple cavitary lesions and consolidation. Bronchoalveolar lavage was negative for bacteria, fungi, and mycobacteria. A serum cryptococcal antigen test result was negative. He was empirically given intravenous imipenem/cilastatin and oral doxycycline, but his symptoms persisted. A right cervical lymph node culture yielded *T. marneffei*. His symptoms and radiologic abnormalities resolved after treatment with intravenous amphotericin B for 2 weeks, followed by oral voriconazole for 6 months.

Case-Patient 4

Patient 4 was a 67-year-old Chinese man with acute myeloid leukemia and hypertension. He had fever and malaise for 2 days without localizing signs. He had been given sorafenib (kinase inhibitor) 8 months earlier for chemotherapy-refractory acute myeloid leukemia (Table 1). His fever did not respond to intravenous meropenem. Subsequently,

Table 2. Laboratory results for 4 case-patients with disseminated *Talaromyces marneffei* infection after targeted therapies*

Laboratory parameter	Case-patient 1	Case-patient 2	Case-patient 3	Case-patient 4
Hematologic†				
Leukocytes, x 10^9 cells/L	12.08	0.91	4.93	33.79
Neutrophils, x 10^9 cells/L	11.01	0.45	3.11	8.45 (with blasts)
Lymphocytes, x 10^9 cells/L	0.83 (CD4+: 315/µL)‡	0.45	1.05	9.12 (with blasts)
Hemoglobin, g/dL	12.3	10.3	8.0	9.2
Platelets, x 10^9/L	250	5	539	15
Biochemical†				
Sodium, mmol/L	136	135	139	138
Potassium, mmol/L	3.5	4.1	3.7	4.4
Creatinine, µmol/L	101	111	78	92
Albumin, g/L	40	32	39	37
Globulin, g/L	34	36	36	39
Total bilirubin, µmol/L	8	9	13	19
ALP, U/L	234	163	112	96
ALT, U/L	79	20	32	61
AST, U/L	38	9	28	123
LDH, U/L	209	97	352	2,069
Immunologic				
Combined HIV antibody/antigen	Negative	Negative	Negative	Negative
Autoantibody against IFN-γ	Negative	Negative	Negative	Negative
Microbiologic				
Blood culture	No bacteria and fungi	*T. marneffei; Mycobacterium chelonae*, *Enterococcus faecium*, MRCNS, and *Candida glabrata*§	*Klebsiella pneumoniae*§	*T. marneffei*
Bone marrow aspirate	ND	*T. marrneffei*	ND	ND
Sputum culture	Negative for pathogenic bacteria, AFB, and fungi	Negative for pathogenic bacteria, AFB, and fungi	Negative for pathogenic bacteria, AFB, and fungi	Negative for pathogenic bacteria, AFB, and fungi
Urine culture	No bacteria and fungi	No bacteria and fungi	No bacteria and fungi	No bacteria and fungi
Stool culture	*T. marneffei*; negative for pathogenic bacteria, including *Clostridium difficile* and AFB	ND	ND	ND
Serum CMV pp65 antigen	Negative	Negative	Negative	Negative
Other	Stool for *C. difficile* toxin (negative); serum for *Entamoeba histolytica* antibody (negative); multiple blood smears for *Plasmodium* sp. (negative)	BAL: *Pneumocystis jiroveci* (smear-positive)	Cervical lymph node: *T. marneffei* (culture-positive)	

*ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; IFN-γ, interferon-γ; MRCNS, methicillin-resistant coagulase-negative *Staphylococcus*; ND, not done; AFB, acid-fast bacilli; CMV, cytomegalovirus; BAL, bronchoalveolar lavage. Reference ranges: leukocytes; 3.89–9.93 × 10^9 cells/L; neutrophils, 2.01–7.42 × 10^9 cells/L; lymphocytes, 1.06–3.61 × 10^9 cells/L; hemoglobin, 13.3–17.7g/dL; platelets, 162–341 × 10^9/L; sodium, 136–148 mmol/L; potassium, 3.6–5.0 mmol/L; creatinine, 67–109 µmol/L; albumin, 39–50 g/L; globulin, 24–37 g/L; total bilirubin, 4–23 µmol/L; ALP, 42–110 U/L; ALT, 8–58 U/L; AST, 5–38 U/L; LDH, 118–221 U/L.
†Results at presentation.
‡Reference range of CD4+ lymphocyte count: 415–1,418 cells/µL.
§Bacteremia caused by *M. chelonae, E. faecium,* MRCNS, and candidemia in case-patient 2, and bacteremia caused by *K. pneumoniae* in case-patient 3 occurred after recovery from *T. marneffei* infection and prolonged hospitalization.

2 sets of blood cultures yielded *T. marneffei*. He was given intravenous amphotericin B for 2 weeks, followed by oral voriconazole. He remained well at follow-up 6 months after symptom onset.

Discussion

T. marneffei infection is an emerging complication in hematology patients receiving targeted therapies. Historically, *T. marneffei* infection has rarely been seen in non-AIDS patients, even in disease-endemic regions. During 1994–2014, only 3 other cases were observed in our hematology patients (*7*,*8*,*11*). None of 47 patients with *T. marneffei* infection in another large local case series during 1994–2004 had hematologic disease (*13*). In the past 20 years, there has been no change in methods for laboratory diagnosis of *T. marneffei* infection or a marked increase

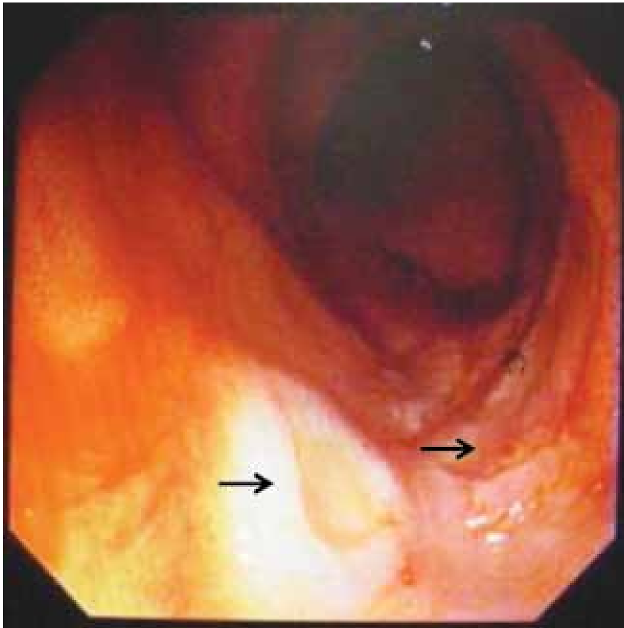

Figure 1. Multiple, shallow, oozing ulcers at the terminal ileum (arrows) detected by colonoscopy on day 4 of hospitalization for case-patient 1, who had a disseminated infection with *Talaromyces marneffei*.

in the number of hematology patients in our hospital. Therefore, these 4 cases indicate an increase in the incidence of *T. marneffei* infection in these patients. Although other immunosuppressants given to case-patients 1, 2, and 4 might have contributed to overall immunosuppression, none of these immunosuppressants, which have been used for years, have been associated with *T. marneffei* infection. Because use of targeted therapies is increasing in diverse patient groups, clinicians should be aware of this emerging complication, especially in patients from disease-endemic regions who have received these therapies with other immunosuppressants.

The exact mechanisms through which these targeted therapies lead to *T. marneffei* infection remain incompletely understood. Rituximab and obinutuzumab (used by case-patients cases 1 and 2) are mAbs against CD20 that predominantly target B cells. Unlike T cells, the role of B cell–mediated humoral response in *T. marneffei* infection is poorly defined. Although case-patient 1 had mild CD4+ lymphopenia probably related to concomitant use of prednisolone and mycophenolate sodium, *T. marneffei* infection is rarely seen in patients with CD4+ counts >300/µL (*1*). We postulate that B cell dysfunction might have impaired production of neutralizing antibodies against key virulence factors of *T. marneffei* or might involve impairment of cytokine-producing B cells, which are essential for T helper cell function (*14*).

More severe infections with fungemeia and bone marrow involvement developed in case-patients 2 and 4, who had undetectable levels of serum antibodies against *T. marneffei*. Correspondingly, case-patients 1 and 3, who had antibody titers >1:320, did not have positive blood culture results (Table). Symptoms developed in case-patient 1 more than a 1 year after he completed therapy with mAbs against CD20. This finding might be related to long-lasting B cell–depleting effects of mAbs against CD20 (*15*).

Regarding kinase inhibitors (used by cases-patients 3 and 4), ruxolitinib is a selective Janus kinase (JAK)-1/2 inhibitor that prevents signal transduction for type I/II cytokines, including IF N-γ, by interfering with the JAK-STAT signaling pathway. Use of ruxolitinib has been associated with opportunistic infections caused by intracellular pathogens, such as *Mycobacterium tuberculsosis* and *Cryptococcus neoformans* (*16*,*17*). Similarly, patients with impaired JAK-STAT signaling, but not those with diabetes mellitus or splenectomy (case-patient 3), are predisposed to *T. marneffei* infection (*6*). Sorafenib is a multikinase inhibitor with various immunomodulatory effects, including impaired T-cell response and proliferation and reduced IFN-γ production (*18*). These immune defects have been associated with reactivation of latent tuberculosis and might also predispose patients to opportunistic infections caused by intracellular organisms such as *T. marneffei* (*18*).

The recognition of disseminated *T. marneffei* infection as an emerging complication in non-AIDS patients treated with targeted therapy has major public health implications.

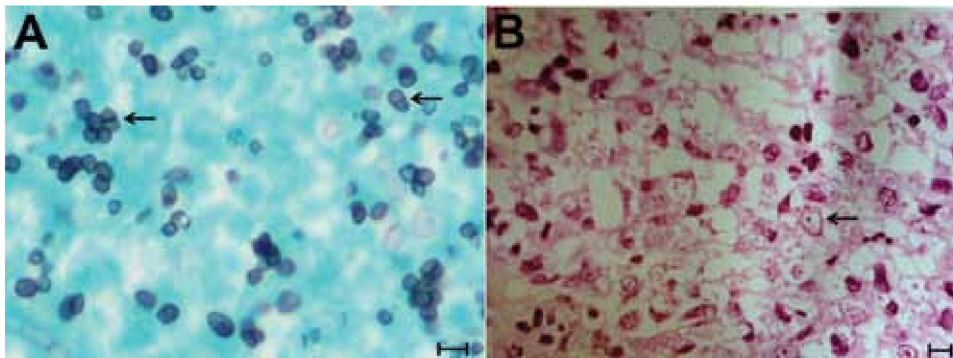

Figure 2. Nasopharyngeal biopsy specimen from case-patient 1, who had a disseminated infection with *Talaromyces marneffei*. A) Grocott silver staining showing abundant yeast cells (arrows) with central septa 4–5 µm in diameter. B) Hematoxylin and eosin staining showing necrotic material admixed with blood and fibrin with aggregates of foamy macrophages (arrow). Scale bars indicate 5 µm.

In regions to which *T. marneffei* infection is endemic, serologic surveillance for patients receiving targeted therapy might be useful in the early diagnosis of *T. marneffei* infection, as in the case of AIDS patients (*19*). In non-endemic regions, such as the United States, clinicians should be vigilant of this infrequent infection in at-risk hematology patients who have resided in or are returning from disease-endemic areas.

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Macacine Herpesvirus 1 in Long-Tailed Macaques, Malaysia, 2009–2011

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Macacine herpesvirus 1 (MaHV1; B virus) naturally infects macaques (*Macaca* spp.) and can cause fatal encephalitis in humans. In Peninsular Malaysia, wild macaques are abundant, and translocation is used to mitigate human–macaque conflict. Most adult macaques are infected with MaHV1, although the risk for transmission to persons who handle them during capture and translocation is unknown. We investigated MaHV1 shedding among 392 long-tailed macaques (*M. fascicularis*) after capture and translocation by the Department of Wildlife and National Parks in Peninsular Malaysia, during 2009–2011. For detection of MaHV1 DNA, PCR was performed on urogenital and oropharyngeal swab samples. Overall, 39% of macaques were shedding MaHV1 DNA; rates of DNA detection did not differ between sample types. This study demonstrates that MaHV1 was shed by a substantial proportion of macaques after capture and transport and suggests that persons handling macaques under these circumstances might be at risk for exposure to MaHV1.

Macacine herpesvirus 1 (MaHV1; also known as B virus) is a zoonotic pathogen that is enzootic among macaque (*Macaca* spp.) populations throughout Asia (*1*,*2*). MaHV1 is an α-herpesvirus related to human herpes simplex viruses (HSV) 1 and 2 (*3*,*4*) and to herpesviruses that infect other nonhuman primates such as baboons (*5*). Like HSV infection in humans, MaHV1 infection in macaques can clinically appear as vesicular lesions on the mucous membranes of the buccal cavity and genital area (*6*,*7*). However, macaques without clinically apparent lesions can still shed MaHV1 (*6*).

Transmission of MaHV1 can occur transcutaneously (via bites) or permucosally (via exposure to macaque body fluids) (*8*,*9*). Among humans, ≈40 cases of MaHV1 encephalitis have been reported; all patients were laboratory workers who had come in contact with rhesus macaques (*M. mulatta*) only or with rhesus macaques and long-tailed macaques (*M. fascicularis*) or their tissues in the research environment (*2*,*3*). For these patients, signs and symptoms of MaHV1 infection included skin ulcers and lesions at the site of injury, influenza-like illness, and infection of the peripheral and central nervous systems (which can develop into brainstem encephalomyelitis and death) (*7*,*9*). The mortality rate for humans with untreated MaHV1 infection is >70% (*7*). This high case-fatality rate has led to strict regulations for handling macaques and macaque clinical samples in laboratories and resulted in the designation of MaHV1 as a Biosafety Level 4 (BSL-4) pathogen and, until recently, a select agent (*2*,*7*,*9*).

In macaques, MaHV1 frequently remains latent in the trigeminal and lumbosacral ganglia; however, in response to stress, it can be asymptomatically reactivated and shed in saliva and urogenital excretions (*10*). Macaques typically acquire MaHV1 at sexual maturity (*11*); previous studies have found IgG against MaHV1 in up to 100% of sexually mature wild or laboratory long-tailed and rhesus macaques (*11*,*12*). As with other viral infections, the presence of IgG indicates previous exposure or infection but does not indicate active virus shedding. During active infection, MaHV1 DNA can be detected in saliva or urogenital samples by use of PCR. Virus culture is also possible but is not routinely performed because doing so safely requires a BSL-4 laboratory (*11*). Using PCR as a diagnostic method has advantages over culture in that it can be performed under BSL-2 conditions, it produces results more rapidly, and its sensitivity and specificity are higher (*13*,*14*).

Little is known about the shedding rate of MaHV1 in macaques outside the laboratory setting, the frequency of transmission to humans, or the incidence of MaHV1 encephalitis among humans (particularly those with frequent contact with macaques). In Asia, at least 50% of cases of encephalitis are never diagnosed to the point of causative agent identification (*15*). Understanding the ecology of MaHV1 among macaques is essential for understanding

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the potential for human infection. Macaques have adapted to urbanized human environments, and contact between humans and macaques can occur in a variety of contexts (e.g., feeding in public recreational areas, capture of wild macaques for the pet trade or biomedical research colonies, consumption, or population management by wildlife authorities). Human–macaque contact can result in bites, scratches, and indirect exposure to macaque body fluids (*16*,*17*). Simian foamy virus, a nonpathogenic retrovirus found in nonhuman primates including macaques, has been transmitted during occupational exposure to macaques via bites and scratches in many of the aforementioned contexts and in agricultural, suburban, and urban environments (*18*–*20*). Zoonotic transmission of simian foamy virus to humans has been demonstrated in Indonesia (*18*) and Bangladesh (*19*). Because exposure to MaHV1 can occur through similar routes (*8*), its transmission under circumstances similar to those of transmission of simian foamy virus in Asia is plausible.

In Peninsular Malaysia, conflict between humans and macaques in residential and public areas results from loss of macaque habitat, successful macaque adaptation to human environments, and subsequent macaque overpopulation. As a result, the Department of Wildlife and National Parks (DWNP) in Peninsular Malaysia implemented a macaque population management program, which includes the removal or translocation of macaques from a conflict area. The possibility of exposure to MaHV1 during macaque capture and transport presents a potential occupational hazard to wildlife personnel.

Our aim with this study was to describe the prevalence of MaHV1 shedding among wild-caught long-tailed macaques after capture and transport in Peninsular Malaysia. This study represents a step toward understanding the potential for zoonotic transmission of MaHV1 outside the laboratory.

Materials and Methods

Capture and Sample Collection

Independently of this study, DWNP, as part of their macaque management program throughout Peninsular Malaysia, captured and transported macaques from 6 states (Johor, Perak, Pahang, Pulau Pinang, Selangor, and Negeri Sembilan) to DWNP holding facilities. Capture and opportunistic blood sampling was performed by DWNP and EcoHealth Alliance during September–November 2009, July–October 2010, and July 2011. The macaques captured had been free ranging and lived in the peripheral vegetation of rural, suburban, and urban communities in several states of Peninsular Malaysia. Trapped animals were transferred into transport cages and taken to the nearest local DWNP facility, where they were held up to 72 h before being transported to the DWNP headquarters in Kuala Lumpur or relocated to a new area. Macaques were kept in groups in cages and provided with food and water throughout the holding period. Animals were captured in accordance with the protocols and guidelines of the Manual for Human–Macaque Conflict Management in Peninsular Malaysia (*21*). This study was conducted under Institutional Animal Care and Use Committee approval no. 18048 from the University of California (Davis, CA, USA). When handling and sampling macaques, personnel involved with this study wore personal protective equipment (PPE; e.g., eye protection, double-layered nitrile gloves, Tyvek coveralls, and P100 respirators) (*22*). Blood and swab samples were collected from each animal at its arrival at the headquarters or at the local DWNP facility before relocation. Macaques were immobilized with an intramuscular injection of a combination of 5 mg/kg ketamine and 5 mg/kg xylazine (*21*). After immobilization, oropharyngeal swab and urogenital swab samples were collected (when possible, urine was also collected by cystocentesis). The samples were placed in 2 mL cryovials (Nalgene Nunc International, Rochester, NY, USA) with 500 µL NucliSens lysis buffer (bioMérieux, Marcy l'Étoile, France) and immediately stored at -80°C. Macaque weight, body condition, sex, and approximate age were recorded. The age of the animals was determined by assessing their weight, body size, and the development of their incisors and genitals (*23*). Macaques were categorized as adult (≥7 years), subadult (3–6 years), or juvenile (1–3 years). The sex and age of 2 animals and the sex of 1 adult animal were not recorded.

Molecular Testing

The samples were vigorously mixed; 100 µL of the sample was used for mechanical nucleic acid extraction by use of the NucliSENS miniMAG system (bioMérieux). The extracted nucleic acid was eluted with 60 µL of buffer. PCR was performed as previously described and validated by Scinicariello et al. (*13*). Briefly, MaHV1 primers (B virus 1, 5′-ACCTCACGTACGACTCCGACT-3′; and B virus 2, 5′-CTGCAGGACCGAGTAGAGGAT-3′; 2.5 µmol/L) were each added to the extraction product and HotStarTaq Plus Master Mix (QIAGEN, Hilden, Germany). The product was placed in a thermocyler at 94°C for 5 min and then underwent 30 cycles as follows: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The products (10 µL) were then analyzed by electrophoresis on 1% agarose gels. Of the ≈10% of samples that were positive by PCR, 14 PCR products were randomly selected and purified with a PCR purification kit (QIAGEN) and sequenced by using the same primers to confirm identity. Sequences were 128 bp and were analyzed by using a BLAST search of GenBank (*24*). Because PCR is reported to be highly specific (*13*) and all 14 PCR products showed 93%–100% nucleotide homology to

MaHV1, we considered the other PCR products with identical amplicon size to also be positive for MaHV1. PCR is more sensitive than culture for detecting HSV (*25*,*26*), and we considered the detection of MaHV1 DNA in a sample as an indication of virus shedding, although viral load was not obtained through culture or quantitative PCR.

The positive control was produced in a BSL-4 facility and removed from containment by use of inactivation procedures approved by the Texas Biomedical Research Institute Biohazard Committee. Briefly, macacine herpesvirus 1 strain E2490 virion "mini-prep" DNA was generated as previously described (*27*). With the same B virus 1 and 2 primers, the region between nt 54886 and 54993 was amplified by PCR from the viral genome (*13*) by using the FailSafe PCR Enzyme Mix (Epicenter, Madison, WI, USA). This region corresponded to a region in the *UL28* open reading frame. The resulting 128-nt fragment was cloned into pCR2.1-TOPO by using the TOPO TA kit (Invitrogen, Carlsbad, CA, USA) to generate the *pMHUL28* gene. The insert was sequenced and confirmed.

PCR sensitivity (limit of detection) was determined by using DNA from *pMHUL28* and 2 confirmed-positive samples by diluting the DNA to copy numbers of 2.71×10^4 for *pMHUL28* and 1.59×10^{11} for the samples. The sensitivity limit for *pMHUL28* by PCR was 9.13×10^2 molecules, and for the swab samples it was $\approx 1 \times 10^4$ molecules. The PCR sensitivity was previously determined to be ≈100 gene copies by using purified viral DNA, and specificity was determined by *SacII* restriction enzyme analysis and Southern blot hybridization by using an MaHV1-specific internal probe (5′-GGAGAAGACGTCGCGGTCGTAC-3′) that discriminates MaHV1 from HSV (*13*).

Immunoassay

A subset of 149 animals, randomly chosen to represent each age group, were tested by the MaHV1 ELISA as described by Ohsawa et al. (*28*). Although the exact specificity and sensitivity of the original MaHV1 ELISA was not determined, it had been validated by testing of known MaHV1-positive (n = 14) and negative (n = 6) serum, and the assay correctly detected 100% of the positive samples and provided negative results for 100% of the negative samples (R. Eberle, pers. comm., 2013). In brief, MaHV1-infected and noninfected cell antigens were added to 96-well round-bottom plates and prepared as previously described (*28*). Wells were blocked with phosphate-buffered saline containing 5% bovine serum albumin and 0.05% Tween 20 (PBS-BSA-Tw) and were incubated at 37°C for 1 h, then rinsed with PBS-Tw. Serum samples were diluted to 1:100 with PBS-BSA-Tw, added to the plate (50 µL/well), and incubated at room temperature for 2 h. The wells were washed 5 times with PBS-Tw. Biotinylated anti-human IgG (Vector Laboratories, Burlingame, CA, USA) was diluted 1:5,000 with PBS-BSA-Tw (50 µL/well) and incubated at room temperature for 1 h. The wells were washed 5 times with PBS-Tw. A complex of avidin and biotinylated peroxidase was prepared according to the manufacturer's instructions, diluted to 1:32, added to washed wells (50 µL/well), and incubated at room temperature for 1 h. The wells were washed 5 times with PBS-Tw. A 3,3′,5,5′-tetramethylbenzidine substrate solution was added (100 µL/well), and the plates were incubated without light for 8–12 min. The reaction was stopped with 2 mol/L sulfuric acid (50 µL/well), and the optical density at 450 nm was measured by using a microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA). An ELISA result was considered positive when the optical density was >0.1 (*28*).

Statistical Analyses

We calculated standard prevalence rates and 95% CIs (*29*) for differences in shedding prevalence based on macaque sample type, sex, and age. For pairwise analysis (2 parameters) of proportions, we conducted a *z*-test. A general linear model was used to investigate the effect of geographic location by state, sex, and age, and the Akaike Information Criterion was used to select the best-fit model (with no significant interactions between the variables). An analysis of variance of the general linear model with a post hoc Tukey HSD (honest significant difference) test was used to assess the significance of each factor. Statistical analyses were conducted by using the R Statistical package (R Core Team, Vienna, Austria). A p value of ≤ 0.05 was considered statistically significant.

Results

Samples from 392 long-tailed macaques from 6 states within Peninsular Malaysia (Figure) were screened by PCR; 149 of these were also screened by ELISA (Table 1). The overall detection of MaHV1 DNA in macaques, in urogenital and/or oropharyngeal samples (n = 392 tested), was 39.3% (95% CI 34.5%–44.1%). All 14 sequenced DNA samples displayed 93%–100% homology with those of MaHV1. Shedding status did not differ significantly among age groups: 37.6% (95% CI 31.2%–43.9%) among 221 adults, 38.8% (95% CI 28.1%–49.4%) among 80 subadults, and 43.8% (95% CI 33.5%–54.1%) among 89 juveniles (for 2 animals, age was not recorded). Male macaques were more likely than females to be shedding the virus at the time of sampling; prevalence was 44.1% (95% CI 37.5%–50.7%) among 220 males and 33.1% (95% CI 26.0%–40.2%) among 169 females; the sex of 3 animals was not recorded (*z*-statistic = 2.192466, degrees of freedom [df] = 1, p = 0.0001) (Table 1). Males were also significantly more likely than females to shed virus in saliva; prevalence was 26.4% (95% CI 20.5%–32.2%) among 220 males and 16.0% (95% CI 10.5%–21.5%)

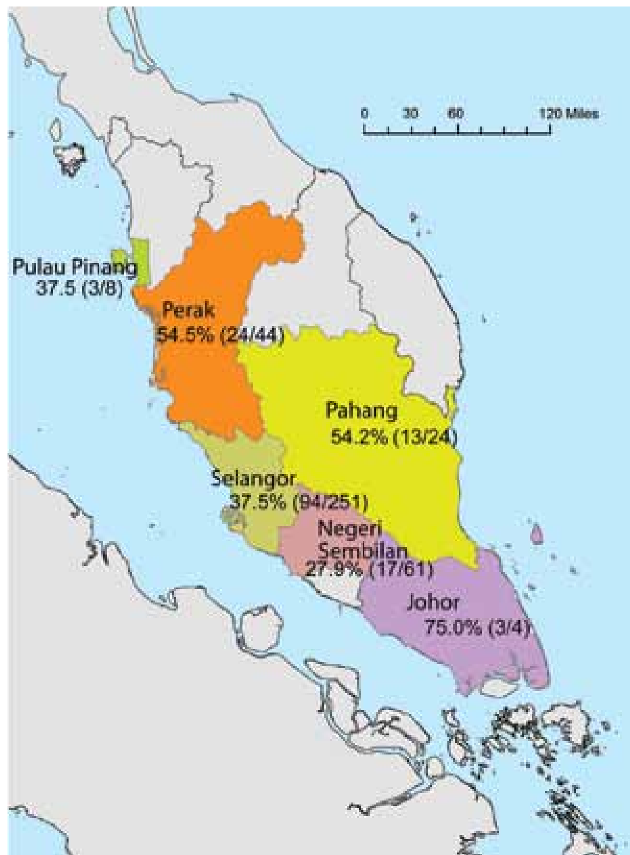

Figure. State of origin and prevalence of macacine herpesvirus 1 shedding within sampled groups of macaques (no. positive/total tested) from Peninsular Malaysia, September 2009–July 2011.

among 169 females (z-statistic = 2.457458, df = 1, p = 0.007). Overall, the proportion of urogenital and oropharyngeal samples positive for MaHV1 DNA did not differ significantly: 24.7% (95% CI 20.5%–29.0%) of urogenital samples and 21.9% (95% CI 17.8%–26.0%) of oropharyngeal samples were positive. We detected viral DNA in oropharyngeal and in urogenital swabs for 18.8% (95% CI 12.7%–25.0%) of the 154 macaques with positive results by PCR (Table 2).

Overall, IgG against MaHV1 was found in 73 (49.0%; 95% CI 38.5%–57.0%) of 149 macaques. We found that seroprevalence differed significantly among age groups: IgG was found in 70.0% of 50 adults, 46.0% of 50 subadults, and 30.6% of 49 juveniles (χ^2 = 15.6333, df = 2, p = 0.0004) (Table 3). Among macaques tested by ELISA, 24.2% (95% CI 17.3%–31.0%) were positive according to PCR but negative according to ELISA results, although these animals did not differ significantly by age, sex, or shedding site (oropharyngeal vs. urogenital) (Table 4).

The geographic origin of macaques that were MaHV1 positive by PCR was as follows: 4 (75.0%; 95% CI 32.6%–100%) were from Johor, 44 (54.5%; 95% CI 39.8%–69.3%) from Perak, 24 (54.2%; 95% CI 34.2%–74.1%) from Pahang, 8 (37.5%; 95% CI 4.0%–71.0%) from Pulau Pinang, 251 (37.5%; 95% CI 31.5%–43.4%) from Selangor, and 61 (27.9%; 95% CI 16.6%–39.1%) from Negeri Sembilan (Figure). An analysis of variance of the general linear model indicated that both sex and geographic location (state) were significantly associated with detection by PCR, but only the effect of sex (F-statistic = 3.97, df = 1, p = 0.047) had enough power to remain significant after a post hoc Tukey HSD (for categorical data) was applied (p = 0.049).

Discussion

We examined MaHV1 shedding among free-ranging macaques after capture and transport, a scenario under which occupational exposure could occur. In addition to the risk that capture and transport poses for handlers, macaques are probably under increased physiologic stress during capture and transport, which might result in increased virus activation and shedding.

Despite little published data for shedding prevalence in free-ranging or recently captured wild macaques with which to compare our findings, serologic evidence from wild-caught macaques transported from India to the United States in the 1950s for polio vaccine testing indicates that the stress of transport probably led to increased MaHV1 seroprevalence from 10% before transit to 70% after transit (*30*). These animals were young (1.0–1.5 years of age) and were kept in groups of 60. PCR studies of laboratory macaques (rhesus and long-tail) have reported a shedding prevalence range of 0%–71% (*11*,*14*,*31*). Sample sizes in these studies were generally very small, and most reported

Table 1. PCR results for macacine herpesvirus 1 in macaques, by age and sex. Malaysia, 2009–2011*

	Male		Female		Unspecified†		Total	
Age group, y	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive	No.	No. (%) positive	No.	No. (%; 95% CI) positive
Adult, >6	120	55 (45.8; 36.9–54.7)	100	28 (28.0; 19.2–36.8)	1	0	221	83 (37.6; 31.2–43.9)
Subadult, 3–6	48	19 (39.6;25.7–53.4)	32	12 (37.5; 20.7–54.3)	0	0	80	31 (38.8; 28.1–49.4)
Juvenile, <3	52	23 (44.2; 30.7–57.7)	37	16 (43.2; 27.3–59.2)	0	0	89	39 (43.8; 33.5–54.1)
Unspecified†	0	0	0	0	2	1 (50.0)	2	1 (50.0; NA)
Total	220	97 (44.1; 37.5–50.7)‡	169	56 (33.1; 26.0–40.2)‡	3	1 (33.3)	392	154 (39.3; 34.5–44.1)

*NA, not applicable.
†2 macaques of unspecified age and sex and 1 adult macaque of unspecified sex were included in the study.
‡Indicates a significant difference (p<0.05) between the 2 groups marked.

Table 2. PCR results for macacine herpesvirus 1 in macaques, by sample type, Malaysia, 2009–2011*

	Oropharyngeal		Urogenital		Oropharyngeal and urogenital	
Sex	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive
M	220	58 (26.4; 20.5–32.2)†	220	56 (25.5; 19.7–31.2)	97	17 (17.5; 10.0–25.1)
F	169	27 (16.0; 10.5–21.5)†	169	41 (24.3; 17.8–30.7)	56	12 (21.4; 10.7–32.2)
Unknown‡	3	1 (33.3; 0–86.7)	3	0	0	0
Total	392	86 (21.9; 17.8–26.0)	392	97 (24.7; 20.5–29.0)	29 (18.8)	29 (18.8; 12.7–25.0)

*For animals with positive results for both oropharyngeal and urogenital samples, percentages are of the total number of positive animals.
†Indicates a significant difference (p<0.05) between the 2 groups marked for oropharyngeal swabs.
‡3 macaques of unspecified sex were included in the results.

prevalence rates were <10%. Shedding prevalence determined in our study certainly falls within these ranges.

We observed that shedding prevalence was significantly higher among male than female macaques, although seroprevalence did not differ. This difference could be related to sociobiological dominance behavior by which females typically remain at the dominance level of their mother, whereas males lose that dominance rank when they leave the group at the time of dispersal. Thus, males continually must earn their ranking as they change groups (*32*). This behavior might predispose males to greater social stress during capture and transport. Unfortunately, we were unable to separate sex-based differences in physiologic stress from the potential effects of the stress of capture and transport, which might have affected male macaques differently than females.

We observed a significant difference in seroprevalence among macaques in different age groups; seroprevalence was highest among adults. This finding was consistent with findings of previous studies (*11*,*12*); however, we did not observe an age-based difference in shedding prevalence. One potential bias in our sampling strategy was that the age groups, which were composed of randomly selected individuals, did not reflect the age ratio of the overall group, which might have contributed to the lack of difference among age groups in shedding prevalence. We had expected to see a lower rate of shedding among juvenile than among adult animals because seroconversion is evident at sexual maturity for most laboratory and free-ranging macaques (*33*). It could be that younger animals were experiencing primary infection from exposure during capture and transport, which could explain the higher than expected shedding prevalence for this age group and in the overall study.

Our primary aim with this study was detection of MaHV1 DNA in macaques. However, we included serologic test results from a subset of animals to identify antibody seroprevalence among macaques in different age groups and to determine whether shedding occurs in the absence of detectable antibodies. We detected viral DNA in 36 seronegative macaques. This finding may have been the result of a recent primary infection before detectable IgG response. Some animals could have been infected by conspecifics during transport or just before capture. It is also possible that these animals were experiencing acute virus reactivation resulting from the stress of capture and transportation during the 6–72 h before sampling because previously infected animals would probably be seropositive. Unfortunately, data for the duration of time between capture and sampling were not available. The incubation period for HSV-1 or HSV-2 in humans is 2–12 days (*34*). Although the time between onset of stress and MaHV1 reactivation has not been determined for macaques, in mice, HSV can reactivate in as little as 14 h after exposure to a stressor (*35*). Approximately 80% of the animals shedding MaHV1 in the absence of detectable IgG were subadults or juveniles, suggesting that this infection was their first.

The low frequency of simultaneous MaHV1 detection in oropharyngeal and urogenital swab samples suggests a variable shedding pattern among individuals, which was not unexpected given the fact that the virus can sequester itself in the ganglia of the trigeminal nerve, sacral nerve (*36*,*37*), or both, which would probably affect the route of virus excretion. Other MaHV1 studies have also reported inconsistent detection of virus in oral and genital secretions from infected laboratory macaques sampled repeatedly over time (*11*,*31*). Sensitivity of the PCR assay we used was lower than that of the one used by Scinicariello et al. (*13*), which might have resulted in underdetection of viral DNA in macaque clinical samples. Use of real-time PCR (not available for this study), such as that developed by Huff et al. (*38*), which has a sensitivity of 10 viral particles,

Table 3. ELISA results for macacine herpesvirus 1 antibodies in , by age and sex, Malaysia, 2009–2011

	Male		Female		Unspecified		Total	
Age group, y	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive	No.	No. (%; 95% CI) positive
Adult, >6*	32	21(65.6; 49.2–82.1)	17	13 (76.5; 60.0–96.6)	1	1 (100.0; NA)	50	35 (70.0; 53.5–82.7)
Subadult, 3–6*	29	12 (41.4; 23.5–59.3)	21	11 (52.4; 34.5–73.7)	0	0 (NA)	50	23 (46.0; 28.1–59.8)
Juvenile, < 3*	26	8 (30.8; 13.0–48.5)	23	7 (30.4; 12.7–49.2)	0	0 (NA)	49	15 (30.6; 12.9–43.5)
Total	87	41 (47.1; 36.6–57.6)	61	31 (50.8; 40.3–63.4)	1	1 (100.0; NA)	149	73 (49.0; 38.5–57.0)

*Indicates a significant difference (p<0.05) between the age groups marked.

Table 4. ELISA and PCR results for macacine herpesvirus 1 in macaques, by age, Malaysia, 2009–2011

Age group, y	No. animals	No. (%) animals			
		PCR positive, ELISA positive	PCR positive, ELISA negative	PCR negative, ELISA positive	PCR negative, ELISA negative
Adult, >6	50	10 (20.0)	8 (16.0)	25 (50.0)	7 (14.0)
Subadult, 3–6	50	9 (18.0)	13 (26.0)	14 (28.0)	14 (28.0)
Juvenile, <3	49	4 (8.2)	15 (30.6)	11 (22.4)	19 (38.8)
Total	149	23 (15.4)	36 (24.2)	50 (33.6)	40 (26.8)

would substantially improve sensitivity of future studies that screen macaques for MaHV1.

The observed MaHV1 shedding patterns suggest that a substantial proportion of animals shed virus after, and potentially during, transport and that the risks for exposure to MaHV1 by wildlife personnel or others handling macaques under these circumstances should be seriously considered. Appropriate PPE, including coveralls, gloves, N95 or P100 respirators, and eye protection, are recommended for wildlife personnel when handling macaques (and any other nonhuman primate) under conditions in which stress and prolonged confinement with other macaques may contribute to increased shedding of MaHV1 and potentially other pathogens. Indeed, as a result of this study, DWNP is strengthening its existing policies requiring personnel handling macaques to wear PPE and use proper work area biosafety and disinfection techniques to reduce the risk for transmission of MaHV1 and other zoonotic pathogens, in accordance with established safety protocols (*22*). Personnel working with macaques have received additional training to increase their awareness of the potential risks for exposure to MaHV1.

Future studies should determine whether zoonotic transmission has occurred among those who have occupational contact with macaques during procedures such as capture, sample collection, treatment, and translocation (e.g., wildlife personnel) and should determine the incidence rate for infection among high-risk populations. Questions remain about the etiology of viral encephalitides throughout Asia and what proportion of these may be caused by MaHV1. Studies that examine the shedding prevalence of MaHV1 in free-ranging macaques will improve our understanding of shedding in the absence of anthropogenic stressors and, coupled with human surveillance, will enable further assessment of the potential risk for zoonotic transmission. These results will be of particular relevance to professionals who are occupationally exposed to macaques.

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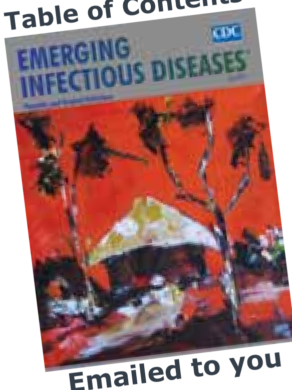

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Malaria Prevalence among Young Infants in Different Transmission Settings, Africa

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The prevalence and consequences of malaria among infants are not well characterized and may be underestimated. A better understanding of the risk for malaria in early infancy is critical for drug development and informed decision making. In a cross-sectional survey in Guinea, The Gambia, and Benin, countries with different malaria transmission intensities, the overall prevalence of malaria among infants ≤6 months of age was 11.8% (Guinea, 21.7%; The Gambia, 3.7%; and Benin, 10.2%). Seroprevalence ranged from 5.7% in The Gambia to 41.6% in Guinea. Mean parasite densities in infants were significantly lower than those in children 1–9 years of age in The Gambia ($p<0.0001$) and Benin ($p = 0.0021$). Malaria in infants was significantly associated with fever or recent history of fever ($p = 0.007$) and anemia ($p = 0.001$). Targeted preventive interventions, adequate drug formulations, and treatment guidelines are needed to address the sizeable prevalence of malaria among young infants in malaria-endemic countries.

Infants are thought to be protected against malaria during the first 6 months of life, largely due to the transfer of maternal antibodies (*1*) and the presence of fetal hemoglobin (*2*). Thus, young infants have received little attention in terms of malaria research and treatment guidelines, and this age group has systematically been excluded from clinical trials. As a consequence, young infants are frequently given off-label antimalarial treatments at dosing schedules recommended for older infants and children (*3*). The lack of attention to case management in this age group is a cause of concern and should be addressed, particularly when considering the widespread use of artemisinin-based combination therapies (ACTs) (*4*) and ongoing antimalarial drug development.

The true prevalence of malaria in young infants is not well characterized, yet defining the prevalence is critical, especially in light of ongoing epidemiologic shifts in populations at risk for malaria (*5*). Data on the prevalence and clinical outcomes of malaria in young infants are limited and contradictory: some studies show minimal risk (*6–8*), and others report that the risk for malaria increases in the first months of life, according to the intensity of transmission (*9*). A few reports indicate that the prevalence of disease is higher than previously thought and that, after birth, the period of protection against malaria is shorter than the widely quoted 6 months (*10*,*11*). However, variations in study designs and challenges related to small sample sizes, lack of details regarding quality control, and varied procedures for sample selection make it difficult to interpret findings from earlier studies (*3*). A better understanding of the risk for malaria in early infancy is needed to develop antimalarial drugs and inform policy decisions for this age group (*4*). To improve our knowledge of malaria in young infants, we used standardized methods and more sensitive diagnostics to better characterize the prevalence of malaria among children ≤6 months of age in different epidemiologic settings.

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Methods

Study Population and Sampling Design

This cross-sectional survey was conducted in 3 countries in western Africa: The Gambia, Benin, and Guinea (also known as Guinea Conakry), representing areas of low,

moderate, and high malaria transmission, respectively. In each country, regions were selected to represent overall malaria transmission trends and surveys were conducted in the catchment areas of 3 health facilities selected by using simple random sampling. In The Gambia, the Essau and the Soma Major Health Centres and the AFPRC General Hospital in Farafenni were selected out of 6 available sentinel surveillance sites. In the southern part of Benin, Bethesda Hospital and the Dodji-Bata and Golo-Djigbe health facilities were selected from a total of 12 available facilities, and in the Farannah district in Guinea, the Nalia, Tiro, and Banian health centers were also selected from 12 available facilities (Figure 1).

Malaria transmission in The Gambia and Benin is seasonal, occurring during the rainy season, whereas transmission in Guinea occurs year-round. Surveys were timed to coincide with the peak of malaria transmission in each country: October–November 2011 in The Gambia and Guinea; July–August 2012 in Benin. Before conducting the surveys, we explained the study objectives to community members in the catchment areas and obtained community approval. We identified households with infants by reviewing delivery records to detect births in the 6 months before the survey; traditional birth attendants assisted with the reviews. An information sheet explaining the objectives of the survey and the study procedures was then distributed to the parents of the infants. After written informed consent was obtained from parents, identified infants were enrolled in the study. In households with ≥2 eligible infants, 1 infant was selected by using simple random sampling. Once the index infant was selected, 2 older children (1–9 and 10–15 years of age) living in the same household were also selected by simple random sampling and included in the study with the objective of estimating the force of transmission and differences in the local risk for infection between infants and older children. If children of the required age group were not available within the infant's household, the nearest households were visited consecutively until eligible children were identified and enrolled.

Data Collection

Study participants underwent a physical examination; axillary temperature and weight were recorded for each child. Information on the use of bed nets, including long-lasting insecticidal nets, and history of fever in the previous 24 hours was collected by using a structured questionnaire. All study participants had a blood sample collected by finger prick (children 1–15 years of age) or heel prick (infants ≤6 months of age). A rapid malaria diagnostic test (RDT) (ICT Malaria P.f. Cassette Test [ML01]; ICT Diagnostics, Cape Town, South Africa) was performed, and children who tested positive were immediately treated according to treatment guidelines for the country in which they lived. Hemoglobin concentration was measured by using a HemoCue Hb 301 System (HemoCue AB, Ängelholm, Sweden) according to the manufacturer's instructions. Thick-film blood slides were stained with 10% Giemsa for 10 min, and the presence of *Plasmodium falciparum* parasites was determined by reading 100 high-power fields under oil immersion. Slides were read independently by 2 microscopists, and parasite density was estimated by counting the numbers of asexual parasites per 200 leukocytes. Results were expressed as the number of parasites per microliter, assuming a total leukocyte count of 8,000 cells/µL. A 20%

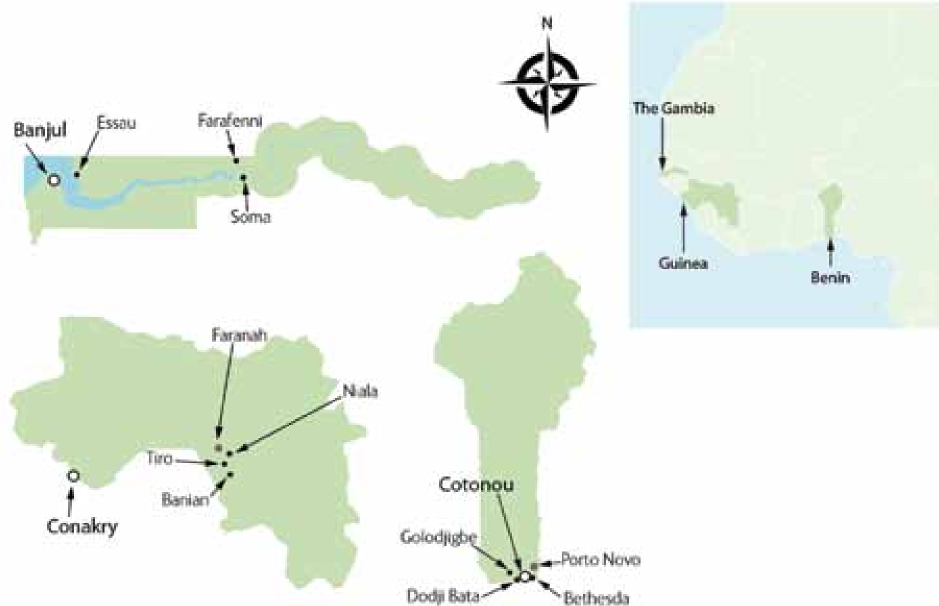

Figure 1. Study sites (arrows) in a study of malaria prevalence among young infants in The Gambia, Benin, and Guinea. Inset shows locations of the 3 countries in western Africa.

error check was used to identify discrepancies between slide readers. All discordant results were read by a senior microscopist, and the result was used as the final read. Blood slides from Guinea and The Gambia were read at the Medical Research Council (MRC) Unit in The Gambia; blood slides from Benin were read, following the same protocol, at Entomological Research Centre of Cotonou. The first 99 slides from Benin were read again in The Gambia; results were comparable.

Molecular diagnosis of malaria parasites and speciation of *Plasmodium* species were conducted by using dry blood-spot samples (DBSs) collected on filter paper (Whatman 3MM; Whatman 3 Corporation, Florham Park, NJ, USA). DNA was extracted from 3 disks (6-mm diameter), which had been punched from DBSs by using a QIA X-tractor robot (QIAGEN, Venlo, Limburg, Netherlands) according to the manufacturer's protocol, and analyzed by using nested PCR as previously described (*12*).

To determine the prevalence of malaria antibodies, we punched disks (6-mm diameter) from DBSs and placed them in 96-well plates. Serum that had been eluted after overnight (18 h) incubation at room temperature in 150 µL of reconstitution buffer (150 µL phosphate-buffered saline/0.05% [vol/vol] Tween 20/0.05% [wt/vol] sodium azide) was used to determine antibodies against the 19-kDa merozoite surface protein 1 ($MSP1_{19}$) by indirect ELISA, as previously described (*13*,*14*). $MSP1_{19}$ used in these assays was obtained from the London School of Hygiene and Tropical Medicine (London, UK).

Sample Size and Statistical Analysis

The sample size was computed on the lowest expected prevalence of infection, assumed to be 2% in The Gambia. For each country, we estimated that 750 children in each of the 3 age categories would be sufficient to determine the prevalence of malaria. Assuming infants ≤6 months of age made up ≈2%–3% of the total population, the required sample size would be found within a population of ≈40,000 persons.

Data from the case record forms were double-entered into an OpenClinica database (https://community.openclinica.com/). After being cleaned, the data were analyzed by using Stata Statistical Software, release 12.1 (StataCorp LP, College Station, TX, USA). Baseline data were analyzed by descriptive methods, and summary statistics were presented as means ±SDs for continuous data and frequencies and proportions for categorical data. The χ^2 test was used to analyze differences in proportions. Two-tailed p values and a 5% significance level were used. Results for infants ≤6 months of age from all 3 countries were pooled together, and univariate and multivariate logistic regression analyses were performed to determine features associated with malaria in this age group. A forward fitting logistic regression model was used to account for confounders and interaction. The odds for malaria with increasing age within the 0- to 6-month-old age group was determined and presented by country.

For the serologic tests, the distributions of log-transformed antibody titers were fitted as the sum of 2 Gaussian distributions, which were assumed to represent a narrower distribution of seronegative results to the left and a broader distribution of seropositive results to the right. The mean concentration of the seronegative distribution (the distribution with the smallest mean) +2 SDs was considered the seropositivity cutoff (*15*).

Ethical Considerations

The study was approved by The Gambia Government/MRC Joint Ethics Committee, National Committee of Ethics for Health Research (Benin), and the National Committee of Ethics for Health Research (Guinea). Written informed consent was obtained from the parents of each participant by a signature or thumbprint.

Results

Characteristics of the Study Population

A total of 6,761 children were included in the survey: 2,270 from The Gambia, 2,276 from Benin, and 2,215 from Guinea. The number of children categorized by age group, sex, and mean weight by age was comparable between countries (Table 1). Almost 40% (838/2,219) of the infants weighed <5 kg; no difference in weight was seen by country. In The Gambia and Benin, bed net coverage (defined as having slept under a bed net the night before the survey) was >90% in children 0–6 months and 1–9 years of age (Table 1). Conversely, bed net coverage was extremely low across all age groups in Guinea; only ≈30% of children <10 years of age and 14% of children 10–15 years age used bed nets (Table 1). Overall, the prevalence of fever was lower among children 10–15 years than among infants 0–6 months of age. The highest percentage of fevers (48.1%, 359/747] and the lowest mean hemoglobin level (10.0 g/dL [SD 1.7]) were among 1- to 9-year-old children in Guinea.

Prevalence of Malaria

By all 3 diagnostic methods, malaria prevalence was lowest in The Gambia and highest in Guinea; Benin had intermediate values. In all 3 countries, malaria prevalence was generally lower in infants 0–6 months of age (Table 2). Results from the RDT and microscopy were comparable, although, with 1 exception, the RDT tended to identify more positive samples. The exception was that microscopy showed a much higher prevalence of malaria among young infants in The Gambia (Table 2).

Table 1. Characteristics of children in a study of malaria prevalence among young infants in different transmission settings, Africa

Characteristic	The Gambia, N = 2,270	Benin, N = 2,276	Guinea, N = 2,215
Age groups, no. (%)			
0–6 mo	734 (32.3)	761 (33.4)	724 (32.7)
1–9 y	768 (33.8)	759 (33.3)	748 (33.8)
10–15 y	768 (33.8)	756 (33.2)	743 (33.5)
Sex, no. (%)			
F	1,222 (53.8)	1,189 (52.2)	1,159 (52.3)
M	1048 (46.2)	1087 (47.8)	1056 (47.7)
Mean weight, kg (SD)			
0–6 mo	5.8 (3.6)	5.2 (1.5)	5.5 (2.5)
1–9 y	12.8 (3.6)	14.3 (4.1)	14.4 (4.1)
10–15 y	31.2 (7.5)	30.1 (7.8)	31.6 (7.6)
Bed net coverage, no./no. total (%)*			
0–6 mo	699/727 (96.1)	678/750 (90.4)	225/723 (31.1)
1–9 y	724/765 (94.6)	656/722 (90.9)	222/746 (29.8)
10–15 y	642/752 (85.4)	581/727 (79.9)	103/740 (13.9)
Fever or history of fever, no./no. total (%)†			
0–6 mo	136/732 (18.6)	129/758 (17.0)	282/724 (38.9)
1–9 y	133/768 (17.3)	119/758 (15.7)	359/747 (48.1)
10–15 y	56/760 (7.4)	83/756 (11.0)	221/743(29.7)
Mean hemoglobin level, g/dL (SD)			
0–6 mo	11.8 (2.2)	11.3 (2.0)	12.1 (3.7)
1–9 y	10.9 (1.5)	11.2 (1.4)	10.0 (1.7)
10–15 y	12.4 (1.4)	12.3 (1.6)	11.8 (1.4)

*Defined as having slept under a bed net the night before survey.
†History of fever refers to fever in the 24 h before the structured questionnaire was completed.

By microscopy, all malaria cases identified in children from The Gambia were determined to be caused by infection with *P. falciparum* parasites. In Benin and Guinea, *P. malariae* and *P. ovale* parasite infections were also identified, predominantly as mixed infections. In Guinea, the prevalence of *P. malariae* parasite infections was 0.3% (2/724) in young infants, 12.0% (90/748) in children 1–9 years of age, and 5.8% (43/743) in children 10–15 years of age. Of these infections, 97% (131/135) were mixed infections with *P. falciparum* parasites. The prevalence of *P. ovale* parasite infection in Guinea was 3.1% (23/748) in children 1–9 years of age and 0.9% (7/743) in children 10–15 years of age; no cases were detected among young infants. In Benin, the prevalence of *P. malariae* parasite infection was 0.1% (1/761) in young infants, 1.7% (13/759) in children 1–9 years of age, and 2.8% (21/756) in children 10–15 years of age. In Benin, 34% (12/35) of the infections were mixed *P. malariae* and *P. falciparum* parasite infections. Overall, the mean parasite density per microliter of blood was 371.5 in The Gambia, 1,688.3 in Benin, and 2,037.9 in Guinea.

Table 2. Prevalence of *Plasmodium* species parasites, by testing method, among children in different transmission settings, Africa

	No./no. total (%)		
Test method, age group	The Gambia, N = 2,270	Benin, N = 2,276	Guinea, N = 2,215
Rapid malaria diagnostic test*			
0–6 mo	3/734 (0.4)	23/761 (3.0)	161/724 (22.2)
1–9 y	11/768 (1.4)	254/759 (33.5)	667/748 (89.2)
10–15 y	35/768 (4.6)	317/756 (41.9)	611/743 (82.2)
Microscopy			
P. falciparum			
0–6 mo	25/734 (3.4)	25/761 (3.3)	133/724 (18.4)
1–9 y	8/768 (1.0)	201/759 (26.5)	574/748 (76.7)
10–15 y	21/768 (2.7)	284/756 (37.6)	612/743 (82.4)
P. falciparum gametocytes			
0–6 mo	1/734 (0.1)	7/761 (0.9)	61/724 (8.4)
1–9 y	2/768 (0.3)	66/759 (8.7)	138/748 (18.4)
10–15 y	7/768 (0.9)	70/756 (9.3)	91/743 (12.2)
PCR			
Plasmodium spp.			
0–6 mo	27/734 (3.7)	78/761 (10.2)	157/724 (21.7)
1–9 y	18/768 (2.3)	243/759 (32.0)	591/748 (79.0)
10–15 y	35/768 (4.6)	324/756 (42.9)	577/743 (77.7)
P. falciparum			
0–6 mo	9/734 (1.2)	41/761 (5.4)	139/724 (19.2)
1–9 y	10/768 (1.3)	193/759 (25.4)	531/748 (71.0)
10–15 y	25/768 (3.3)	234/756 (30.9)	502/743 (67.6)

*ICT Malaria P.f. Cassette Test (ML01) (ICT Diagnostics, Cape Town, South Africa).

For the 3 countries, *Plasmodium* spp.–specific PCR also showed a higher prevalence of malaria in all age groups with increasing malaria transmission intensity (Table 2). Prevalence of malaria in infants, as determined by molecular methods, was higher in Guinea (21.7%, 95% CI 18.7%–24.7%) than in Benin (10.2%, 95% CI 8.1%–12.4%) and The Gambia (3.7%, 95% CI 2.3%–5.0%) (Table 2). Species-specific PCR results, compared with microscopy results, showed a lower prevalence of *P. falciparum* parasite infection in children >10 years of age in Benin (30.9% [234/756] vs. 37.6% [284/756]; p = 0.006) and Guinea (67.6% [502/743] vs. 82.4% [612/743]; p<0.0001) (Table 2). However, in The Gambia, species-specific PCR results, compared with microscopy results, showed a lower prevalence of *P. falciparum* infection only in young infants (1.2% [9/734] vs. 3.4% [25/734]; p = 0.005). Gametocyte prevalence by microscopy was lower in infants and increased with age and transmission intensity across the 3 countries (Table 2).

Prevalence of Malaria Antibodies

Overall, the prevalence of $MSP1_{19}$ antibodies varied from 5.7% among young infants in The Gambia to 45.9% among 1- to 9-year-old children in Guinea (Table 3). Antibody seroprevalence generally increased with age and with transmission intensity across the 3 countries. With the exception of results for children ≥1 year of age in Guinea, antibody seroprevalence was higher than the prevalence of infection as determined by microscopy (Table 3). For young infants, antibody seroprevalence was also higher than parasite prevalence and increased with transmission intensity from 5.7% in the Gambia to 36.5% in Benin and 41.6% in Guinea.

Malaria in Infants 0–6 Months of Age

The overall prevalence of malaria among infants was 8.2% (183/2,219) as determined by microscopy and 11.8% (262/2,219) as determined by PCR; the prevalence was substantially higher in Guinea than in Benin or The Gambia (Table 2). Mean parasite densities per milliliter of blood were significantly lower in infants than in 1- to 9-year-old children in The Gambia (68/µL [SD 168] vs. 26,708/µL [SD 32,074]; p<0.0001]) and in Benin (6,894/µL [SD 20,567] vs. 12,933/µL [SD 49,895]; p = 0.0021) but not in Guinea (5,725/µL [SD 11,423] vs. 5,479/µL [SD 21,689]; p = 0.89).

Malaria in infants was significantly associated with fever or history of fever in the previous 24 hours (adjusted odds ratio [aOR] 1.65, 95% CI 1.15–2.37; p = 0.007), axillary temperature ≥37.5°C (aOR 2.07, 95% CI 1.08–3.98; p = 0.029), and anemia (aOR 5.54, 95% CI 3.91–7.84; p = 0.001) (Table 4). Infants weighing <5 kg had significantly higher odds for having malaria (aOR 3.45, 95% CI 2.22–5.26, p = 0.001).

In The Gambia and Benin, the odds for having malaria remained almost stable across the 0- to 6-month-old age group (Figure 2). In contrast, lower odds for having malaria was seen in infants 0–2 months of age in Guinea and markedly increasing odds for having malaria was seen in infants 2–6 months of age. The overall trend was lower odds for malaria in infants 0–3 months of age and subsequently increasing odds for malaria from ≈3 to 6 months of age (Figure 2). In Guinea and Benin, antibody titers were higher early in infancy, declined steadily over the 0- to 4-month age range, and then increased slightly after 4 months of age. Conversely, in The Gambia, infants had lower antibody titers and little evidence of increasing titers over the 0- to 6-month age range (Figure 3).

Discussion

The prevalence of malaria among infants 0- to 6-months of age was not trivial (range 3.7%–22%, by PCR) and increased with transmission intensity, as documented by the prevalence among older children. This variability in prevalence may be due to differences in transmission intensity but may also be due to differences in the use of preventive measures, as illustrated by the extremely low use of bed nets in Guinea. Such low intervention coverage may be an indicator of weak health systems with limited access to

Table 3. Anti–$MSP1_{19}$ antibody seropositivity and *Plasmodium falciparum* parasite prevalence among children in different transmission settings, Africa*

Country, age group	Parasite prevalence, no. (%)†	Antibody seropositive, no. (%)	p value
The Gambia, N = 2,270			
0–6 mo	25 (3.4)	33 (5.7)	0.044
1–9 y	8 (1.0)	53 (7.0)	<0.001
10–15 y	21 (2.7)	57 (8.1)	<0.001
Benin, N = 2,276			
0–6 mo	25 (3.3)	269 (36.5)	<0.001
1–9 y	201 (26.5)	303 (41.5)	<0.001
10–15 y	284 (37.6)	311 (42.5)	0.054
Guinea, N = 2,215			
0–6 mo	133 (18.4)	299 (41.6)	<0.001
1–9 y	574 (76.7)	339 (45.9)	<0.001
10–15 y	612 (82.4)	320 (44.2)	<0.001

*$MSP1_{19}$, 19-kDa merozoite surface protein.
†Prevalence as determined by microscopy.

Table 4. Characteristics of 0- to 6-month-old infants with malaria in 3 African countries with different transmission settings

Variable	No malaria, n (%), N = 2,036	Malaria, n (%), N = 183	Odds ratio (95% CI) Crude	Odds ratio (95% CI) Adjusted*	p value
Country					
The Gambia	709 (96.6)	25 (3.4)	Reference	Reference	
Benin	736 (96.7)	25 (3.3)	0.96 (0.55–1.69)	0.74 (0.41–1.32)	0.307
Guinea	591 (81.6)	133 (18.4)	6.38 (4.11–9.92)	5.28 (3.30–8.44)	0.001
Sex					
F	1,040 (92.8)	81 (7.2)			
M	996 (90.7)	102 (9.3)	1.31 (0.97-1.78)	1.13 (0.80–1.59)	0.491
Fever or history of fever†					
No	1,573 (94.4)	94 (5.6)			
Yes	458 (83.7)	89 (16.3)	3.25 (2.38–4.44)	1.65 (1.15–2.37)	0.007
Anemia‡					
No	1,724 (95.5)	81 (4.5)			
Yes	312 (75.4)	102 (24.6)	6.96 (5.01–9.67)	5.54 (3.91–7.84)	0.001
Weight, kg					
<5	1,225 (88.7)	156 (11.3)			
≥5	811 (96.8)	27 (3.2)	0.26 (0.17–0.40)	0.29 (0.19–0.45)	0.001

*Adjusted for country, sex, fever, anemia, age, and weight.
†History of fever refers to fever in the 24 h before the structured questionnaire was completed.
‡Defined as hemoglobin level of <10 g/dL.

other malaria control interventions (e.g., prompt and efficacious treatment and intermittent preventive treatment for pregnant women), which enable an efficient cycle of malaria transmission in local populations, including infants.

In the low-transmission setting in The Gambia, the risk for malaria did not vary substantially between age groups. This finding was in obvious contrast to those in Benin and Guinea, where prevalence among infants was substantially lower than that among older children. This suggests that in areas where transmission has decreased substantially to low levels, the risk for infection may be shared by the entire population, including infants, and in high-transmission settings, infection in infants may be relatively limited by passively transferred maternal antibodies or possibly by lower attractiveness of infants to mosquitoes (*16*,*17*). The prevalence figures reported are consistent with those of earlier studies, which were limited by smaller sample sizes, different selection criteria, and small geographic areas (*4*,*6*,*11*,*18–20*). The results of our surveys in these 3 countries in western Africa provide a regional estimate of the current prevalence of malaria among infants. The survey was conducted by using a relatively large sample size and robust methods of malaria diagnosis, factors that enhance the generalizability of the findings to other settings.

It is not surprising that $MSP1_{19}$ antibody seroprevalence was generally higher than parasite prevalence in young infants: this finding may be a reflection of maternal antibodies passively transferred to the fetus during the last trimester of pregnancy (*21*) and not necessarily a reflection of the infant's own responses. Prenatal transfer of antibodies may also explain the dynamics of $MSP1_{19}$ antibody titers in young infants. In Guinea and Benin, high titers were observed in children in early in infancy, followed by a rapid decline in mean antibody titers until 4 months of age and then a subsequent slight increase. In contrast, infants in The Gambia had lower antibody titers and little evidence of an increase over the 0- to 6-month age range, indicating no substantial ongoing endogenous antibody production.

P. falciparum was the dominant parasite species in all age groups, but a few cases of *P. malariae* and *P. ovale* infection (mostly mixed infections with *P. falciparum* parasites) were found among infants in Guinea and Benin. Therefore, currently available ACTs should suffice for the management of these cases, although failed parasite clearance has been reported in some *P. malariae* and *P. ovale* parasite–infected persons treated with ACTs (*22*).

Malaria in infants was significantly associated with fever or with a history of fever in the 24 hours before the survey, but only 10% of infants with malaria had an axillary

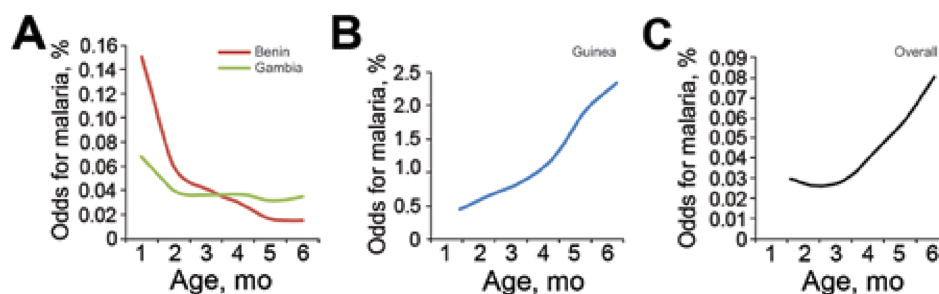

Figure 2. Odds of having malaria, with increasing age, in infants 0–6 months of age in Benin and The Gambia (A), Guinea (B), and in the 3 countries overall (C). Note that the scale of the *y*-axis in panel B differs from that in panels A and C.

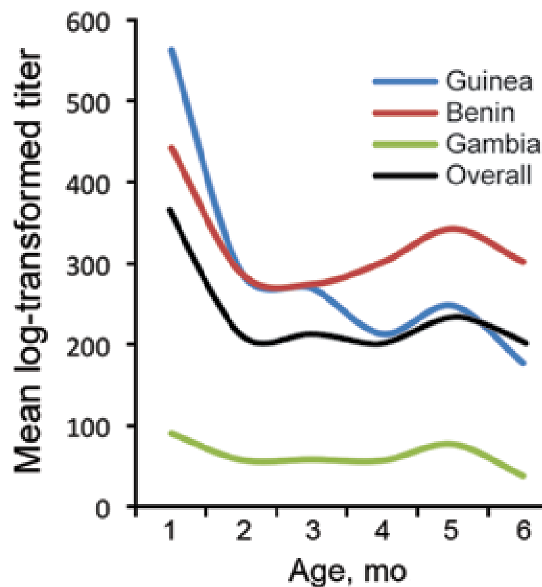

Figure 3. Dynamics of 19-kDa merozoite surface protein antibody titers by infant age in Benin, The Gambia, and Guinea and in the 3 countries overall.

temperature ≥37.5°C at the time of the survey. Although parasite densities in infants were lower than those in older children, about half of the infants with malaria were symptomatic. This finding contrasts with the long-held belief that malaria in young infants is not associated with clinical symptoms (*23*,*24*). The findings from this survey therefore provide evidence that malaria in young infants may be symptomatic and should be evaluated for and treated. In addition, malaria in this age group was significantly associated with anemia, indicating that malaria can have a major negative effect on the health of infants. Other previously reported clinical manifestations (e.g., splenomegaly, hepatomegaly, jaundice, vomiting, diarrhea, poor feeding, restlessness, drowsiness, pallor, respiratory distress, and convulsions) (*25*,*26*) were not consistently documented in this survey. A study systematically investigating for malaria in all 0- to 6-month-old infants attending health facilities in the same areas as this survey has recently been completed and should provide more information on the clinical signs and symptoms of malaria in this age group; that study used RDTs, microscopy, PCR, and hemoglobin measurements.

We have shown that malaria in young infants is not rare, can be symptomatic, and has major health consequences, most notably anemia. Current World Health Organization guidelines recommend the use of ACTs in infants, but they specify that for young infants weighing <5 kg, the available evidence is insufficient to confidently recommend this treatment. Thus, many of the ACTs carry label restrictions saying they should not be used for infants weighing <5 kg (*27*). This restriction is problematic because a substantial proportion (40%) of the infants in these surveys weighed <5 kg and would therefore not meet standard criteria for treatment with ACTs. In addition, there are few pediatric ACT formulations, and the dosing is often difficult. Therefore, data on the efficacy and safety of ACTs in young infants is urgently needed to inform optimal treatment.

The tools used in our study provided comparable estimates of the prevalence of malaria in young infants, with the exception of RDTs, which greatly underestimated the prevalence of malaria in The Gambia, possibly because of the low parasite densities (*28*,*29*). Prevalence estimates determined by microscopy and PCR were surprisingly similar, which may be due to the high sensitivity of microscopy readings conducted in a research institution with strict quality-control procedures. Using microscopy, we were able to detect parasite densities as low as 2 parasites/µL of blood; it is estimated, however, that in an average health care facility with standard microscopy, the detection threshold would be 50–100 parasites/µL of blood (*30*,*31*). In some sites, the lower prevalence by PCR, compared with RDT, may be due to persistent antigenemia from past infections in the absence of current parasitemia.

The overall dynamics of infection across the 3 countries suggests that the period of protection against malaria may be the first 3 months of life, and thereafter the odds for malaria rise increasingly by age. Our findings therefore provide evidence that the period of perinatal protection may be shorter than 6 months and that the 0- to 6-month-old age group is not a homogenous group in terms of malaria susceptibility. Thus, the challenge is that young infants are not adequately protected against malaria because of their limited coverage by current preventive strategies, such as seasonal malaria chemoprevention and intermittent preventive treatment during infancy, which are not widely implemented. This inadequate coverage is critical because our findings show that young infants can be affected by malaria and subsequently become anemic, which would also potentially increase their vulnerability to other pathogens (*32*). Other interventions, such as the RTS,S/AS01 malaria vaccine, which will soon be registered for use, resulted in modest protection against clinical malaria in this age group and did not have any effect on preventing anemia (*33*).

In conclusion, the prevalence of malaria is sizeable among young infants living in malaria-endemic countries. This problem must be addressed through the development of adequate pediatric drug formulations, targeted preventive interventions, and treatment guidelines for young infants.

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Mr. Ceesay is a higher scientific officer and research coordinator at the Medical Research Unit, The Gambia. His research interests have focused mainly on the epidemiology of malaria in The Gambia and, more recently, on determining the risk for malaria in early infancy and in different transmission settings.

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Transdermal Diagnosis of Malaria Using Vapor Nanobubbles

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A fast, precise, noninvasive, high-throughput, and simple approach for detecting malaria in humans and mosquitoes is not possible with current techniques that depend on blood sampling, reagents, facilities, tedious procedures, and trained personnel. We designed a device for rapid (20-second) noninvasive diagnosis of *Plasmodium falciparum* infection in a malaria patient without drawing blood or using any reagent. This method uses transdermal optical excitation and acoustic detection of vapor nanobubbles around intraparasite hemozoin. The same device also identified individual malaria parasite–infected *Anopheles* mosquitoes in a few seconds and can be realized as a low-cost universal tool for clinical and field diagnoses.

Malaria control and elimination would benefit greatly from an efficient and universal diagnostic tool that is fast (provides results in seconds), noninvasive and safe (uses no blood sampling or reagents), simple to use (can be operated by nonmedical personnel), sensitive and specific (detects low-level asymptomatic infections), and inexpensive and that detects malarial infection in humans and in mosquitoes (*1–21*). We recently proposed a transdermal blood- and reagent-free approach based on hemozoin-generated vapor nanobubbles (H-VNBs) (*22*) in which malaria parasite–specific endogenous nanocrystals of hemozoin are optically excited in vivo with a safe and short laser pulse (delivered to blood vessels through the skin). The light is converted into nonstationary localized heat that evaporates the adjacent nanovolume of liquid and thus generates an expanding and collapsing vapor nanobubble inside the parasite. The nanosize and high optical absorbance of hemozoin provide higher malaria infection specificity of these H-VNBs than does any normal blood and tissue components (*23–26*). Their transient expansion and collapse result in a noninvasive pressure pulse that is easily detected through the skin with an ultrasound sensor. In our preliminary studies (*22*), H-VNBs detected parasitemia as low as 0.0001% in vitro (human blood), and 0.00034% in vivo (transdermal detection in animals), with no false-positive signals. Therefore, H-VNB might be able to detect extremely low parasite densities provided the method can be applied to humans or mosquitoes simply and inexpensively.

To determine the technical and medical feasibility of H-VNBs for malaria diagnosis and screening, we prototyped a diagnostic device and evaluated it in a patient with confirmed malaria and in noninfected persons as controls. We also evaluated the device in *Plasmodium falciparum*–infected mosquitoes.

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Materials and Methods

Prototype Design and Algorithms

The laboratory prototype (Figure 1, panel A) comprised the newly designed compact low-cost pulsed laser (532 nm, 10 μJ, 200 ps; Standa, Vilnius, Lithuania). The laser pulse is delivered to the skin by the combination probe at the fluence 36 mJ/cm^2 (Figure 1, panel B). The probe was developed for transdermal diagnostics and includes an optical fiber guide and a custom acoustic sensor with a preamplifier that is integrated in 1 compact hand-held unit. In response to each laser pulse, the probe detects an acoustic pulse and generates an output electrical signal as an acoustic trace (Figure 1). Its output signals were collected and analyzed with custom-designed software (NI LabVIEW, Austin, TX, USA) by using a signal amplifier, digital oscilloscope (LeCroy 42X; Teledyne LeCroy, NY, USA), and computer. The peak-to-peak amplitude A of the acoustic trace obtained in response to each laser pulse was measured and presented as a histogram for 400 sequential laser pulses. A malarial infection–negative trace histogram was used to determine "the malaria threshold" T as the maximum amplitude for the malarial infection–negative signal. Any trace with an amplitude above that threshold was considered to be hemozoin (malarial infection)–positive. To quantify the infection, we counted the incidence rate of malarial infection–positive traces IR (the probability

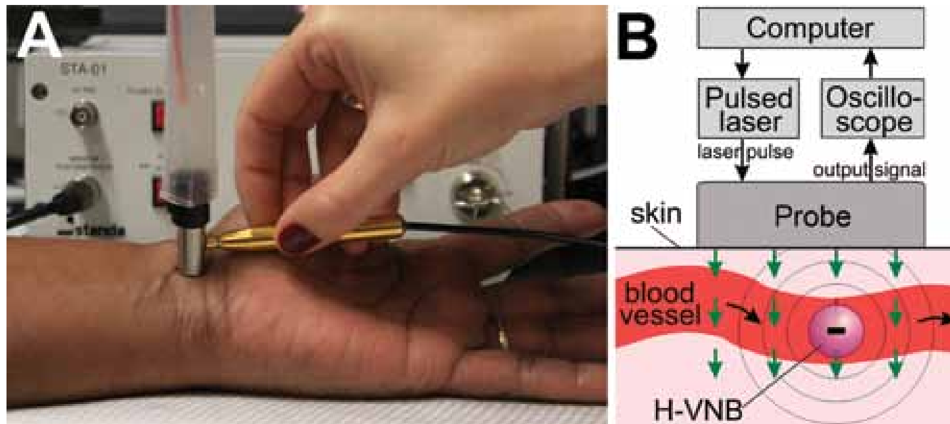

Figure 1. A) Experimental laboratory prototype of a malaria diagnostic device with the pulsed laser and the integrated probe shown being scanned across a human wrist. B) Functional diagram of the prototype and the principle of transdermal optical excitation and acoustic detection of vapor nanobubbles around hemozoin in malaria-infected cells exposed to the laser pulses (green arrows). H-VNB, hemozoin-generated vapor nanobubble.

of the trace incidence with an amplitude above the malaria threshold calculated for 400 laser pulses) and calculated the Hemozoin Index (HI) (22): HI = IR(A – T).

Monitoring of Transient Vapor Nanobubbles

The direct monitoring of transient vapor nanobubbles in response to a single laser pulse uses our optical scattering method (*27*,*28*). This method used time-resolved optical scattering of a probe continuous laser beam of very low power (633 nm, 0.1 mW). The probe laser beam was focused on the blood sample collinearly with the excitation laser pulse. The axial intensity of the probe laser beam that passed through the blood sample was monitored with a photodetector. The response to the laser pulse included bulk heating and generation of a transient vapor nanobubble. The bulk heating of the exposed blood volume (without generation of a vapor nanobubble) was detected optically by using a thermal lensing effect that revealed the fast heating and gradual cooling of the exposed volume (Figure 2, panel A, black line in inset). The generation of an expanding and collapsing vapor nanobubble created a strong localized scattering of the probe laser beam by the vapor–liquid boundary, and this effect reduced the probe beam intensity with the bubble diameter (Figure 2, panel A, red line in inset). A vapor nanobubble–specific signal typically is shaped like an inverted bell and represents the growth and collapse of the bubble.

Patient with Confirmed Malaria

The patient was admitted to Ben Taub General Hospital, Harris Health System (Houston, TX, USA), with fever, myalgia, abdominal pain, nausea, and vomiting for the previous 4 days and no history of malaria chemoprophylaxis. Malaria identification and speciation was done by microscopy (thin, Wright-Giemsa–stained, peripheral blood smears) and a rapid malaria antigen test (BinaxNow Malaria; Alere Scarborough, Inc., Scarborough, ME, USA). Both tests confirmed a *P. falciparum* malaria infection. By the time of the H-VNB test, the patient had already received antimalarial drugs (doxycycline, malarone, quinidine, and quinine) for 24 h. During hospitalization, the patient had mild hemolytic anemia and thrombocytopenia, neither of which required transfusion of blood products. In addition to the antimalarial medications and to provide symptomatic relief, the patient received intravenous fluids, antiemetics, and antipyretics.

Diagnostic Locations

We found wrist and ear lobe veins to be the optimal location for the test. Fingertips were also explored but were inadequate because some persons develop very thick and rigid skin patterns that prevent efficient transdermal delivery of a laser pulse to the blood vessels.

Influence of Skin Tone

The difference between the amplitudes of the in vitro background traces from intact blood (Figure 2, panel B, black bars) and those in vivo from a malaria-negative volunteer (Figure 2, panel D, black bars) accounts for the additional contribution of the optical absorbance by melanin in dark skin. The increase in the bulk optical absorbance consequently increased the bulk transient heating (without the generation of H-VNBs) and thus increased the average trace amplitude of the background trace from 10.7 ± 1.7 mV in blood alone (in vitro) to 18.1 ± 5.4 mV in blood and skin (in human) as can be seen by comparing the black histograms in Figure 2, panels B and D. We further studied the influence of skin tone on the background trace in 5 healthy volunteers with different skin tones (Figure 2, panel F). For 532-nm wavelength light, the predictable increase in the background trace amplitude resulted from the higher concentration of the skin pigment, melanin, which determines skin darkness (tone). Therefore, in malaria diagnosis, reference malaria-negative data (histogram and threshold) should be linked to the same level of skin tone as in the malaria patient. Although the optical absorbance

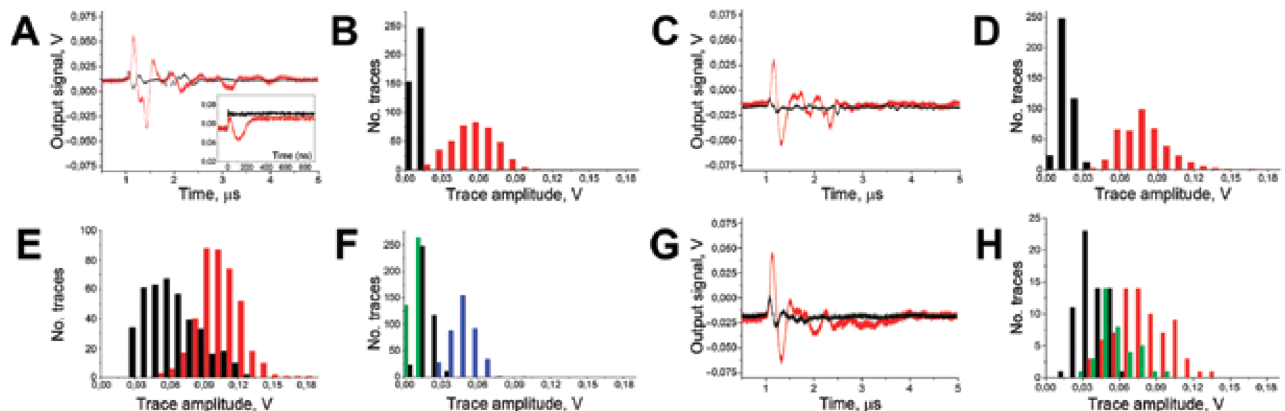

Figure 2. A) Acoustic traces obtained in vitro in response to a single laser pulse exposure (532 nm, 36 mJ/cm^2 delivered at 0 time point) from the sample of whole human blood (black) and for the same blood sample after the addition of hemozoin in the concentration corresponding to the parasitemia level of 0.8% (red). y-axis shows the output signal of the acoustic sensor, Inset: optical scattering time-responses obtained for these 2 samples show the transient bulk heating of the blood (black) and the generation of transient vapor nanobubble (red). B) Histograms of the trace amplitudes for 400 traces obtained under identical conditions for the above 2 samples. y-axis indicates number of traces with the amplitude in the specific range. C) Acoustic traces obtained from the wrist veins of an uninfected volunteer with light dark skin (black) and from a malaria-diagnosed patient with light dark skin (red). D) Histograms of the acoustic trace amplitudes obtained from the wrist veins of an uninfected volunteer with light dark skin (black) and for a malaria-diagnosed patient with similar light dark skin (red). E) Histograms of the acoustic trace amplitudes obtained from the earlobes of an uninfected volunteer (black) and for a malaria-diagnosed patient (red). F) Histograms of the acoustic trace amplitudes obtained for healthy volunteers with different skin darkness: green, pale light skin; black, light dark skin; blue, dark skin. G) Acoustic traces obtained transcuticle from individual mosquitoes: fed with uninfected blood and oocyst negative (black) and fed with malaria-infected blood and oocyst positive (red). H) Histograms of the acoustic trace amplitudes obtained from oocyst-negative (black) and oocyst-positive (red) mosquitoes and mosquitoes fed with uninfected blood mixed with hemozoin at 60 µg/mL (green).

of skin pigment and hemoglobin is not as high as that for hemozoin (*23*,*24*) and is not sufficient to generate vapor nanobubbles under the laser pulse duration and fluence used (*29*), the bulk transient heat released by hemoglobin and melanin generates a background acoustic trace, which limits the signal-to-noise ratio for the H-VNB method and thus increases the detection threshold for malaria infection. This limitation will be alleviated by developing a "malaria-specific" pulsed laser with a wavelength ≈672 nm—the focus of our ongoing effort because such lasers are not currently available. The optical absorbance of hemoglobin and melanin at 672 nm is much lower than that at 532 nm, and the amplitudes of the background signals and their dependence on skin tone will be reduced. At the same time, the laser pulse energy efficacy of H-VNB generation at 672 nm is similar to that at 532 nm, as we demonstrated previously (*22*).

Mosquito Model

For infection in mosquitos, female *Anopheles gambiae* mosquitoes were fasted for 6 hours and fed on infected blood by using jacketed membrane feeders warmed to 37°C by a circulating water bath. Briefly, cultured *P. falciparum* (NF54 strain) gametocytes were diluted to 0.3% gametocytemia, 50% normal human erythrocytes and human serum for 15 min. After removing unfed mosquitoes, blood-fed mosquitoes were maintained at 26°C and 70%–80% relative humidity on 10% dextrose. Ten days later, 15 randomly selected mosquitoes were dissected to determine the oocyst numbers, and 2 other groups were used for the device evaluation. Midguts were stained with 0.1% mercurochrome and oocysts counted microscopically. Each of the 15 mosquitoes had 9–151 oocysts (median 50). The remaining mosquitoes were killed by freezing at -20°C before analysis using the prototype device. Mosquitoes fed on uninfected blood and maintained as described were used as negative controls (uninfected and oocyst negative). For further use as positive controls, mosquitoes were fed on uninfected blood containing 60 µg/mL hemozoin.

All procedures were approved by the corresponding internal review board committees at Rice University and Baylor College of Medicine (for Ben Taub Hospital). The patient and the volunteers provided informed consent.

Results and Discussion

We have prototyped a diagnostic device (Figure 1) and evaluated it in a malaria patient and uninfected controls and in malaria infection–positive mosquitoes. Initial in vitro validation of the designed prototype used samples of whole human blood without (uninfected blood) and with hemozoin (#tlrl-hz; InvivoGen, San Diego, CA, USA)

(a proxy for malaria parasite–infected blood [22]). The sample cuvette modeled the blood vessel by using a skin-colored film, a channel with blood 1 mm deep, and an acoustically dampening bottom. The bulk optical absorption of the laser pulse at 532 nm by normal whole blood (mainly by hemoglobin) produced the background trace (Figure 2, panel A, black line) associated with the thermo-elastic effect (*30*) (a heat-driven transient pressure rise). However, no vapor nanobubbles were generated because the laser fluence applied was well below the vapor nanobubble generation threshold for any normal blood components (*22*). The absence of vapor nanobubbles was confirmed experimentally by monitoring the laser pulse-exposed sample volume with an optical scattering method (*22*,*27*) by using a low-power probe laser beam at 633 nm and monitoring its time-response to the excitation laser pulse (Figure 2, panel A, black line in inset). In normal blood, the optical scattering time-response to a single laser pulse indicated incremental transient bulk heating without generating a vapor nanobubble. Previously we have shown that such a bulk photothermal effect does not cause any detectable detrimental effects at the molecular and cellular levels (*22*). Adding hemozoin nanocrystals to the blood at a concentration of 23 μg/mL, (which corresponds to ≈0.8% of parasitemia [*9*]), resulted in a completely different acoustic trace under the same excitation and detection conditions (Figure 2, panel A, red line). This trace was attributed to H-VNBs, which were directly detected in the same sample by optical scattering with optical time-responses of 50–100 ns duration and the H-VNB-specific shape, which revealed the vapor bubble expansion and collapse without any recoil (Figure 2, panel A, red line in inset). Unlike the background acoustic traces obtained from the normal blood, the H-VNB acoustic traces yielded 5-fold higher peak-to-peak amplitudes and thus were easily differentiated from the blood background traces in the trace amplitude histograms (Figure 2, panel B).

To obtain proof of the device feasibility, the prototype was further tested in human volunteers who did not have malaria and on a malaria patient with a similar skin tone (dark). We applied the probe to wrists and earlobes and positioned it over subcutaneous vessels. Thus, laser pulses were delivered to blood through the skin. No blood samples were taken, and no reagents were applied. Acoustic traces in response to each of 400 laser pulses (of the same fluence as described earlier) were collected simultaneously with laser irradiation (within 20 seconds total) and analyzed statistically in real time. In the malaria patient, the parasitemia (percentage of infected erythrocytes) was determined by microscopy (thin blood film) and varied from 2% (corresponding to ≈69,000 parasites/μL) 4 hours before the device test to 0.3% (corresponding to ≈8,600 parasites/μL) 9 hours after the device test. As malaria-negative controls, we used healthy volunteers with similar skin tone and under the conditions and procedure applied to the malaria patient. Acoustic traces from the wrist of the malaria patient (Figure 2, panel C, red line) showed the H-VNB–specific pattern similar to that of the hemozoin-positive samples in vitro (Figure 2, panel A, red line) and had much higher amplitudes than those obtained from a healthy volunteer with a similar skin tone (Figure 2, panel C, black line). The amplitudes for the traces from the malaria patient were significantly higher than those from the control, and the 2 histograms barely overlapped (Figure 2, panel D). Therefore, these acoustic traces indicated malarial infection in a clinically ill patient. Similar traces were obtained when the device was applied to the earlobes of the malaria patient and volunteers (3 volunteers with dark skin tone were studied) (Figure 2, panel E). The similarity between the wrist and ear lobe results further validates the successful detection of malarial infection by the H-VNB method. A quantitative analysis used the volunteers' histograms to determine the malaria threshold amplitude and revealed HI values as 42.4 mV and 1.3 mV in the malaria patient for the wrist and earlobe, respectively.

The safety of H-VNB generation in humans is ensured by the safe level of the laser fluence applied, 36 mJ/cm^2, which is considered to be skin-safe according to federal regulations (*31*). In addition, no short-term (10 min) or long-term (3–4 d) signs of skin damage or irritation were observed in the study participants. These observations are also in line with our previous observation of no damage to the laser-exposed malaria parasite–negative blood cells (*22*). Therefore, the device and procedure developed appear to be safe, and coupled with their blood- and reagent-free nature, deliver a completely noninvasive diagnosis of malaria in humans.

We further studied the influence of skin tone on the background trace for 5 healthy volunteers with different skin tones (Figure 2, panel F). For 532-nm wavelength light, we observed a predictable increase in the background trace amplitude resulting from the increase in the concentration of melanin.

In the third model, we evaluated the device for the rapid noninvasive transcuticle analysis of individual malaria-infected (oocyst-positive) *Anopheles* mosquitoes. Ten acoustic traces were obtained for each mosquito (7 in each group) by scanning the body across the probe under the same laser pulse fluence as described for the human studies. The negative control group (fed with uninfected human blood, no oocysts) returned acoustic traces similar to those for hemozoin- and malaria parasite–negative human blood (Figure 2, panel G, black line). In the malaria-infected mosquitoes, the trace shape and amplitude (Figure 2, panel G, red line) were similar to those obtained for hemozoin- and malaria parasite–positive traces. The histogram

of malaria-infected mosquitoes significantly shifted to the right, compared with the negative control group (Figure 2, panel H). Finally, the positive control group of mosquitoes fed with the blood mixed with hemozoin (60 μg/mL) also showed traces of a high amplitude (Figure 2, panel H, green bars). This experiment demonstrated the feasibility of the rapid noninvasive detection of individual oocyst-carrying mosquitoes. We propose that vapor nanobubbles can be generated around residual hemozoin in developing oocysts or similar dense forms of heme formed by the malaria parasites at this stage (*32*,*33*).

These results provide a proof-of-principle for the H-VNB technology as a unique noninvasive transdermal diagnostic tool for malarial infection in humans and mosquitoes. The next step is to optimize the prototype with a malaria-specific laser operating at 672 nm for a better sensitivity (*22*). Such prototype will be evaluated in large-scale studies in humans in clinical and field settings in malaria-endemic countries. Although the estimated cost of a battery-powered device (size of a shoebox) is US $15,000, a single unit will be able to test ≈200,000 persons each year without any additional cost (e.g., specialized staff, facilities, and diagnostic reagents); thus, the cost of the individual test may be well below that of a rapid diagnostic test. The device may be able to diagnose asymptomatic and low-density infections, undetectable by microscopy and rapid diagnostic tests and could be deployed for mass screening and treatment or at border control points (a major advantage would be the speed at which results will be available). The presence of hemozoin in all blood stages and types of malaria parasites (*25*,*26*) ensures the broad and universal application of our method, even without differentiation of the malaria species. The rapid and simple detection of malaria-infected mosquitoes could provide an easy tool to estimate the transmission intensity, contributing to the efforts of malaria transmission reduction and local elimination.

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Dr. Lukianova-Hleb is a research scientist at Rice University. Her research interests include nanophotonics and nanomedicine with the focus on cancer and infectious diseases.

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etymologia

Quinine [kwin'in]

From the Quechua *kina*, "bark," quinine is an alkaloid of cinchona that has antimalarial properties. In the 1620s, Jesuit missionaries living in Peru learned of the healing powers of the bark of "fever trees" that grew in the high forests of Peru and Bolivia.

"Cinchona officinalis 001" by H. Zell. Own work. Licensed under CC BY-SA 3.0 via Wikimedia Commons.

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Lack of Transmission among Close Contacts of Patient with Case of Middle East Respiratory Syndrome Imported into the United States, 2014

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In May 2014, a traveler from the Kingdom of Saudi Arabia was the first person identified with Middle East respiratory syndrome coronavirus (MERS-CoV) infection in the United States. To evaluate transmission risk, we determined the type, duration, and frequency of patient contact among health care personnel (HCP), household, and community contacts by using standard questionnaires and, for HCP, global positioning system (GPS) tracer tag logs. Respiratory and serum samples from all contacts were tested for MERS-CoV. Of 61 identified contacts, 56 were interviewed. HCP exposures occurred most frequently in the emergency department (69%) and among nurses (47%); some HCP had contact with respiratory secretions. Household and community contacts had brief contact (e.g., hugging). All laboratory test results were negative for MERS-CoV. This contact investigation found no secondary cases, despite case-patient contact by 61 persons, and provides useful information about MERS-CoV transmission risk. Compared with GPS tracer tag recordings, self-reported contact may not be as accurate.

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Middle East respiratory syndrome coronavirus (MERS-CoV) is a lineage C betacoronavirus that was first reported in September 2012 in a patient from the Kingdom of Saudi Arabia (*1*). By September 8, 2014, a total of 837 laboratory-confirmed cases and 292 associated deaths had been reported by the World Health Organization. All reported case-patients have resided in or had recent travel to the Arabian Peninsula and neighboring countries (*2*).

Clusters of MERS-CoV infection have occurred within extended families, households, and healthcare settings (*3–6*). Contact investigations around imported cases in the United Kingdom, France, and Tunisia identified cases among household and healthcare contacts, suggesting person-to-person transmission (*7–9*). However, these investigations found limited onward transmission: a maximum of 3 second-generation cases were found among investigations with total contacts ranging from 7–163 persons (*7–9*). Other contact investigations of imported cases outside of the Middle East have found no secondary transmission (*10–13*).

On April 29, 2014, the Indiana State Department of Health (ISDH) informed the Centers for Disease Control and Prevention (CDC) of a patient under investigation for MERS-CoV infection. A clinical specimen from the patient was confirmed positive by CDC on May 2, 2014 (*5*); this infection was identified as the first imported MERS case in the United States. The case-patient, a physician and resident of Saudi Arabia, traveled by airplane to Chicago, Illinois, USA, via London, United Kingdom, then by bus to Indiana, USA. He stayed with his family in Indiana for 4 days, during which time he twice met with a business

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associate in Illinois before seeking medical care at an Indiana hospital; multiple healthcare personnel (HCP) at the hospital were exposed to the patient (*14*). Given the uncertainty around how MERS-CoV is transmitted, we conducted a comprehensive contact investigation of this case to characterize exposures in household, community, and hospital settings and to quantify the risk of transmission. We also compared contact reported by HCP during standardized interviews with those in global positioning system (GPS) tracer tag recordings.

Methods

Ethical Review

This investigation was part of a public health response, so it was determined by CDC to be a nonresearch investigation and not subject to review by the CDC Institutional Review Board. All participants provided verbal consent before interview; parental permission and assent from from minors were obtained as appropriate.

Definitions and Identification of Contacts

For the purpose of this investigation, we defined contacts as all persons who had potential exposure to the case-patient before airborne and contact precautions were instituted. More specifically, we defined HCP contacts as all persons who had a face-to-face (within 1 meter) interaction with the case-patient or who entered the case-patient's room without appropriate personal protective equipment (PPE; i.e., gloves, N95 respirator, gown, and eye protection) before airborne and contact precautions were instituted. HCP contacts were identified by reviewing GPS tracer tag logs, the case-patient's medical chart, and emergency department (ED) security video footage or through the hospital hotline, on which personnel could self-identify. GPS tracer tags were worn routinely by registered nurses (RNs) and certified nursing assistants (CNAs). The tags track the date and time that staff enter and exit a patient's room. We reviewed hospital GPS records to determine the exposure time and number of patient visits for attending RNs and CNAs.

Hospital visitor contacts were defined as all persons who visited the case-patient at the hospital before airborne and contact isolation precautions were instituted. Household contacts were defined as all persons who stayed overnight in the same household as the case-patient between his arrival in the United States and his admission to the hospital. Community contacts were defined as all persons, other than household or HCP contacts, who had face-to-face exposure to the case-patient. Hospital visitor, household, and community contacts were identified from interviews with the case-patient, family members, and hospital staff.

Duration of Exposure, Infection Monitoring, and Quarantine

Duration of exposure was determined by asking contacts how much time they had spent with the case-patient. Duration of exposure was also calculated from GPS records.

Following confirmation (on May 2, 2014) that the patient was infected with MERS-CoV, HCP and household contacts checked their body temperature twice daily and self-monitored for respiratory or gastrointestinal symptoms for a total of 14 days after their last exposure to the case-patient. HCP also reported to the hospital's Employee Health Services each day. In addition, nonphysician HCP contacts were requested to self-quarantine at home or wear surgical masks in the community, and physician HCP contacts were requested to wear surgical masks at work.

Interviews

The case-patient was asked to report his medical and exposure history, health care–seeking behaviors, job-related activities, and social activities during the 14 days before illness onset. HCP, household, and community contacts answered standard questionnaires covering basic demographic information; infection control practices when in contact with the case-patient; type, length, and frequency of contacts with the case-patient; chronic medical conditions; and symptoms since first exposure to the patient.

Biologic Specimen Collection

Serum, nasopharyngeal swab, oropharyngeal swab, stool, and urine samples were collected from the case-patient on various dates (*15*). Two sets of nasopharyngeal and oropharyngeal swab samples and serum samples were collected from all contacts. The initial and follow-up sets of specimens were collected on postexposure days 3–8 and 12–14, respectively. An additional set of specimens was collected within 48 hours from any contacts who became symptomatic.

Nasopharyngeal and oropharyngeal swab samples were tested at the ISDH laboratory, Massachusetts Department of Public Health, Illinois Department of Public Health, or CDC within 72 hours of collection. Stool and urine samples were tested at the ISDH laboratory, and serum samples were tested at CDC.

Laboratory Testing

Nasopharyngeal, oropharyngeal, urine, serum, and stool specimens were tested by using a MERS-CoV real-time reverse transcription PCR (rRT-PCR) developed by CDC, as previously described (*15*). Serum specimens collected on postexposure days 12–14 were screened for MERS-CoV–specific IgG, IgM, and IgA by using a recombinant nucleocapsid–based ELISA. Positive ELISA results were confirmed by MERS-CoV immunofluorescence assay

(IFA) and microneutralization assay (*14*). A specimen positive by ELISA, indeterminate or negative by IFA, and negative by microneutralization was determined to be negative. A positive serologic result required a positive ELISA result and confirmation by IFA or microneutralization assay. On the basis of clinical discretion, a multiplex PCR assay virus panel (Biofire Diagnostics, Salt Lake City, UT, USA) was performed on samples from the case-patient and 3 contacts.

Data Analyses

Basic descriptive analyses were conducted for all contacts. When available, self-reported and GPS-monitored exposure time and number of visits were compared by calculating Pearson correlation coefficients.

Results

Case-Patient

The case-patient worked at a Saudi Arabia hospital where patients infected with MERS-CoV had been treated in April 2014. He did not recall caring for known MERS patients or patients with respiratory symptoms, but he did perform noninvasive procedures, using appropriate PPE, on 3 or 4 intubated patients. None of his colleagues, friends, or household members had respiratory symptoms during April. Beginning on April 18 (i.e., day of illness [DOI] 1), he had low-grade fever, fatigue, and myalgias. On DOI 6, he departed for the United States; on DOI 10, a mild, nonproductive cough and shortness of breath developed. The case-patient was admitted to the hospital on DOI 11 for right lower lobe pneumonia with hypoxia. On DOI 12, he was suspected of having MERS-CoV infection, so airborne precautions (i.e. N95 respirator and patient isolation in an airborne infection isolation room) were instituted. At 11:00 AM on DOI 13, after MERS-CoV infection was confirmed, contact precautions were initiated and the case-patient was moved to another airborne infection isolation room with an anteroom. Test results for sputum, oropharyngeal swab, and plasma samples continued to be positive for MERS-CoV until DOI 16. A detailed report of the case-patient's clinical course is published elsewhere (*14*). The case-patient was discharged from the hospital 11 days after admission (DOI 21).

Contact Investigation

HCP Contacts

Fifty-three HCP self-identified as contacts of the case-patient or were identified as contacts from security video footage, GPS tracer tag logs, or the case-patient's medical record. Two HCP declined to be interviewed, and 3 could not be reached. Of the 48 HCP contacts interviewed within 1 week of exposure, 3 were determined to not to be contacts and were excluded from the analyses. Of the remaining 45 HCP contacts, 23 were exposed to the patient on hospitalization day 1 (13 in the ED and 10 in the patient's room or the computerized tomography suite), 19 were exposed on hospitalization day 2, and 9 were exposed on hospitalization day 3; several HCP were exposed on multiple days (Figure 1).

Of the 45 HCP contacts, 7 (16%) were men and 38 (84%) were women. The median age was 41.5 years (range 22.0–61.0 years). HCP in several job classifications were exposed to the case-patient, but most (47%) were RNs or CNAs. Most HCP contacts (71%) were assigned to work in the ED (n = 21 [47%]) or the ward in which the case-patient was hospitalized (11 [24%]); however, 12 (27%) HCP contacts worked in multiple departments (Table).

Six HCP contacts were nonclinical staff (administration, housekeeping, or social services) who had direct contact with the case-patient's surroundings but never touched the case-patient. Thirty-three HCP contacts (physicians, RNs, CNAs, phlebotomists, and radiology technicians) touched the case-patient while performing activities such as recording vital signs, listening to his lungs, and drawing blood. RNs and CNAs had the most frequent exposures; the median number of self-reported visits for each RN and CNA were 7 and 2, respectively (Table). Six respiratory therapists touched the case-patient and administered nebulizer treatments or spirometry tests.

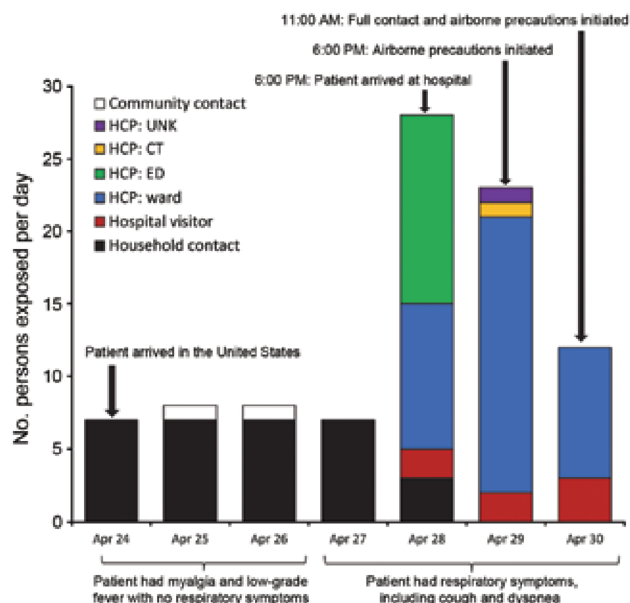

Figure 1. Number and type of contacts exposed to a Middle East respiratory syndrome coronavirus case-patient per day after his arrival in the United States on April 24, 2014. The same persons could be counted on multiple days of exposure. CT, computed tomography department; ED, emergency department; UNK, unknown; ward, patient's hospital floor.

Because airborne precautions began ≈24 hours after admission, most HCP contacts (39 [86.7%]) did not use a respirator or surgical mask while attending to the case-patient. Four HCP contacts had underlying medical conditions (current pregnancy, diabetes, or chronic steroid use), which might increase their risk for MERS-CoV infection or disease. Most HCP contacts (26 [58%]) were exposed to the case-patient 1 time; 18 were exposed >2 times, and 4 were exposed ≥10 times (Table). Overall, the median total self-reported exposure time was 11 minutes 30 seconds (range 15 s to 69 min 45 s). Two HCP contacts were excluded from length and frequency of exposure analyses because they could not remember their exposure to the case-patient.

The following symptoms most commonly developed in 9 HCP contacts: rhinorrhea (33%), odynophagia (22%), or headache (22%) within postexposure day 14; more than 1 symptom developed in some contacts. Fever did not develop in any of these contacts.

Hospital Visitor Contacts

Three family members were identified as hospital visitor contacts: 2 were also household contacts, and the other was an out-of-town family member who had not been exposed in the household. Two of these contacts were exposed on hospitalization days 1 and 2 without wearing any PPE, and all 3 were exposed on hospital day 3 while wearing N95 masks but no other PPE (Figure 1).

Household Contacts

Of the 7 household contacts, 5 permanently resided in the house where the case-patient stayed in the United States, and 2 were visiting from Massachusetts. One household contact was also an HCP contact and was included in both categories. All household contacts had minimal exposure to the case-patient during DOI 7–10 because he had isolated himself during most of his stay. Three household contacts reported hugging and kissing him on the day he arrived (DOI 7) and spending a few hours in the car with him before hospital admission (DOI 7–10). Coryza, but not fever, developed in 2 household contacts; 1 of these contacts tested positive for rhinovirus.

Community Contact

The 1 community contact was a business associate of the case-patient. The contact shook hands with the case-patient and had 2 face-to-face meetings with him on April 25 (2.0 h in length) and April 26 (1.5 h in length). At that time, the case-patient had mild myalgias and fever without any respiratory symptoms. On May 14, the contact had a runny nose and mild cough, but fever did not develop, and the contact had test results positive for rhinovirus.

Table. Demographic, employment, and exposure information for health care personnel contacts of patient with the first imported case of Middle East respiratory syndrome into the United States, 2014*

Health care personnel data	No. (%)
Sex	
M	7 (16)
F	38 (84)
Age group, y	
<30	13 (29)
30–39	9 (20)
40–65	23 (51)
Occupation	
Administration	3 (7)
Housekeeping	2 (4)
Medical doctor	3 (7)
Nurse practitioner	1 (2)
Nursing assistant	10 (22)
Phlebotomist	4 (9)
Radiology technician	4 (9)
Respiratory therapist	6 (13)
Registered nurse	11 (24)
Social personnel	1 (2)
Primary employment location in hospital	
Ward	21 (47)
Emergency department	11 (24)
Multiple locations	12 (27)
Computed tomography suite	1 (2)
Personal protective equipment worn while in contact with the patient†	
Gown	0
Goggles	2 (5)
N95 respirator	6 (14)
Surgical mask	2 (5)
Pre-existing condition‡	
Yes	4 (9)
No	40 (89)
No. self-reported times HCP visited the patient's room between 6:00 PM April 28 and 11:00 PM April 30	
0	1 (2)
1	26 (58)
2–5	11 (24)
6–9	3 (7)
>10	4 (9)

*HCP, health care personnel; Ward, patient's hospital floor.
†Full personal protective equipment includes N95 respirator, goggles, gown and gloves.
‡Pre-existing conditions that may increase the risk of infection included current pregnancy, chronic steroid use and diabetes.

Laboratory Results

For 60 contacts, both initial and follow-up nasopharyngeal and oropharyngeal swab samples and serum samples were negative for MERS-CoV by rRT-PCR and for MERS-CoV–specific antibodies by serologic testing. For the community contact, MERS-CoV test results for initial and follow-up nasopharyngeal and oropharyngeal swab samples and serum samples were negative by rRT-PCR, low titer–antibody positive by ELISA, indeterminate by IFA, and negative by microneutralization assay. His MERS-CoV antibody status was determined to be negative because the ELISA result could not be confirmed by either IFA or microneutralization assay. Additional nasopharyngeal,

oropharyngeal, and serum samples from 8 symptomatic HCP contacts were negative for MERS-CoV by rRT-PCR and serologic testing.

Self-Reported Versus Monitored HCP Exposure Duration and Number of Visits

Of the 45 HCP contacts, 11 (24%; 3 RNs and 8 CNAs) wore GPS tracer tags. Of those 11 contacts, 8 reported a number of visits to the patient's room similar (±2) to that recorded by the tracer tag; 1 underestimated the number by 22 visits; 1 underestimated the number by 16 visits; and 1 did not recollect the tag-recorded visits (Figure 2, panel A). There was no consistent pattern in the way HCP reported their number of visits: some overestimated and others underestimated the number. The total exposure time was more difficult for HCP to recall. Five estimated their exposure time within 10 minutes of the tracer tag–reported time, and 4 estimated within 20 minutes (Figure 2, panel B). The maximum time difference between cumulative self-reported and tracer tag–recorded time was 39 minutes. No significant correlation was found between self-reported and GPS-measured time (R^2 = 0.47) and number of visits (R^2 = 0.45) with the case-patient.

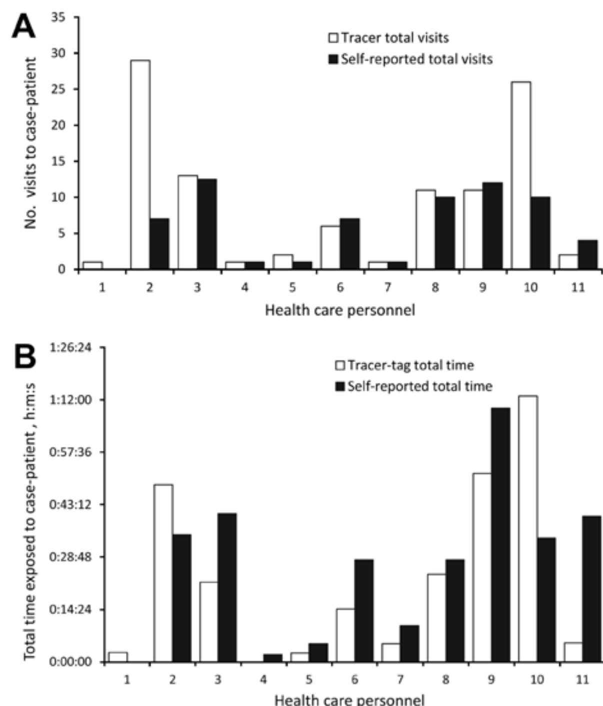

Figure 2. Comparison of self-reported and global positioning system (GPS) tracer tag–reported visits (A) and exposure times (B) for health care personal (HCP) who had contact with a Middle East respiratory syndrome coronavirus case-patient during his hospitalization, United States, 2014. Visits and exposures could be reported for 8 certified nursing assistants and 3 registered nurses who wore GPS tracer tags. The self-reported number of visits to the patient's room was derived from interviews held 5–7 days after exposure to the case-patient.

Discussion

We describe the contact investigation of the first identified MERS patient in the United States. All 61 identified contacts had negative test results for MERS-CoV even though some had face-to-face interactions with or prolonged exposure to the case-patient or administered nebulizer treatments and spirometry tests to the case-patient.

The absence of transmission to household contacts could be explained by the case-patient's mild initial respiratory symptoms, his hospital admission <24 hours after respiratory symptom onset (DOI 11), his self-isolation at home, and his lack of need for caregiving assistance before admission, all of which served to limit household members' exposure. Similarly, the absence of transmission to the community contact may have been due to the case-patient's lack of respiratory symptoms during the 2 meetings. The absence of transmission to household and community contacts in this investigation is similar to that seen in contact investigations of several other patients with MERS (*11–13*); however, in other settings, transmission to household members who provided care to persons with MERS-CoV infection have been reported, and household clusters have been documented (*3–7*,*16*).

When the case-patient was admitted during the second week of illness, the virus load in his sputum was high (*14*). However, none of the HCP contacts became infected. Serologic results may become positive >10–14 days after exposure, so we minimized the possibility of missing any asymptomatic infections by combining serologic results with clinical evaluation and PCR results. The absence of transmission to the HCP contacts may have been due to the absence of high-risk procedures (e.g., intubation, respiratory suctioning, and bronchoscopy), the short duration of exposure, and the few HCP contacts with underlying medical conditions. In addition, the hospital implemented strict infection control practices soon after the case-patient was suspected of having MERS-CoV infection, limiting the number and duration of exposures. These findings are similar to those from some previously documented contact investigations (*12*). However, there have been reports of transmission to HCP contacts in hospitals with multiple MERS patients or delayed implementation of appropriate infection control practices (*5*,*7*). The different findings from reported investigations illustrate that the specific activities that lead to an increased risk for MERS-CoV transmission still need to be clearly defined.

Severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 in China and spread globally in 2003. SARS-CoV shares similar characteristics with MERS-CoV, including likely zoonotic origin and transmission (*17–20*). Recent research on MERS-CoV has demonstrated plausibility for zoonotic transmission from dromedary camels to humans (*21*,*22*). MERS-CoV

seems less able than SARS-CoV to spread from person-to-person (*23–26*). Reports from the SARS-CoV epidemic showed tertiary transmission to >100 people, and 20% of health care workers become infected from the index patient (*24*,*27*). Most documented clusters of MERS-CoV infection show limited spread outside certain hospital settings, and unlike transmission in the SARS-CoV epidemic, there have been no foci of sustained transmission outside of the MERS-CoV infection epicenter in and near the Arabian Peninsula (*23*). However, as with SARS-CoV, the risk for MERS-CoV transmission may vary by patient, and health care facilities must maintain a high index of suspicion and immediately institute appropriate infection control practices for suspected cases.

This investigation is unique because we had independent documentation of duration of exposure from GPS-based tracer tags for 20% of HCP contacts. Most HCP contacts accurately reported case-patient exposure. However, HCP with the most contact had poorer recall accuracy, and 20 minutes' difference in exposure may alter the HCP contact risk, given that each visit was generally <3 minutes in duration. These findings have important implications for future contact investigations, and we recommend using objective measures of exposure, such as surveillance footage or GPS tracer tags, when available. In addition, we note that self-reported exposures are not always accurate because the accuracy of recalled time versus actual time spent with case-patients may be less reliable for HCP contacts that see a patient regularly for short periods of time.

This investigation had some limitations. First, risk factors for transmission could not be analyzed because none of the contacts were infected. Second, the use of the GPS tracer tag system to monitor HCP interaction with the case-patient might not always have given accurate results because HCP may not have been wearing their assigned tag when entering the room or, conversely, may have stood close to but not in the room, causing the tracking system to record incorrectly that the HCP had entered the room. Use of the GPS system also does not account for changes in risk to HCP contacts, such as if they entered the room while the case-patient was having a computed tomography scan.

In summary, we conducted a thorough contact investigation of this MERS case, including a detailed characterization of the type, duration, and frequency of exposures among HCP, household, and community contacts and testing of contacts for acute disease and asymptomatic infection. We documented the absence of transmission of MERS-CoV from the first identified imported case-patient in the United States despite his having multiple contacts at home and in the hospital before the implementation of appropriate infection control procedures. In addition, our comparison of GPS-monitored contact with HCP recall of contact calls into question the accuracy of information collected by recall during a contact investigation because not all HCP reported information could be confirmed by the GPS tracer tag logs. Although factors leading to MERS-CoV transmission are likely to be complex, additional information is needed regarding the natural history of the illness, in terms of virus shedding, modes of transmission, the role of asymptomatic infections in transmission, effective infection control practices, and the length and types of exposures that do and do not lead to transmission of the virus.

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Monitoring of Ebola Virus Makona Evolution through Establishment of Advanced Genomic Capability in Liberia

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To support Liberia's response to the ongoing Ebola virus (EBOV) disease epidemic in Western Africa, we established in-country advanced genomic capabilities to monitor EBOV evolution. Twenty-five EBOV genomes were sequenced at the Liberian Institute for Biomedical Research, which provided an in-depth view of EBOV diversity in Liberia during September 2014–February 2015. These sequences were consistent with a single virus introduction to Liberia; however, shared ancestry with isolates from Mali indicated at least 1 additional instance of movement into or out of Liberia. The pace of change is generally consistent with previous estimates of mutation rate. We observed 23 nonsynonymous mutations and 1 nonsense mutation. Six of these changes are within known binding sites for sequence-based EBOV medical countermeasures; however, the diagnostic and therapeutic impact of EBOV evolution within Liberia appears to be low.

The outbreak of Ebola virus disease (EVD) in Western Africa that started in November 2013 (*1*) is the largest recorded filovirus disease outbreak. As the outbreak continues, public health and emerging infectious disease officials have declared a continuing need for real-time monitoring of Ebola virus (EBOV) evolution (*2*,*3*). As of March 11, 2015, a total of 41% of reported cases had been fatal (*4*). By the end of March 2015, the intensity of the outbreak, which throughout its course affected 6 Western Africa countries, appeared to be receding, with near 0 activity in Liberia and no cases in Mali, Nigeria, and Senegal. However, EBOV continues to spread in Guinea and Sierra Leone. The epidemic is still causing more infections per week than have been recorded in previous EVD outbreaks (*5*). Therefore, public health officials continue to use media to maintain public awareness, to advocate for diligent handwashing and use of other protective measures, and to avoid complacency that could lead to reemergence (*5*). Vigilance is of paramount importance because currently used assays for EVD diagnosis, and many medical countermeasures in development, were designed using EBOV reference genome variants from previous outbreaks (*6*–*9*). Therefore, monitoring EBOV genomic drift is crucial because genetic changes can affect the efficacy of sequence-based therapeutics and diagnostics.

The size and spread of the current EVD outbreak reinforces the need to build public health infrastructure, including state-of-the-art diagnostic and surveillance capabilities, to implement and maintain effective EVD monitoring, treatment, and prevention platforms. The Liberian Institute for Biomedical Research (LIBR), established in 1975, is located in Charlesville, 50 km southeast of Liberia's capital, Monrovia. As of April 2, 2015, it is one of the few local facilities within Liberia processing clinical samples from persons suspected to have EVD. A consortium comprising US government and nongovernment agencies has been working with the Liberian government to equip LIBR with

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advanced genomic sequencing capabilities. These capabilities are dedicated primarily to EVD surveillance activities, including genome sequencing of EBOV-positive samples. The new LIBR Genome Center has a Miseq sequencer (Illumina, San Diego, CA, USA) and ancillary supporting capabilities, including electrophoresis for qualification, fluorometry for quantitation, PCR for amplification, and fully functional computational analysis capabilities to perform pathogen discovery and microbial genome characterization. The US Army Medical Research Institute of Infectious Diseases (USAMRIID) Center for Genome Sciences supports LIBR operation and development. Sample preparation procedures under biosafety containment are provided within the same building complex by the Liberian National Reference Laboratories, operated by USAMRIID and the National Institutes of Health Integrated Research Facility Ebola Response Team (Fort Detrick, Frederick, MD, USA). Throughput at the LIBR Genome Center is 10–20 samples (≈10 billion bases of sequence data) per week, with a target turnaround time of 7 days from sample receipt for high-priority samples. To ensure long-term sustainment of surveillance-based sequencing capabilities, local biomedical scientists have been trained and can proficiently perform all daily activities.

Here we demonstrate the utility and capabilities of the LIBR Genome Center. With the immediate goal of continuing the natural history characterization of the EBOV Makona variant (EBOV/Mak [*10*]) currently circulating in Western Africa and to support ongoing clinical trials to evaluate candidate medical countermeasures, we describe 25 EBOV genome sequences from the first 5 sequencing runs conducted at the LIBR Genome Center. We chose these samples for full-genome characterization from ≈1,700 available samples on the basis of high viral load (cycle threshold [C_t]) value ≤24) and date of collection to ensure up-to-date temporal coverage.

Materials and Methods

Samples

We chose samples from 25 patients from the larger collection (≈1,700 positive cases) on the basis of diagnostic C_t values that indicated a high enough viral load to provide a full genome (C_t≤24), beginning with the most recent available at the time of preparation in February 2015. Sampling continued with progressively older samples to describe the lineages most likely to still be circulating at the time. These patients were treated in 7 different Ebola treatment units and had resided in 7 of the 15 counties in Liberia (Table 1; online Technical Appendix 1 Figure 1, http://wwwnc.cdc.gov/EID/article/21/7/15-0522-Techapp1.pdf). Plasma or oral swab samples from which viral RNA was recovered and sequenced were tested at LIBR during September 23, 2014–February 14, 2015. Patients' ages were as follows: 1 infant (1 year), 6 children (2–15 years), 8 young adults (18–35 years), and 10 middle-aged adults (42–67 years). The male:female ratio was 2:1. However, among ≈1,700 samples at LIBR from persons with EVD, the ratio was close to 1:1 (48%/52%), and viral load did not differ by patient sex, which demonstrates that our higher ratio is a sampling artifact.

Sample Processing

RNA was converted to cDNA and amplified by using sequence-independent single-primer amplification (*11*). Amplified cDNA was quantified with a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and used as the starting material for the Illumina Nextera XT DNA library preparation kit (Illumina). Sequencing was performed on an Illumina Miseq by using either V2 or V3 reagent kits (Illumina) with a minimum of 2 × 151 cycles per run.

Genome Assembly

We assembled EBOV genomes by aligning reads to the genome of Ebola virus/H.sapiens-wt/SLE/2014/Makona-G3686.1 (GenBank accession no. KM034562.1) (*12*). Amplification primers were removed from the sequencing reads by using Cutadapt version 1.21 (*13*), and low-quality reads/bases were filtered by using Prinseq-lite version 0.20.4 (-min_qual_mean 25 -trim_left 20 -min_len 50) (*14*). Reads were aligned to the reference genome by using DNAStar Lasergene nGen (DNAStar, Madison, WI, USA), and a new consensus was generated by using a combination of Samtools v0.1.18 (*15*) and custom scripts. Only bases with Phred quality score ≥20 were used in consensus calling, and a minimum of 3× read-depth coverage, in support of the consensus, was required to make a call; positions lacking this depth of coverage were treated as missing (i.e., called as "N").

Genetic Analysis

Consensus sequences generated here were aligned with additional publically available EBOV genomes by using Sequencher version 5.2.3 (Gene Codes, Ann Arbor, MI, USA). SnpEff version 4.1b (build 2015-02-13) was used to annotate all single-nucleotide polymorphisms (SNPs) by using the genome of Ebola virus/H.sapiens-wt/GIN/2014/Makona-C15 (GenBank accession no. KJ660346.2) as a reference (*16*). All 25 genomes from Liberia were used to identify variable sites. For the rest of the genetic analysis, we used only the 14 sequences with >90% genome coverage. A median-joining haplotype network was constructed in PopART version 1.7.2 (http://popart.otago.ac.nz). Path-O-Gen version 1.4 (*17*) was used to calculate the root-to-tip distances by using a maximum-likelihood phylogeny (PhyML version 3.0 (*18*); general time reversible model)

Table 1. Characteristics of Ebola virus samples from selected patients, Liberia, September 2014–February 2015*

Sample ID	Patient age, y/sex	County of residence	Test date	Sample type	Average C_t value†
LIBR10054	53/M	Bomi	2014 Sep 23	Plasma	20.5
LIBR10053	42/NA	Not Available	2014 Oct 1	NA	22
LIBR0058	67/M	Rivercess	2014 Nov 5	NA	22
LIBR0059	27/M	Rivercess	2014 Nov 5	NA	22
LIBR0073	27/M	Grand Bassa	2014 Nov 6	Plasma	18.5
LIBR0067	29/NA	Bomi	2014 Nov 6	Plasma	21
LIBR0063	3/F	Montesserrado	2014 Nov 6	Oral swab	17.5
LIBR0093	47/M	Montesserrado	2014 Nov 6	Plasma	15.5
LIBR0092	18/F	Montesserrado	2014 Nov 8	Plasma	21
LIBR0090	62/F	Margibi	2014 Nov 8	Plasma	22
LIBR0116	4/F	Grand Bassa	2014 Nov 10	Plasma	19
LIBR0168	15/M	Bomi	2014 Nov 13	Plasma	22.5
LIBR0176	42/M	Montesserrado	2014 Nov 14	Oral swab	22.5
LIBR0173	64/M	Montesserrado	2014 Nov 14	Oral swab	22
LIBR0286	9/F	Grand Cape Mount	2014 Nov 22	Plasma	22
LIBR0333	35/F	Grand Cape Mount	2014 Nov 25	Plasma	19.5
LIBR0423	45/F	Montesserrado	2014 Dec 3	Plasma	21.5
LIBR0430	1/M	Grand Bassa	2014 Dec 3	Oral swab	23.5
LIBR0503	8/F	Sinoe	2014 Dec 10	Plasma	23
LIBR0505	29/F	Sinoe	2014 Dec 10	Plasma	25
LIBR0605	2/M	Montesserrado	2014 Dec 20	Oral swab	23
LIBR0624	53/M	Montesserrado	2014 Dec 22	Plasma	19.5
LIBR0993	33/M	Montesserrado	2015 Jan 20	Plasma	19.5
LIBR1195	35/M	Margibi	2015 Feb 2	Oral swab	22.5
LIBR1413	56 M	Montesserrado	2015 Feb 14	Plasma	22.5

*C_t, cycle threshold; ID, identification; NA, not available.
†C_t values used as indicator of viral load obtained from 2 diagnostic assays performed on all samples (Kulesh-TM and Kulesh-MGB [*9*]).

with rooting based on the EBOV phylogeny published by Gire et al. (*12*). BEAST version 1.8.2 (*17*) was used to estimate the mutation rate and the time to the most recent common ancestor for several evolutionary lineages that included Liberia EBOV isolates. For analysis, we divided the alignment into 3 partitions (i.e., first + second codon sites, third codon site, and noncoding sites). The substitution process was modeled independently for each by using the Hasegawa, Kishino, and Yano model with 4 gamma categories. An exponential growth coalescent model was used with a strict clock. The XML input file is available on request from the authors.

Results

From the first 5 sequencing runs, we obtained 25 EBOV genomes with >50% coverage; 6 of these were coding complete (Table 2) (*19*). These genomes contained 97 new sequence variants: 47 synonymous, 23 nonsynonymous, 1 nonsense, and 26 noncoding mutations (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/21/7/15-0522-Techapp2.xlsx). Multiple distinct evolutionary lineages were detected, but all were consistent with a single introduction of a cluster 2–type (*12*) virus into Liberia followed by within-country diversification (Figure 1, panel A). Because 19 of the 25 genomes had calls at all 5 positions that discriminate clusters 1, 2, and 3, we have high confidence in cluster attribution.

Molecular dating places the common ancestor to all of the sampled isolates from Liberia during May 2–July 9, 2014 (95% highest posterior density [HPD] interval), which corresponds with the early days of the outbreak in Monrovia (*3*). However, we cannot rule out ongoing EBOV exchange among EVD-infected countries. In fact, shared ancestry among 3 isolates from Liberia and the 4 available sequences from Mali suggests some level of international movement. We estimated dates associated with 2 nodes along the shared Liberia/Mali EBOV lineage (labeled * and ** in Figure 1, panel A); these estimates ranged from July 6 through September 15, 2014, and from July 26 through September 27, 2014, respectively (95% HPD). Overall, collection dates correlated well with root-to-tip distances within the Western Africa EVD outbreak (Figure 1, panel B). Linear regression analysis (using the lm function in R version 3.1.1; http://www.r-project.org/) estimated an overall rate of change of 9.17×10^{-4} substitutions/site/year ($\pm 5.23 \times 10^{-5}$). Bayesian analysis estimated a similar rate of change of $9.44–15.67 \times 10^{-4}$ substitutions/site/year (95% HPD).

We reviewed all publicly available genomic information for EBOV/Mak (122 genome sequences [*1*,*12*]) to evaluate the effect of genomic drift on biomedical countermeasures (drugs and diagnostic assays). We assessed the potential impact of intra-outbreak genetic divergence on 13 drugs and 2 diagnostic assays (known to be used in Liberia) with the same approach previously used (*6*). Two sequence-binding treatment modalities are available for postexposure treatment of EVD: small interfering RNAs (siRNAs) (*20*) and phosphorodiamidate morpholino oligomers (*21*) targeting *L*, *VP24*, and/or *VP35* gene transcripts, and passive immunotherapy based on antibodies or

Table 2. Next-generation sequencing of 25 Ebola virus isolates derived from selected patients sampled, Liberia, September 2014–February 2015

Sample ID	Coverage, %*	No. reads	Finishing category†	GenBank accession no.
LIBR0093	99.4	169,000	Coding complete	KR006947
LIBR0116	97.9	710,168	Coding complete	KR006948
LIBR10054	98	2,150,725	Coding complete	KR006964
LIBR0073	98.5	3,351,831	Coding complete	KR006944
LIBR0503	98.9	3,193,168	Coding complete	KR006956
LIBR0286	98.3	1,731,953	Coding complete	KR006952
LIBR0993	96.5	750,000	Standard draft	KR006960
LIBR0423	97.1	2,676,454	Standard draft	KR006954
LIBR0333	97.1	1,775,653	Standard draft	KR006953
LIBR10053	98	1,691,652	Standard draft	KR006963
LIBR0067	97	2,403,590	Standard draft	KR006943
LIBR0092	93.9	2,758,142	Standard draft	KR006946
LIBR0090	93.1	1,422,271	Standard draft	KR006945
LIBR1413	88.2	2,500,000	Standard draft	KR006962
LIBR0058	91.4	1,632,978	Standard draft	KR006940
LIBR0176	89.4	1,907,863	Standard draft	KR006951
LIBR0168	89.2	1,221,075	Standard draft	KR006949
LIBR0505	83.8	741,165	Standard draft	KR006957
LIBR1195	73.1	2,200,773	Standard draft	KR006961
LIBR0624	68	1,550,511	Standard draft	KR006959
LIBR0063	69	2,883,384	Standard draft	KR006942
LIBR0173	72.3	1,456,490	Standard draft	KR006950
LIBR0059	59.1	851,606	Standard draft	KR006941
LIBR0605	64.7	1,587,732	Standard draft	KR006958
LIBR0430	56.2	3,139,009	Standard draft	KR006955

*Percentage of genome bases (of 18,959 total bases) called in the consensus sequences (requires ≥3× coverage with base quality ≥20).
†Categories are defined in (*19*).

antibody cocktails targeting EBOV glycoprotein (*22–26*). These treatments inhibit viral replication by targeting viral transcripts for degradation (siRNA) or by blocking translation (phosphorodiamidate morpholino oligomers), or they acutely neutralize the virus to enable the host to mount an effective immune response (passive immunotherapy). These countermeasures were originally designed specifically against sequences obtained during previous outbreaks (*20*,*27*) or were generated against their glycoproteins (e.g., the monoclonal antibodies [mAbs] were obtained after immunization with Ebola virus/H.sapiens-tc/COD/1995/Kikwit-9510621 [EBOV/Kik-9510621] [*28*]).

Since the Western Africa outbreak began, at least 33 viral mutations have occurred that could affect countermeasures. We previously reported 27 of these mutations (*6*). Twenty-six (79%) mutations induced nonsynonymous changes to epitopes recognized by mAbs included in passive immunotherapy cocktails. Another 5 (15%) were located in published binding regions of siRNA-based therapeutic drugs. Tekmira has adjusted its siRNAs to account for 4 of these 5 changes since its initial publication (*29*; E.P. Thi et al., unpub. data). The final 2 mutations were located in the published binding region of primers or probes for quantitative PCR diagnostic tests that have been used during outbreak control activities in Liberia: 1 change each in the binding sites of the Kulesh-TM assay and the Kulesh-MGB assay (*9*). Nevertheless, reassessment of the assays at USAMRIID has suggested that the changes will be tolerated without loss in sensitivity (data not shown). Changes in all EBOV/Mak sequences are considered "interoutbreak" (n = 23); changes observed only in some sequences from Western Africa are considered "intraoutbreak" sites (n = 10, EBOV-WA <100%). We also examined the binding sites of an additional 18 publicly available EBOV quantitative PCRs, which might (or might not) also be used in Western Africa (online Technical Appendix 1 Figure 2, online Technical Appendix 1 Table). We observed 25 changes, of which 6 were reported previously (*12*). Each SNP has the potential to affect the efficacy of available therapeutic drugs (original and updated versions) or diagnostic assays (Table 3; Figure 2; online Technical Appendix 1 Figure 2, online Technical Appendix 1 Table; nucleotide positions are reported relative to EBOV/Kik-9510621, for consistency [*6*]).

Several of the 27 previously identified changes (green in Figure 2) already have been demonstrated to be tolerated while maintaining efficacy (*24*,*30*,*32–34*), thus minimizing their potential effect (*6*). Six of these 33 SNPs (EBOV-LIB <100%; orange in Figure 2) appeared during the surveillance period of this study (September 23, 2014–February 14, 2015) in samples obtained in Liberia (*12*). None of these changes have been previously associated with EBOV resistance to any therapeutic drug. Five of the new changes might affect 1 of the components of the ZMapp antibody cocktail (mAb 13C6). However, the conformational target site for this antibody (positions 1–295, soluble glycoprotein) is broader in length and more poorly defined than the other sequence-based countermeasure targets considered in our risk assessment. The sixth mutation might affect the

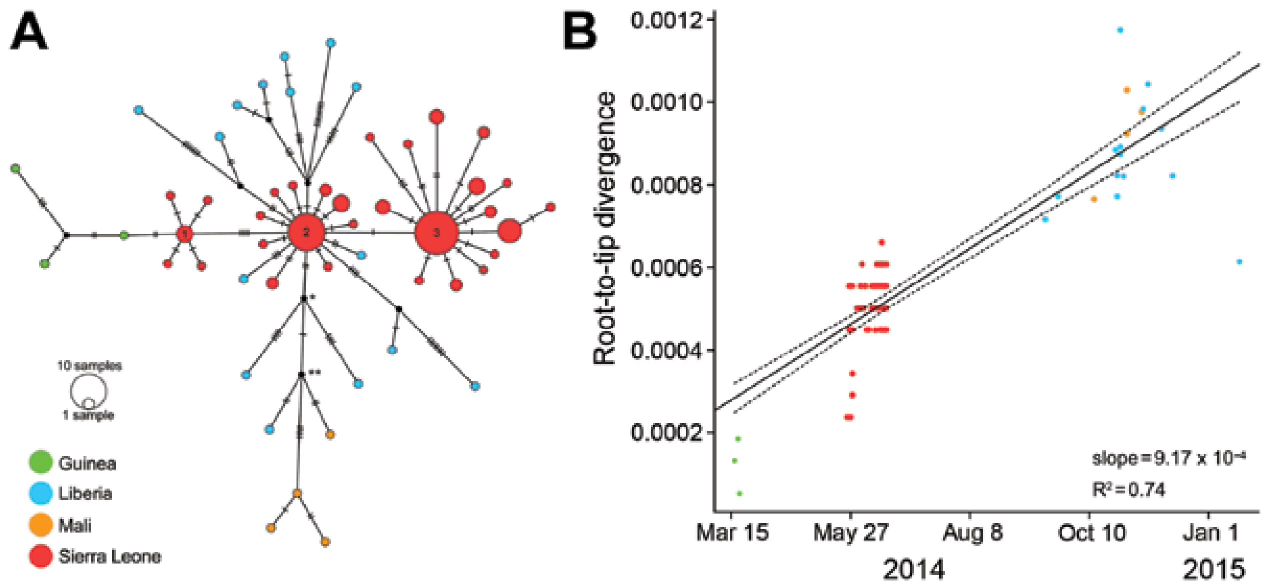

Figure 1. A) Median-joining haplotype network constructed from a full-genome alignment of 122 clinical Ebola virus Makona (EBOV/Mak) isolates (list of isolates in online Technical Appendix 3, http://wwwnc.cdc.gov/EID/article/21/7/15-0522-Techapp3.xlsx). Each colored vertex represents a sampled viral haplotype, with the numbered vertices representing the centers of the 3 clusters described in (*12*). All sampled isolates from Liberia originated from cluster 2. The size of each vertex is relative to the number of sampled isolates, and the colors indicate country of origin. Hatch marks indicate the number of mutations along each edge. Because of missing data, 2,764 sites (14.6% of total genome) were excluded from the analysis, including 26 sites with variability among isolates (16.7% of all variable sites). B) Root-to-tip distance correlates well with test date and estimates a rate of evolution equal to 9.17×10^{-4} substitutions/site/year. This analysis comprises 110 clinical EBOV/Mak isolates collected during March 17, 2014–January 20, 2015 (online Technical Appendix 3, isolates with dates).

binding site of the siRNA viral protein (VP) 35 target (for that particular sample, the mutation appears in an area of low sequencing coverage depth). Thus, when these new changes are combined with the changes observed previously (yellow in Figure 2), we can conclude that retesting several therapeutic drugs against isolates currently circulating might be necessary to determine whether any of these mutations impact their efficacy. In particular, it is important to reevaluate drugs that include mAb 13C6 (part of the ZMapp, ZMAb, and MB-003 antibody cocktails), mAb 13F6 (part of MB-003), mAb 1H3 (part of ZMAb), and the siRNA VP35 targets (Table 3, Figure 2) (*6*).

Discussion

Our study details the establishment of a genomic sequencing and analysis center within Liberia for real-time monitoring of viral evolution. The initial sequences generated at this facility have provided a first glimpse into EBOV/Mak evolution from the end of 2014 to the beginning of 2015. Although genetically diverse, the viruses circulating in Liberia during this period are consistent with a single introduction event followed by diversification within Liberia. The cluster 2 haplotype from which all the sampled Liberia sequences radiate is thought to have been circulating in Guinea and Sierra Leone during late May 2014 (*12*). Moreover, it was the second most common sequence detected in Sierra Leone during late May through mid-June (*12*). Introduction of this haplotype from either of these neighboring countries could have resulted in the sampled diversity; however, we cannot rule out the possibility of multiple introductions. Additional spatial and temporal sampling within Liberia, Guinea, and Sierra Leone will help to differentiate these 2 scenarios.

The 25 Liberia EBOV/Mak genomes included 23 nonsynonymous mutations and 1 nonsense mutation that have not previously been seen in Western Africa (although some of these mutations have been observed in EBOV isolates from previous EVD outbreaks). A nonsense mutation, which is present within 2 of the 25 sequences, is predicted to result in premature truncation (6 aa) of VP30. VP30 is an essential protein for viral transcription; it is needed for the RNA-dependent RNA polymerase (L) to read beyond a *cis*-RNA element in the nucleoprotein mRNA 5′ untranslated region (*35*) and is required to reinitiate transcription at gene junctions (*36*). Moreover, VP30 phosphorylation modulates the composition and function of the RNA synthesis machinery (*37*). To our knowledge, no functional domains have been described in the truncated region. Further characterization is needed to determine whether this or any of the other detected mutations impacted the relative fitness of the affected EBOV isolates. Within Liberia, geography showed little correlation with phylogeny; most

Table 3. Mutation analysis of candidate therapeutic drug and diagnostic binding sites for EBOV*

Reference position	Type	Reference base	Called base	EBOV-WA, %	EBOV-LIB, %	Codon	Feature name
850	SNP	A	G	100	100	G:GGA @ 127 → G:GGg	NP
852	SNP	A	G	100	100	K:AAA @ 128 → R:AgA	NP
895	SNP	A	G	100	100	T:ACA @ 142 → T:ACg	NP
907	SNP	T	C	1	0	N:AAT @ 146 → N:AAc	NP
919	SNP	T	C	100	100	F:TTT @ 150 → F:TTc	NP
1288	SNP	A	T	1	0	V:GTA @ 273 → V:GTt	NP
1495	SNP	A	G	100	100	Q:CAA @ 342 → Q:CAg	NP
1498	SNP	C	T	1	4	L:CTC @ 343 → L:CTt	NP
1507	SNP	T	A	100	100	A:GCT @ 346 → A:GCa	NP
1552	SNP	C	T	100	100	R:CGC @ 361 → R:CGt	NP
1862	SNP	A	G	100	100	S:AGC @ 465 → G:gGC	NP
6359	SNP	T	C	100	100	N:AAT @ 107 → N:AAc	GP
6909	SNP	T	A	1	0	W:TGG @ 291 → R:aGG	GP
7730	SNP	G	A	100	100	E:GAG @ 564 → E:GAa	GP
7775	SNP	A	G	100	100	L:CTA @ 579 → L:CTg	GP
7778	SNP	C	A	100	100	R:CGC @ 580 → R:CGa	GP
10252	SNP	A	T	1	4		
10253	SNP	A	G	1	0		
12694	SNP	T	A	100	100	I:ATT @ 371 → I:ATa	L
12886	SNP	A	C	2	0	L:CTA @ 435 → L:CTc	L
12952	SNP	A	G	100	100	L:CTA @ 457 → L:CTg	L
13267	SNP	C	T	100	100	T:ACC @ 562 → T:ACt	L
13607	SNP	G	A	1	4	V:GTC @ 676 → I:aTC	L
13624	SNP	T	G	1	0	N:AAT @ 681 → K:AAg	L
13630	SNP	A	G	100	100	P:CCA @ 683 → P:CCg	L

*EBOV, Ebola virus; GP; glycoprotein, ; L, RNA-dependent RNA polymerase; LIB, Liberia; NP; nucleoprotein; SNP, single-nucleotide polymorphism; WA, Western Africa.

EBOV lineages within Liberia appear to be geographically widespread within the sampled regions.

Previous analysis of EBOV/Mak genomes from Sierra Leone and Guinea suggests that the evolutionary rate within the current EVD outbreak might be higher than the rate between outbreaks (*12*). After incorporation of sequences from Liberia, which were collected later in the outbreak, our estimates of substitution rate fell between the previous estimates for EBOV/Mak only and for all EBOV (*12*,*38*). As more sequence data become available, it will be interesting to see whether a significant change in the evolutionary rate can be detected within the current EVD outbreak.

Our ability to quantify international EBOV exchange is limited because few isolates from other countries were available during the sampled timeframe. However, shared ancestry between isolates from Mali and 3 isolates from Liberia suggests at least 1 transmission event across national borders (*3*). All EVD cases in Mali have been attributed to movement of infected persons into Mali from Guinea (*39*). With the current dataset, it is impossible to say whether the shared Liberia/Mali lineage originated in Liberia and was then transported to Mali through Guinea or whether the lineage emerged in Guinea and later moved independently to Liberia and Mali. Active EBOV outbreaks were occurring in both Liberia and Guinea during the period estimated for the emergence of this shared lineage (July–September 2014).

The genomic changes observed for EBOV/Mak during its circulation in Liberia append 5 additional mutations to the list of changes that might affect the binding of the 13C6 mAb, a component of ZMapp. All of these changes, however, were present at relatively low frequency (≤12%) in our current sample, and none of the sampled lineages have accumulated >1 change per therapeutic drug type. We observed no significant changes (i.e., likely to affect efficacy) in the binding sites for the 2 diagnostic assays known to be used in Liberia. Overall, no dramatic changes were observed in the samples evaluated; the risk assessment for the impact of genomic drift during the outbreak should remain low. As previously stated (*6*), our analysis is not without caveats. Our current analysis covers only the late period of the outbreak in Liberia; no analysis has yet been published with data for similar time points from Guinea or Sierra Leone. In addition, to complete our assessment of the evolution of EBOV in Liberia, an earlier period of time from the introduction of the virus in March 2014 to early September 2014 needs to be investigated.

Our findings offer a concise evaluation of the potential impact of the evolution of EBOV/Mak based on genome reconstruction of 25 isolates from Liberia obtained during September 2014–February 2015. This work would not have been possible without the establishment of a genomic surveillance capability in Liberia, which emphasizes the

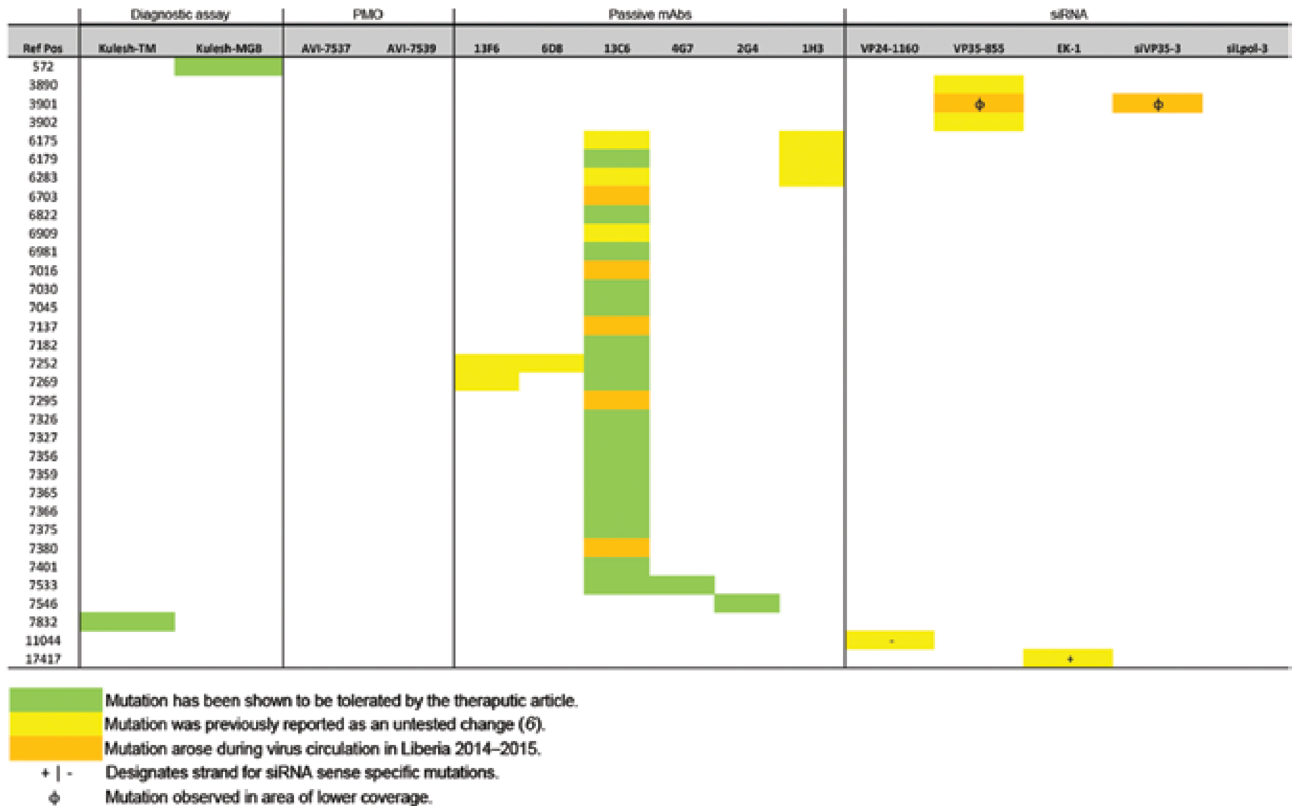

Figure 2. Mutation analysis of candidate therapeutic drug and diagnostic binding sites used in outbreak of Ebola virus (EBOV) disease, Western Africa. A single-nucleotide polymorphism (SNP) table is combined with a heat map based on 2 categories: 1) mutations tolerated by the therapeutic drug or diagnostic target (highlighted in green); 2) mutations within the binding region of a therapeutic drug or diagnostic assay that have not yet been tested (highlighted in yellow/orange) (*20–24,27,30,31*). Changes previously described are highlighted in yellow; changes that appeared during circulation in Liberia are highlighted in orange. The reference nucleotide positions reported here are in relation to EBOV/Kik-9510621 (GenBank accession no. AY354458), which is one of the primary isolates used as reference for developing these therapeutic drugs and diagnostic assays. A summary of the changes to the probes is available in online Technical Appendix 1 Table (http://wwwnc.cdc.gov/EID/article/21/7/15-0522-Techapp1.pdf). PMO, phosphorodiaminate morpholino oligomer; mAB, monoclonal antibody; siRNA, small interfering RNA; Ref pos, reference positive; VP, viral protein.

need for global sequencing capabilities to be part of the first response during future virus outbreaks.

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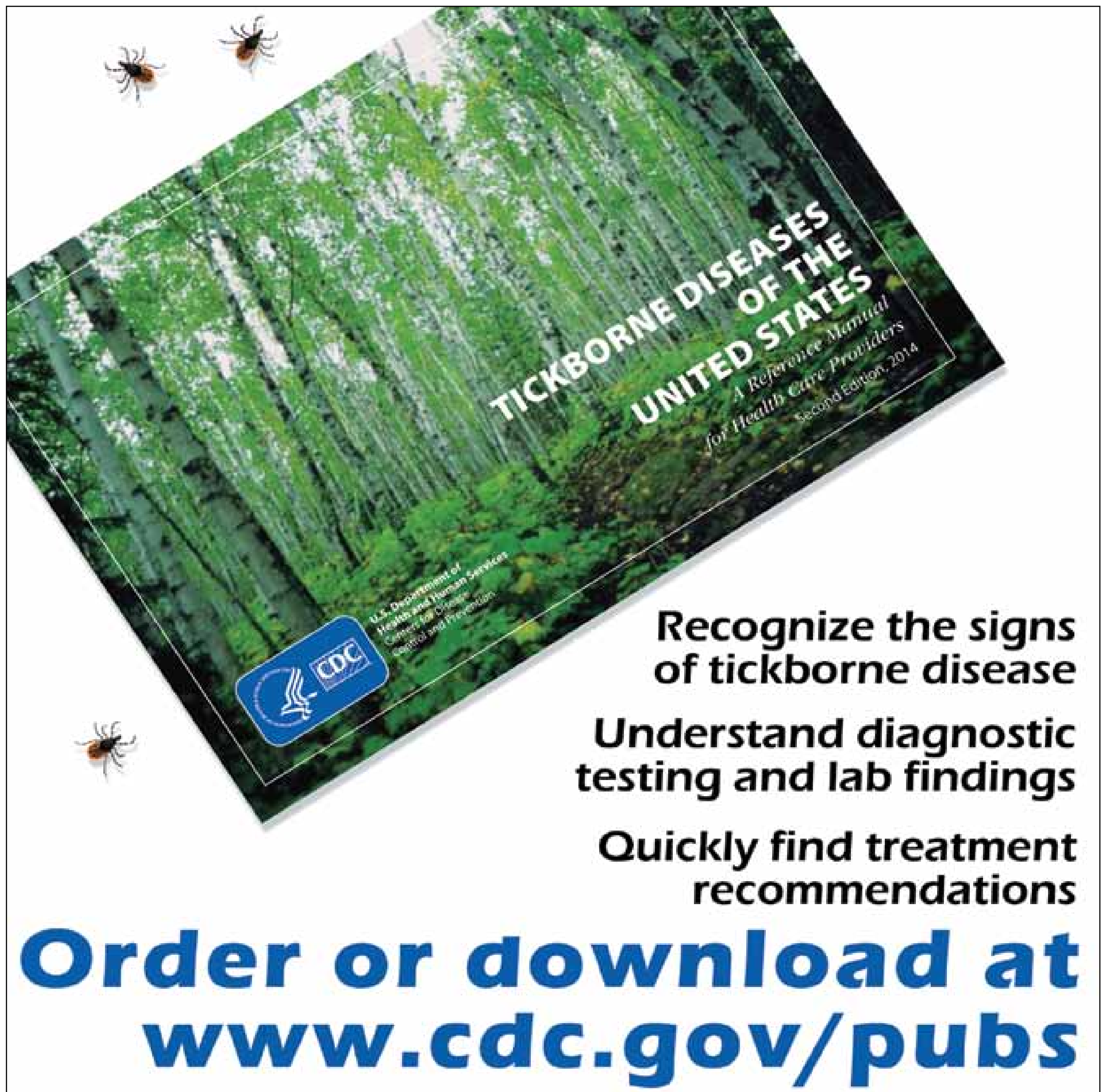

Parechovirus Genotype 3 Outbreak among Infants, New South Wales, Australia, 2013–2014

Germaine Cumming,[1] Ameneh Khatami, Brendan J. McMullan, Jennie Musto, Kit Leung, Oanh Nguyen, Mark J. Ferson, Georgina Papadakis, Vicky Sheppeard

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Release date: June 15, 2015; Expiration date: June 15, 2016

Learning Objectives

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

1. Describe the clinical and epidemiologic features of an HPeV3 outbreak among Australian infants
2. Compare the presentation of the HPeV3 outbreak in Australia with that in the northern hemisphere
3. Determine the efficacy of active surveillance in detecting and monitoring the HPeV3 outbreak among Australian infants.

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From October 2013 through February 2014, human parechovirus genotype 3 infection was identified in 183 infants in New South Wales, Australia. Of those infants, 57% were male and 95% required hospitalization. Common signs and symptoms were fever >38°C (86%), irritability (80%), tachycardia (68%), and rash (62%). Compared with affected infants in the Northern Hemisphere, infants in New South Wales were slightly older, both sexes were affected more equally, and rash occurred with considerably higher frequency. The New South Wales syndromic surveillance system, which uses near real-time emergency department and ambulance data, was useful for monitoring the outbreak. An alert distributed to clinicians reduced unnecessary hospitalization for patients with suspected sepsis.

The clinical manifestations of infection with human parechoviruses (HPeVs), members of the family *Picornaviridiae*, are often indistinguishable from those caused by human enterovirus infections. Over the past decade, outbreaks of human parechovirus genotype 3 (HPeV3) have been reported from the Northern Hemisphere and are particularly well documented in Japan (where the virus was discovered), Canada, the United Kingdom, Denmark, and the Netherlands (*1–4*). Of the 16 HPeV genotypes, HPeV3 is the most aggressive and causes a sepsis-like syndrome in neonates (*5*). HPeV infection seems to follow a seasonal pattern; incidence is higher in summer and autumn (*2*,*3*). It can be spread by the fecal–oral and respiratory routes (*4*).

On November 22, 2013, Health Protection New South Wales (NSW), Australia, was notified of a possible cluster of HPeV cases at The Children's Hospital at Westmead in Sydney. At that time, 7 neonates had experienced rapid onset of acute sepsis-like illness with fever >38°C and a combination of irritability/pain, diarrhea, confluent erythematous rash, tachycardia, tachypnea, encephalitis, myoclonic jerks, and hepatitis. Inquiries revealed that neonates described as "red, hot, angry" had also been admitted to other tertiary children's hospitals in NSW (*6*). An expert advisory group comprising staff from the NSW Ministry of Health, Health Protection NSW, public health units, and the Sydney Children's Hospital Network was convened to coordinate the investigation.

On November 25, 2013, PCR detection of HPeV RNA confirmed HPeV infection in 2 of the children. The NSW public health network and clinicians agreed that a surveillance program should be initiated to gather information on the epidemiologic and clinical characteristics and outcomes of children with HPeV infection.

In addition to the public health response, Health Protection NSW issued a media release to alert members of the public to the outbreak. On November 29, 2013, HPeV3 information including a case definition, instructions for accessing diagnostic testing, and recommended clinical management was distributed to all emergency departments, pediatricians, and early childhood health services in NSW. During the outbreak, the expert advisory group met regularly via teleconference to discuss and address any emerging issues. HPeV3 active surveillance activities were concluded on January 31, 2014, while other forms of surveillance continued into February 2014. We describe the epidemiology of the outbreak as observed through several surveillance mechanisms.

Methods

HPeV infection is not a notifiable disease under the Public Health Act 2010 (NSW). This HPeV3 outbreak was detected and reported by clinicians alert to unusual clusters and patterns of disease. Other forms of surveillance were developed as a result of this alert. Surveillance consisted of 3 components: 1) active surveillance (case finding at the sentinel sites); 2) passive surveillance (laboratory reporting of all positive HPeV specimens from sentinel sites and elsewhere in NSW to Health Protection NSW); and 3) syndromic surveillance (reporting of infants seen in emergency departments by the NSW syndromic surveillance system that uses near real-time emergency department and ambulance data [*7*]) (Figure 1). The sentinel sites were 3 tertiary children's hospitals in NSW: The Children's Hospital at Westmead, The Sydney Children's Hospital Randwick, and John Hunter Children's Hospital Newcastle. Passive and syndromic surveillance continued into February 2014. In addition to surveillance, public health communication in the form of an HPeV information sheet for clinicians was distributed on November 29, 2013, alerting emergency department staff, pediatricians, and early childhood health service staff of current HPeV activity in NSW, providing a description of HPeV infection, and recommending management options (i.e., early laboratory testing and provision of supportive care after receipt of confirmation of HPeV infection).

Active Surveillance

Active surveillance activities commenced at the 3 hospitals (sentinel sites) on November 25, 2013, and continued through January 19, 2014; however, some retrospective case finding was included for cases with onset dating back to October 1, 2013, when the outbreak was thought to have started. A patient with a suspected case of HPeV was defined as a neonate or young infant <3 months of age with sepsis-like illness and fever >38.0°C and ≥2 of the following: irritability/pain, rash, diarrhea, tachycardia, tachypnea, encephalitis, myoclonic jerks, or hepatitis. A patient with a confirmed case of HPeV was a suspected case-patient for whom PCR was positive for HPeV. Clinicians at the sentinel sites collected case information by using an HPeV case investigation form and PCR testing of patient stool, cerebrospinal fluid (CSF), nasopharyngeal aspirate, throat

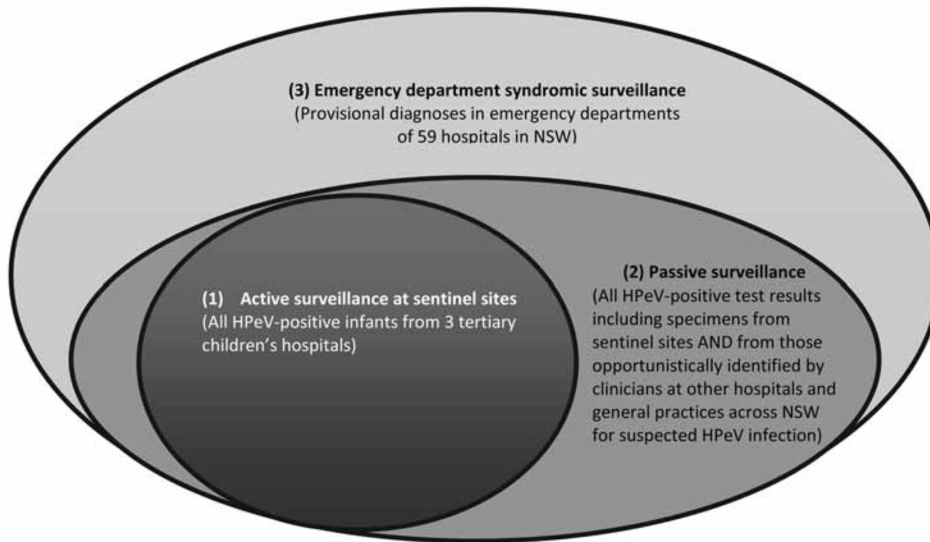

Figure 1. Venn diagram showing capture and overlaps in human parechovirus (HPeV) case identification/reporting resulting from the 3 surveillance mechanisms used during the HPeV outbreak in New South Wales (NSW), Australia, during October 2013–February 2014.

swab, rectal swab, or whole blood samples for HPeV; stool and CSF samples were preferred. (*3*,*8*,*9*). These data were entered into the NSW Notifiable Conditions Information Management System. Weekly reports informed the NSW public health network of outbreak progression.

Passive Surveillance

All positive HPeV test results from NSW residents referred to the Victorian Infectious Diseases Reference Laboratory (VIDRL) in Melbourne, Victoria (the only laboratory in the region testing for HPeV), from October 1, 2013, through February 2, 2014, were reported to NSW Health. Specimen date; estimated illness onset date; sample type; and patient date of birth, sex, and postcode were recorded in the NSW Notifiable Conditions Information Management System.

Syndromic Surveillance

The NSW emergency department syndromic surveillance system monitored the number of infants <1 year of age for whom a provisional diagnosis of fever/unspecified infection was made in the emergency department and the number of patients who required hospital admission, including admission to critical care wards. A diagnosis of fever/unspecified infection can include fever symptoms, unspecified viral infection, unspecified viremia, unspecified bacteremia, unspecified bacterial infection, or unspecified infection. Weekly reports compared recent data with historical data from the previous 5 years.

Laboratory Methods

From all clinical samples, nucleic acid was extracted by using QIAGEN DX reagents (QIAGEN, Hilden, Germany) on a QIAxtractor NA extraction robot (QIAGEN). cDNA was synthesized by using a method previously described (*10*) and was tested in an HPeV real-time PCR selective for the 5′ untranslated region, which was developed at VIDRL (*10*). (The primer and probe sequence details for this assay can be supplied upon request to G.C.)

Molecular analysis to obtain the HPeV genotype was performed on selected samples that had been positive by real-time PCR. Specimens from 41 patients were selected for genotyping on the basis of ensuring representation of infants' geographic locations, ages, sex, illness onset dates, specimen types, and sex. Identification of specific HPeV genotypes was obtained through amplification of the viral protein 1 gene by use of a gel-based seminested PCR (*11*). The generated PCR products were sequenced and compared with reference sequences by using the primers and methods described elsewhere (*12*).

Statistical Analyses

Descriptive analysis of epidemiologic variables and patient demographic characteristics were performed. Characteristics of infants <3 months of age, such as length of stay (LOS), were compared by using *t*-tests to determine the effects of public health messaging. Analyses was performed by using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA).

Results

Laboratory Findings

From November 1, 2013, through February 28, 2014, a total of 420 specimens were submitted for HPeV PCR testing; for some patients, >1 specimen was submitted. PCR results were HPeV positive for 289 (69%) specimens from 198 patients (Table 1). In addition to confirming HPeV RNA

Table 1. Results of PCR testing of specimens from patients from New South Wales received at the VIDRL, Melbourne, Victoria, Australia, November 1, 2013–February 28, 2014 *

Test	Specimens, no. (%)	Patients, no. (%)
Total no.	420 (100)	308 (100)
PCR+ for HPeV	289 (69)	198 (64)
PCR+ for HPeV only	285 (68)	194 (63)
PCR+ for HPeV and EV†	4 (1)	4 (1)
PCR+ for EV only	15 (4)	14 (5)
PCR+ for EV	19 (5)	18 (6)
PCR– for HPeV	131 (31)	110 (36)
PCR– for HPeV and EV	116 (28)	96 (31)

*EV, enterovirus; HPeV, human parechovirus;VIDRL, Victorian Infectious Disease Reference Laboratory: +, positive; –, negative.
†Because of how the testing algorithm is set up at VIDRL, all samples tested for HPeV are also tested for EV. EV results are shown to demonstrate the small amount of EV detected in this HPeV outbreak period, with even fewer co-infections.

in samples from 198 patients in NSW, HPeV type 3 was identified from all 41 (21%) positive samples for which molecular analysis was subsequently performed. The phylogenetic tree demonstrating all HPeV3 isolates genotyped at VIDRL during the outbreak is reported elsewhere (*6*).

Because of the algorithms used in the testing, enterovirus results were also available for all samples submitted (Table 1). A total of 194 patients had HPeV infection only, 4 had dual infections (HPeV and enterovirus), and 14 had enterovirus infection only (Figure 2). Results for the rest of the patients were negative. Focusing on CSF and fecal samples, 123 (73%) of 168 CSF samples were HPeV positive by PCR (mean cycle threshold [C_t] detection value 31.6), and 114 (73%) of 156 fecal samples were positive (mean C_t 27.2) (Table 2). PCR was run for 45 cycles; therefore, C_t values >45 were considered negative.

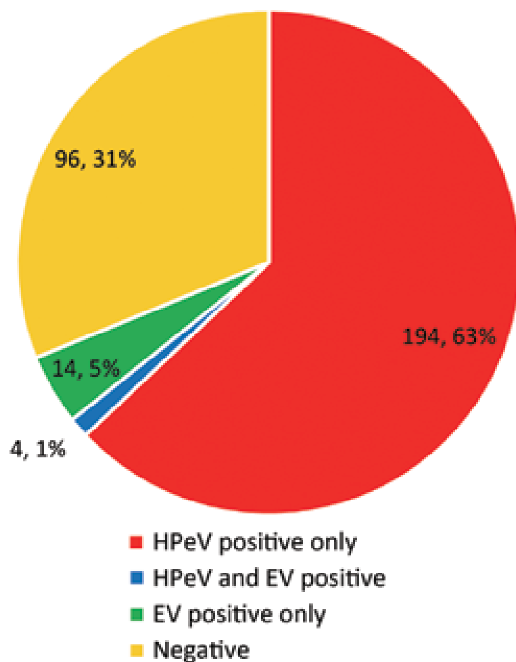

Figure 2. Patient human parechovirus (HPeV) and enterovirus (EV) results for all 198 patients in New South Wales, Australia, tested by the Victorian Infectious Diseases Reference Laboratory during November 1, 2013–February 28, 2014.

Active and Passive Surveillance Findings

Active surveillance identified 94 infants whose illness met the definition of a confirmed case (patient <3 months of age and HPeV-positive laboratory results, originating from sentinel sites). Passive surveillance spanning specimen collection dates from October 1, 2013, through February 2, 2014, identified another 89 laboratory-confirmed cases in NSW in patients 0–17 months of age. The outbreak peaked during the first 2 weeks of December 2013 (Figure 3).

More cases (105 [57%]) were in male than female patients; median patient age was 1.51 months (or median 46 days, range 0–537 days) (Figure 4; Table 3). Intrafamily HPeV3 transmission was identified in twins, 2 parent–child pairs, and a set of cousins. A descriptive case series of the infants infected with HPeV3 during this outbreak, containing further clinical details on select cases, is reported elsewhere (*6*).

Analysis of case investigation forms from the sentinel sites reporting HPeV signs and symptoms showed that the most commonly reported signs for infants <3 months of age were fever (86%), irritability/pain (80%), tachycardia (68%), and rash (62%) (Table 3). Similar signs were displayed by those ≥3 months of age; however, 20% fewer in this age group had fever and tachycardia (Table 3). As described previously, all infants at the sentinel sites were well at the time of hospital discharge, and further longitudinal follow-up studies are examining the long-term outcomes of these infections having occurred in early life (*6*).

Of the 183 confirmed cases, 108 (59%) were captured by the 3 sentinel surveillance sites, and another 75 (41%) were diagnosed at other hospitals. Most (57%) patients resided in the Sydney metropolitan area, and the remaining 43% were from regional or rural areas of NSW. This finding compares with 64% and 36% of the NSW population residing in metropolitan Sydney and regional/rural areas, respectively (*13*).

Analysis of case investigation forms for the 108 patients at the sentinel sites also showed that 103 (95%) patients were admitted to hospital and had an average LOS of 4.4 (1–13) days (Table 3). Mean LOS was greater for infants <3 months of age (4.5, range 1–13 days) than for

Table 2. HPeV testing of specimens from patients from New South Wales, received at the VIDRL, Melbourne, Victoria, Australia, November 1, 2013–February 28, 2014, by specimen type*

Sample and result	Specimens, no. (%)	Patients, no. (%)
Total	420 (100)	308 (100)
CSF		
Total samples tested	168 (40)	161 (52)
HPeV+ (mean C_t 31.6)†	123 (73)	116 (72)
PCR+ for HPeV and EV	1 (1)	1 (1)
Stool		
Total samples tested	156 (37)	147 (48)
HPeV+ (mean C_t 27.2)	114 (73)	106 (72)
PCR+ for HPeV and EV	2 (1)	2 (1)
Other		
Total samples tested	96 (23)	8 (28)
HPeV+	52 (54)	45 (52)
PCR+ for HPeV and EV	1 (1)	1 (1)
Sequenced: HPeV3	41 (14)	41 (21)

*CSF, cerebrospinal fluid; C_t, cycle threshold; EV, enterovirus; HPeV, human parechovirus; VIDRL, Victorian Infectious Disease Reference Laboratory: +, positive.
†At VIDRL, PCRs are run for 45 cycles, so any C_t value greater than 45 is considered negative.

those ≥3 months of age (3.7, range 1–11 days). The rate of admission to an intensive care unit was lower for older infants (14%) than for those <3 months of age (30%) (Table 3).

The trend statistic on the distribution of LOS in infants <3 months of age showed that LOS was significantly reduced among infants <3 months of age who became ill after the HPeV alert was sent on November 29, 2013, to emergency departments and pediatricians. Mean LOS at the sentinel sites was 5.7 days before and 4.0 days after sending of the alert (Satterthwaite *t*-test, $p<0.05$) (Figure 5).

Syndromic Surveillance Findings

The number of infants <1 year of age with a provisional emergency department diagnosis of fever/unspecified requiring hospital admission began to rise sharply in mid-November 2013 and peaked during the first week of December (Figure 6, panel A). At the peak, the number of admissions was 83, compared with an average of 52 for the same week in previous years. Admissions remained elevated until mid-January 2014, when they returned to background levels. Admissions to critical care wards spiked during the second week of December, when 9 patients were admitted, well above the average of 1 admission per week for the same week in previous years (Figure 6, panel B). During the surveillance period, most admissions were to the 2 children's hospitals in metropolitan Sydney.

Discussion

This outbreak is probably one of the first large parechovirus outbreaks to be reported in Australia. We observed a large number of cases over a relatively short period (≈4 months), peaking around late spring/early summer, which is earlier than documented seasonality for parechovirus in the Northern Hemisphere (*3*). Although sequencing in this series was incomplete (21% of patients), this parechovirus outbreak was determined to have been caused by HPeV3 for the following reasons: all sequenced HPeV-positive samples were genotype 3; the epidemiology and spectrum of illness seen by clinicians at the sentinel sites was relatively

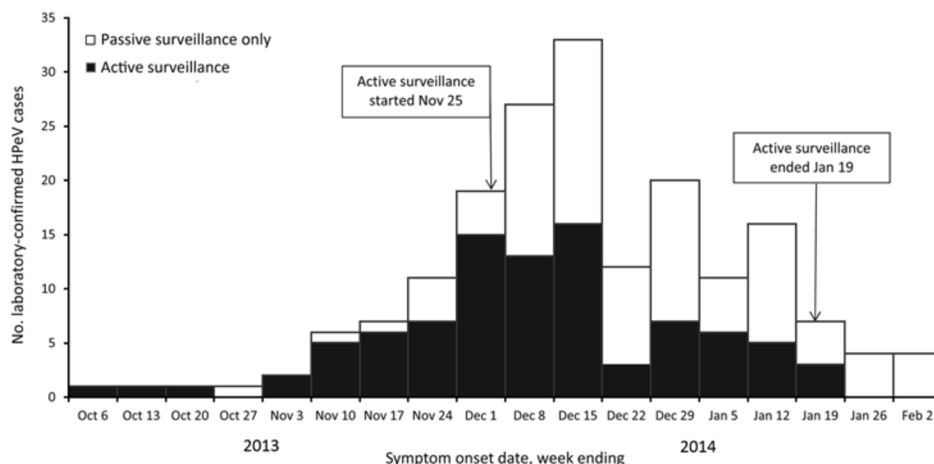

Figure 3. Number of laboratory-confirmed human parechovirus (HPeV) cases identified by active and passive surveillance, by week of symptom onset, in New South Wales (NSW), Australia, during the October 2013–early February 2014 outbreak (total = 183 cases). Source: NSW Notifiable Conditions Information Management System data (http://www.health.nsw.au/epidemiology/Pages/Notifiable-diseases.aspx), February 18, 2014. Source: NSW Notifiable Conditions Information Management System data (http://www.health.nsw.au/epidemiology/Pages/Notifiable-diseases.aspx), February 18, 2014.

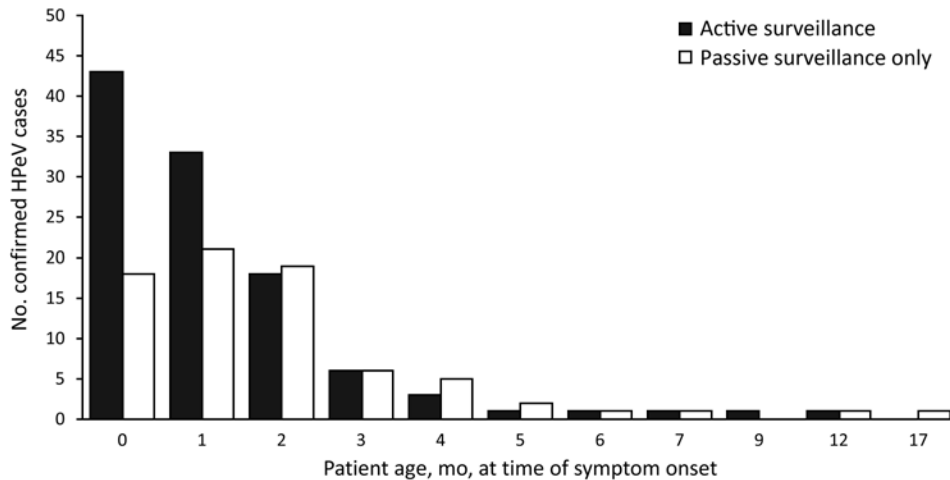

Figure 4. Age distribution of 183 patients with confirmed human parechovirus (HPeV) infection at time of symptom onset, detected by active and passive surveillance, New South Wales (NSW), Australia, October 1, 2013–February 2, 2014. Source: NSW Notifiable Conditions Information Management System data (http://www.health.nsw.au/epidemiology/Pages/Notifiable-diseases.aspx), February 18, 2014.

homogenous and in keeping with other reports of HPeV3 infections in infants (*3*,*14*); and PCR was enterovirus positive for only 18 patients but HPeV positive for 198 patients.

Infected infants in this outbreak were older than those reported elsewhere. The median age of 46 days was higher than that reported in Denmark (39 days) and the Netherlands (40 days) (*3*,*14*). The occurrence of a substantial proportion (17%) of HPeV3 infections in infants ≥3 months of age was consistent with results from a US study reporting 18% of cases in infants >60 days of age but contrary to other data indicating that HPeV3 infection occurs almost exclusively in infants <3 months of age (*2*,*4*).

The age cutoff in the case definition for active surveillance at the sentinel sites may have introduced a bias toward HPeV infection being more frequently suspected in infants <3 months of age; thus, these infants might have undergone more HPeV testing than older infants, thereby underestimating the true mean age of infants affected in this outbreak. Had the age cutoff in the case definition been set at 12 months (all infants), bias toward younger infants

Table 3. Characteristics of patients with laboratory-confirmed HPeV detected by active surveillance, New South Wales, Australia, October 1, 2013–February 2, 2014*

Characteristic	Passive surveillance for all laboratory-confirmed HPeV case-patients,† no. (%)	Active surveillance, enhanced data for all HPeV case-patients from sentinel sites,‡ no. (%), n = 108		
		<3 mo	≥3 mo	All ages
Total	183 (100)	94 (87)	14 (13)	108 (100)
Sex				
F	78 (43)	45 (48)	6 (43)	51 (47)
M	105 (57)	49 (52)	8 (57)	57 (53)
Admitted to ward	NA	89 (95)	14 (100)	103 (95)
Length of stay, days				
Minimum	NA	1	1	1
Maximum	NA	13	11	13
Mean	NA	4.5	3.7	4.4
Admitted to ICU	NA	28 (30)	2 (14)	30 (28)
Clinical features				
Fever	NA	81 (86)	9 (64)	90 (83)
Irritability/pain	NA	75 (80)	11 (79)	86 (80)
Diarrhea/loose stool	NA	29 (31)	2 (14)	31 (29)
Tachypnea	NA	26 (28)	3 (21)	29 (27)
Tachycardia	NA	64 (68)	7 (50)	71 (66)
Hepatitis	NA	9 (10)	1 (7)	10 (9)
Rash	NA	58 (62)	9 (64)	67 (62)
Encephalitis	NA	7 (7)	0 (0)	7 (6)
Myoclonic jerks	NA	4 (4)	1 (7)	5 (5)

*Table source: NSW Notifiable Conditions Information Management System data (http://www.health.nsw.au/epidemiology/Pages/Notifiable-diseases.aspx), February 18, 2014. HPeV, human parechovirus; ICU, intensive care unit; NA, not available; NSW, New South Wales; VIDRL, Victorian Infectious Disease Reference Laboratory, Melbourne, Victoria, Australia.
†Passive laboratory surveillance ceased at NSW Health on February 2, 2014, leaving at a total of 183 laboratory-confirmed HPeV cases. This number differs from the total of 198 confirmed HPeV cases reported by VIDRL surveillance, which continued through to February 28, 2014, and identified another 15 cases.
‡Sentinel surveillance sites collected additional demographic, hospital, and clinical data onto the HPeV case investigation forms, which were entered into the NSW Notifiable Conditions Information Management System. No information on hospital stay and clinical features was available for cases identified by laboratory-only surveillance. Surveillance at sentinel sites is a subset (59%) of the outbreak total of 183 laboratory-confirmed cases.

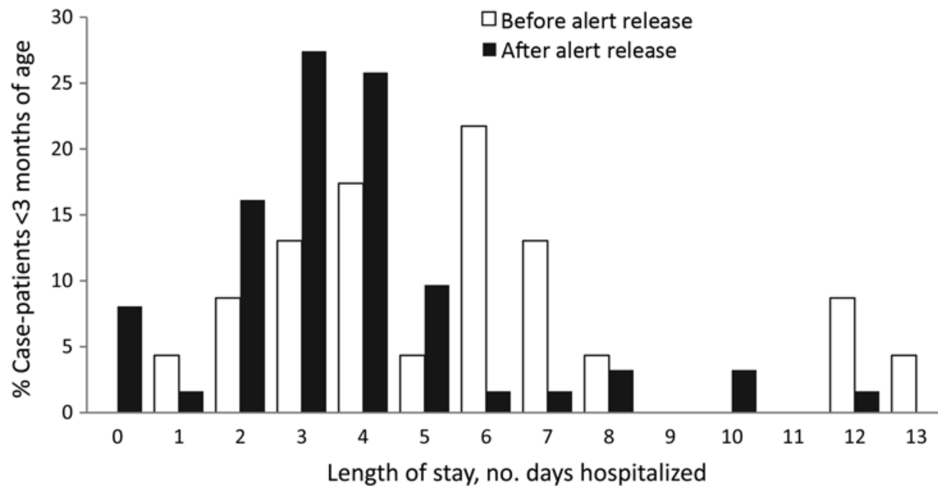

Figure 5. Distribution of hospital length of stay for infants <3 months of age at sentinel sites during active surveillance of the human parechovirus outbreak in New South Wales (NSW), Australia, October 1, 2013–February 2, 2014. Source: NSW Notifiable Conditions Information Management System data (http://www.health.nsw.au/epidemiology/Pages/Notifiable-diseases.aspx), February 18, 2014.

would have been avoided, and the median age might have been older than that reported here.

After the November 29, 2013, release of the HPeV alert to emergency department staff and pediatricians, mean LOS among infants <3 months of age decreased by 30%, from ≈5.7 days at the start of the outbreak to 4.0 days ($p = 0.0250$). This statistically significant finding reflects a degree of effectiveness of public health messaging in raising clinician awareness of HPeV. During the latter part of the outbreak, after Health Protection NSW issued the alert, informed clinicians may have felt more comfortable discontinuing treatment and discharging infants sooner if their illness met the criteria of the HPeV case definition. The alert seemed to result in improved clinical management of cases, avoidance of unnecessary prolonged exposure to empirically prescribed antimicrobial drugs, provision of appropriate supportive treatment, and earlier discharge from hospital. The mean LOS during the NSW outbreak (4.4 days) was lower than that reported in the Netherlands (7 days), according to an analysis of retrospective diagnoses when no public health intervention would have taken place (*8*). Another factor that could contribute to reduced LOS in the latter part of an outbreak is the increasing age of young infants becoming ill with HPeV infection in the second half of the outbreak, although this factor was not statistically significant in this dataset (*15*).

The male:female split in this outbreak was less pronounced than that reported for other studies, although more cases consistently occurred in boys. Others have reported a higher preponderance of infection in boys,

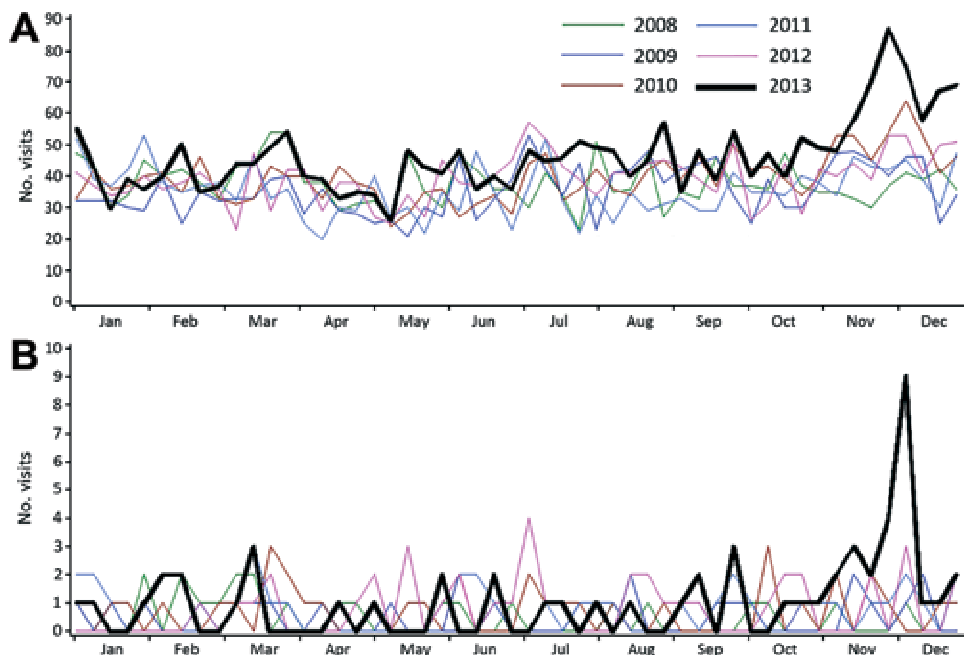

Figure 6. Total weekly counts of visits to the emergency department for fever or unspecified infection for which patients were (A) admitted to ward and (B) admitted to critical care for 2013, compared with each of the 5 previous years, children <1 year of age, for 59 hospitals in New South Wales, Australia. Source: Emergency department syndromic surveillance report produced on June 3, 2014.

ranging from 70% to 90%, compared with our finding of 57% (*2*,*5*,*8*,*14*).

Clinical signs reported in the literature for HPeV3-infected infants were consistent with our findings of fever, irritability, and encephalitis (*2*,*6*,*8*). However, rash occurred with much higher frequency (62% vs. 17%) in the NSW outbreak than in other outbreaks (*8*).

Syndromic surveillance that used emergency department data proved to be a useful and timely way to monitor emergency department hospital admissions temporally associated with the HPeV3 outbreak. Increased presentation of infants <1 year of age with a provisional emergency department diagnosis of fever/unspecified infection requiring hospital admission, in particular admission to critical care, were associated with increased detection of HPeV3 at the clinical level. Emergency department syndromic surveillance reports also helped confirm an overall decline in admissions from emergency departments in early 2014, supporting the eventual withdrawal of active surveillance. Emergency department syndromic surveillance in NSW does not routinely monitor age-specific or admission-specific (hospital ward or critical care unit) aberrations; that is, all children <5 years of age are monitored as a group. In the future, data generated through the emergency department syndromic surveillance system may continue to be useful for monitoring the evolution of an HPeV or similar outbreak. The cost of maintaining emergency department syndromic surveillance like that used during this outbreak (fever/unspecified infection among children <1 year of age and admission to hospital) would need to be considered. Costs of doing so include personnel time for checking reports and investigating signals, infrastructure costs, and opportunity costs; choosing to monitor HPeV3-related signals indirectly means that signals for diseases that are not prioritized are not monitored. In addition, the sensitivity of this grouping will need to be tested in future outbreaks before it can be considered a reliable proxy indicator of a seasonal outbreak

We initiated sentinel surveillance on the assumption that nearly all neonates with severe HPeV3 disease would be referred to 1 of the 3 tertiary children's hospitals in NSW. Through passive surveillance we identified an additional 41% of HPeV patients who had been seen at other health facilities. Enhanced data were not collected for these presumably milder cases, which has limited our capacity to conduct more extensive significance testing across the observed differences in clinical features. Recording more information on potential exposures (e.g., infants' daycare attendance, existence of older siblings, and occurrence of family illness in weeks preceding infants' admission) would have further aided our understanding of HPeV3 transmission in the community.

Conclusion

The objectives of HPeV surveillance were achieved: document the outbreak, describe the clinical features of cases, help inform clinicians and the public, monitor the evolution of the outbreak, and add to the knowledge base. The HPeV3 infection outbreak in NSW, Australia, differed slightly from that documented in the Northern Hemisphere; the NSW outbreak apparently affected slightly older infants (as well as neonates and young infants), cases were more evenly split between boys and girls, and rash occurred at a considerably higher frequency. The value of awareness-raising communication strategies was demonstrated by the statistically significant 30% reduction in LOS during the outbreak immediately after release of the alert to emergency department staff and pediatricians. This alert helped to minimize unneeded use of antimicrobial drugs and reduce unnecessary hospitalization. Although active surveillance is resource intensive, it has helped to define HPeV3 infection in NSW and link it with a syndromic surveillance indicator in the emergency department syndromic surveillance system. Syndromic surveillance is a potentially useful proxy indicator that should be considered for future detection and surveillance of seasonal outbreaks of viral infections.

Acknowledgments

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Ms. Cumming is a public health specialist working with the NSW health system. Her research areas of interest include population health surveillance and evaluations and public health response preparedness.

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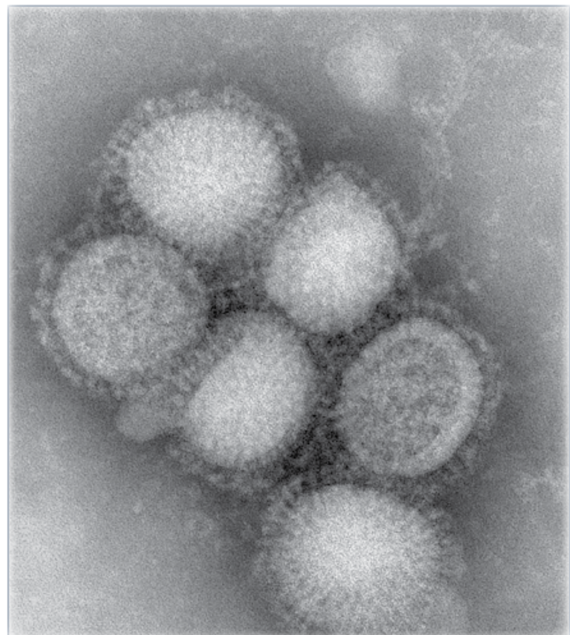
Outbreak of a New Strain of Flu at a Fair

Dr. Karen Wong, an EIS officer with the Centers for Disease Control and Prevention, discusses her study about flu outbreaks at agricultural fairs.



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MERS-CoV in Upper Respiratory Tract and Lungs of Dromedary Camels, Saudi Arabia, 2013–2014

Abdelmalik I. Khalafalla, Xiaoyan Lu, Abdullah I.A. Al-Mubarak, Abdul Hafeed S. Dalab, Khalid A.S. Al-Busadah, Dean D. Erdman

To assess the temporal dynamics of Middle East respiratory syndrome coronavirus (MERS-CoV) infection in dromedary camels, specimens were collected at 1–2 month intervals from 2 independent groups of animals during April 2013–May 2014 in Al-Ahsa Province, Saudi Arabia, and tested for MERS-CoV RNA by reverse transcription PCR. Of 96 live camels, 28 (29.2%) nasal swab samples were positive; of 91 camel carcasses, 56 (61.5%) lung tissue samples were positive. Positive samples were more commonly found among young animals (<4 years of age) than adults (≥4 years of age). The proportions of positive samples varied by month for both groups; detection peaked during November 2013 and January 2014 and declined in March and May 2014. These findings further our understanding of MERS-CoV infection in dromedary camels and may help inform intervention strategies to reduce zoonotic infections.

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging pathogen associated with severe respiratory symptoms and renal failure in infected persons (*1*,*2*). Saudi Arabia is the country most severely affected by the virus and is where the first recognized case was identified in 2012. The origin of MERS-CoV remains a mystery. Bats seem to be the reservoir host of the virus (*3*) but are probably not the source of the ongoing MERS-CoV outbreak because of limited contact with humans in the Arabian Peninsula. Early observations that some MERS-CoV–infected persons had been exposed to camels suggested a possible role of these animals as intermediate reservoir hosts (*2*,*4*). Serologic surveys subsequently conducted in several countries in the Arabian Peninsula and Africa identified high rates of MERS-CoV–specific antibodies in dromedary camels (*5*–*12*). Furthermore, MERS-CoV infection in dromedary camels was definitively proven by the detection of virus and virus sequences in respiratory specimens, feces, and milk collected from camels in Qatar (*9*,*13*), Oman (*14*), Saudi Arabia (*5*,*15*,*16*), and Egypt (*17*).

The few published studies that looked for MERS-CoV in the respiratory tract of naturally infected dromedary camels examined nasal or ocular swab samples but not samples from the lower respiratory tract. Moreover, several studies relied on only a few specimens or collected specimens at only 1 time point (*9*,*13*–*15*). To address these limitations and to clarify the dynamics of MERS-CoV infection in these animals, we conducted a year-round study in which we collected a large number of specimens from the upper respiratory tracts of live dromedary camels and from the lungs of dromedary camel carcasses.

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Materials and Methods

Sample Collection

This study was approved by the Institutional Review Board of the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia. Respiratory specimens were collected from 2 independent groups of mixed-age dromedary camels (*Camelus dromedaruis*). The first collection was obtained during April 2013–May 2014 at the Al Omran Abattoir, Al Omran City, in Al-Ahsa Province in the eastern region of Saudi Arabia. Livestock slaughtered at this abattoir include cattle, goats, sheep, and camels originating from Al-Ahsa and neighboring provinces. Animals selected for slaughter were mainly from the livestock market and from herds located around Al-Ahsa Province. At the livestock market in Al-Ahsa, dromedary camels are housed in small groups (10–15 animals), where they may stay for no more than 4 days. They are then transported in vehicles to the abattoir, where they are kept for no more than 24 hours before slaughter.

Samples were taken from slaughtered dromedary camels on 8 occasions (every 1–2 months). On each particular collection date, tissue specimens were collected from the lungs of all slaughtered dromedary camels. A total of 91 animal carcasses were sampled; 28 had been young animals (<4 years of age) and 63 had been adults (≥4 years of age). Lung lobes that showed pulmonary lesions were sampled; if both lobes showed lesions or if no lesions were visible,

the left lobe was sampled because of its close proximity to the person collecting the sample. The tissue samples (≈1–2 g) were collected aseptically from inside the lung lobes by using sterile surgical instruments (scalpels, forceps, and scissors). To avoid cross-contamination, lungs were moved to a clean room adjacent to the slaughtering hall and examined on a freshly disinfected table by a person wearing a newly donned gown, face mask, and sterile gloves and using a new set of sterile surgical instruments. Collected tissue samples were immediately deposited in labeled sterile plastic bags and placed in a cooler containing ice packs for transport to the laboratory.

A second sample was collected from age-matched animals over the same period and consisted of 96 nasal swab specimens (36 young animals and 60 adults), 94 from visually healthy dromedary camels and 2 from camels with nasal and lachrymal discharge. Nasal swabs were collected from animals at 3 locations in Al Ahsa Province (Al Omran abattoir, Al Ahsa livestock market, and the veterinary hospital of King Faisal University). For this procedure, a long sterile flexible swab was inserted into 1 nostril until slight resistance was felt; the swab was then rotated, held in place for 5 seconds, withdrawn, and placed in 1 mL of cold viral transport medium containing antibiotics (this medium was chosen to enable future attempts to isolate the virus).

Both swab and lung specimens were transported on ice to the laboratory within 1–2 hours of collection and stored at −80°C until testing. Collection dates and numbers of samples are listed in Table 1.

Sample Processing and RNA Extraction

Swab specimens in transport media were mixed and then clarified by centrifugation at 350 × *g* for 10 minutes; the supernatants were recovered for extraction. Lung samples were thawed and homogenized by using a TissueRuptor homogenizer (QIAGEN, Hilden, Germany), and 20% suspensions were prepared in 5 mL of transport medium. The resulting homogenates were subjected to centrifugation as above, and the supernatants were recovered for extraction. Total RNA was extracted from 140 µL of each nasal swab or lung sample by using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions.

Reverse Transcription PCR

Extracted RNA was tested by using a gel-based pan-coronavirus reverse transcription PCR (RT-PCR) assay according to the protocol of Vijgen et al. (*18*). Real-time RT-PCR (rRT-PCR) was performed by using an assay kit provided by the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). This assay panel targets the MERS-CoV nucleocapsid protein gene (*19*) and a region upstream of the envelop protein gene described by Corman et al. (*20*). All samples were screened by using gel-based RT-PCR and 2 rRT-PCR assays and were considered positive for MERS-CoV if a positive result was obtained with at least 2 of the 3 tests following World Health Organization recommendations (http://www.who.int/csr/disease/coronavirus_infections/WHO_interim_recommendations_lab_detection_MERSCoV_092014.pdf). All RT-PCRs included no-template negative controls and quantified MERS-CoV transcript as positive control. cDNA was prepared from 20 positive samples and shipped to CDC for independent confirmation and sequencing.

Nucleotide Sequencing and Phylogenetic Analyses

To assess the genetic variability of MERS-CoV, we sequenced the spike protein gene coding region (4,062 nt) on the 20 positive samples. Sequencing was performed on an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Grand Island, NY, USA) by using Sequencher version 4.8 software (Gene Codes, Ann Arbor, MI, USA) for sequence assembly and editing. Sequence alignments were performed by using ClustalX version 1.83 implemented in BioEdit version 7.2.5 (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). Phylogenetic analyses were performed by using MEGA version 6.06 (http://www.megasoftware.net). The neighbor-joining method (tree algorithm inferred with the Kimura 2-parameter substitution model of sequence evolution) was used to construct

Table 1. Middle East respiratory syndrome coronavirus in dromedary camels, by sample group and collection date, Al-Ahsa Province, Saudi Arabia, 2013–2014*

Sample collection	Nasal swab samples, live camels		Lung tissue samples, camel carcasses		Total samples	
date	No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive
2013 Apr	NA	NA	12	8 (66.6)	12	8 (66.6)
2013 May	16	5 (31.3)	11	6 (54.5)	27	11 (40.7)
2013 Jun	10	3 (30.0)	NA	NA	10	3 (30.0)
2013 Sep	NA	NA	12	7 (58.3)	12	7 (58.3)
2013 Nov	16	6 (37.5)	13	10 (76.9)	29	16 (55.2)
2013 Dec	10	4 (40.0)	11	9 (81.8)	21	13 (61.9)
2014 Jan	12	4 (33.3)	10	8 (80.0)	22	12 (54.5)
2014 Mar	14	4 (28.6)	11	5 (45.4)	25	9 (36.0)
2014 May	18	2 (11.1)	11	3 (27.3)	29	5 (17.2)
Total samples	96	28 (29.2)	91	56 (61.5)	187	84 (44.9)

*Tested by reverse transcription PCR. NA, not applicable.

phylogenetic trees, and bootstrap resampling analyses were performed (1,000 replicates) to test tree reliability.

Results

During the study, a total of 91 lung tissue samples and 96 nasal swabs were obtained from the 2 groups of camels (Table 1). Overall, 84 (44.9%) of 187 animals were MERS-CoV positive by RT-PCR. The proportion of MERS-CoV–positive animals sampled varied by month and year. For months when specimens were available from both groups, the proportion of positive samples from both groups was highest during the cool months (November 2013–January 2014), then steadily declined, reaching the lowest point during the warm month of May 2014.

MERS-CoV RNA was detected by RT-PCR in a high proportion (56 [61.5%] of 91) of lung tissue samples from animal carcasses. In contrast, MERS-CoV RNA was detected in 28 (29.2%) nasal swab samples collected from the 96 live animals (Table 1).

All animals from both groups appeared healthy on visual inspection except for 2. These 8-month-old dromedary camel calves, located outside of the Al Omran abattoir, exhibited purulent nasal and lachrymal discharge; MERS-CoV RNA was detected in nasal swab specimens from these 2 calves (Figure 1). MERS-CoV RNA was more often detected in the lung and nasal cavity of young camels than adult camels (Table 2).

cDNA prepared from 20 samples positive for MERS-CoV by RT-PCR were shipped to CDC for independent confirmation. All 20 samples were confirmed MERS-CoV positive by multiple rRT-PCRs selective for independent regions of the MERS-CoV genome. However, attempts to amplify larger regions of the genome for sequencing were less successful. Despite repeated attempts, only 4 samples had cDNA of sufficient quality for successful sequencing. Sequences of the full MERS-CoV spike gene coding region were obtained from nasal swabs collected from 3 live animals in December 2013 (camels C8, C9) and May 2014 (camel C23) and from a lung sample collected from 1 animal carcass (camel C7) in November 2013 (GenBank accession nos. KP405225 [camel C8], KP405226 [camel C7], KP405227 [camel C9], KP966104 [camel C23]). The spike sequences differed from each other and clustered with published MERS-CoV sequences from humans and dromedary camels with no clear correlation in time or location. Sequences from the sample from camel C7 most closely matched sequences obtained from a human in Hafar Al-Batin in 2013; sequences from camel C9 most closely matched sequences obtained from a human in Riyadh in 2014; and the sequence from camel C23 was identical to a sequence obtained from a dromedary camel in an unidentified region of Saudi Arabia in 2014 (Figure 2). No coding differences from consensus were identified in the spike protein receptor binding domain region (residues 484–567) that directly interacts with the dipeptidyl peptidase-4 receptor (*21*).

Figure 1. Mucopurulent nasal discharge and lacrymation in 8-month-old dromedary camel naturally infected with Middle East respiratory syndrome coronavirus, Ahsa, Saudi Arabia, December 2013.

Discussion

Our results confirm previous reports documenting wide circulation of MERS-CoV in dromedary camel populations in the Middle East. In other studies, RT-PCR detection of MERS-CoV in nasal swab specimens from these animals has ranged from 1.6% to 41.7%. Studies conducted in Qatar detected MERS-CoV in 4 (35.7%) of 14 (*13*) and 5 (41.7%) of 12 (*9*) animals tested; in Saudi Arabia, 9 (22%) of 41 (*16*) and 51 (25%) of 202 (*5*); in Oman, 5 (6.6%) of 76 (*14*); and in Egypt, 4 (3.6%) of 110 (*17*). A recent large study of 7,803 dromedary camels in the United Arab Emirates identified MERS-CoV RNA in only 1.6% of animals (*22*). Of note, these authors found proportionately more positive animals near the border with Saudi Arabia and detected >5-fold more among animals sampled from slaughter houses.

Overall, we detected MERS-CoV in the upper respiratory tract of a higher proportion of animals tested in Al-Ahsa, but this proportion was within the upper range previously reported. In contrast, Alagaili et al. (*5*), in a comprehensive survey conducted in November and December 2013, sampled 5 regions of Saudi Arabia (Gizan in the south, Taif in the west, Tabuk in the north, Uniza in the center, and Hofuf [Al-Ahsa] in the east) and reported 66% positivity by rRT-PCR in animals from Taif versus only 5% from Al-Ahsa, despite seroprevalence of 92% in the latter. During the same period and in the same region, we detected MERS-CoV in 38.5% of nasal swab samples. This difference may be because of differences in the numbers and ages of animals sampled, time

Table 2. Middle East respiratory syndrome coronavirus among dromedary camels, by sample group and camel age, Al-Ahsa Province, Saudi Arabia, 2013–2014*

	Nasal swab samples, live camels		Lung tissue samples, camel carcasses		Total samples	
Age, y	No. tested	No. (%) positive	No. tested	No. (%) positive	No. tested	No. (%) positive
Young, <4	36	15 (41.7)	28	23 (82.1)	64	38 (59.4)
Adult, $\geq$4	60	13 (21.7)	63	33 (52.4)	123	46 (37.4)
Total samples	96	28 (29.2)	91	56 (61.5)	187	84 (44.9)

*Tested by reverse transcription PCR.

of specimen collection, or even between geographically proximate dromedary camel herds where rates of MERS-CoV detection can vary dramatically (*9*).

Of note, detection of MERS-CoV RNA by RT-PCR does not necessarily indicate active virus replication. When 3 dromedary camels were experimentally inoculated, infectious MERS-CoV was detected in the upper respiratory tract for only 7 days, but RNA could be detected by RT-PCR for up to 35 days after inoculation (*23*). We were unable to perform virus isolation studies because of lack of suitable biosafety infrastructure.

We also found that a high proportion of lung tissues from slaughtered dromedary camels at the Al Omran abattoir were MERS-CoV positive by RT-PCR. In their experimental inoculation study, Adney et al. (*23*) observed histologic lesions in the epithelium of the upper and lower (trachea, bronchi, and bronchioles) respiratory tract and recovered viable virus from these tissues and from 1 of 4 lung lobes of an animal euthanized 5 days after inoculation; viable virus was not recovered from tissues of 2 other animals at 28 and 42 days after inoculation. Although that limited study found infection extending to the lung of 1 animal, the authors found that the upper respiratory tract was the predominant site of virus replication and offered that finding as an explanation for the lack of observed systemic illness among naturally infected dromedary camels. An alternative hypothesis posits that, in the natural setting, subclinical MERS-CoV infection of the lower respiratory tract also occurs, possibly enhanced by crowding and stress endured during transport and corralling before slaughter. Although we did not collect matching premortem nasal swab samples from slaughtered animals to determine how many were also positive for MERS-CoV in the upper respiratory tract, our findings raise the possibility that testing upper respiratory tract samples alone may underestimate the true number of actively infected animals. In humans, MERS-CoV was detected in the lower respiratory tract of infected patients for ≈1 month while oronasal swab samples were negative (*24*). Likewise, MERS-CoV detection has been found to be enhanced from lower respiratory tract specimens, and therefore these specimens are recommended by the World Health Organization for diagnosis of MERS-CoV infection (*2*,*24*,*25*). Although great care was taken to avoid contamination with ambient MERS-CoV present in the abattoir, the possibility that sample contamination occurred cannot be entirely ruled out. Further studies that include immunohistologic examination and virus isolation from the lower respiratory tract of naturally infected dromedary camels will be needed to substantiate these findings.

Our detection of MERS-CoV RNA in 2 camel calves with purulent nasal discharge was consistent with those of Hemida et al. (*16*), who also observed mild clinical signs characterized by nasal discharge in some naturally infected young dromedary camels, and of Adney et al. (*23*), who documented appearance of purulent nasal discharge in the 3 experimentally infected adult dromedary camels. We also detected MERS-CoV RNA in a higher proportion of specimens from younger than from older adult dromedary camels, consistent with findings of previous studies that MERS-CoV infection is more common among young camels (*5*,*16*).

Our study also investigated temporal variation in MERS-CoV infection in dromedary camels. Although data interpretation was complicated by discontinuity in the months sampled and sampling from only 1 animal group in some months, a temporal pattern in MERS-CoV prevalence was apparent. For both animal groups, peak detection occurred during November 2013–January 2014, followed by a steady decline, reaching the lowest point in May 2014. Although we observed no clear temporal differences in the geographic origins or ages of dromedary camels brought to slaughter, which might bias these results, our data are nevertheless limited and should not be used to imply a general pattern of MERS-CoV circulation in dromedary camels in Saudi Arabia. Nevertheless, these findings would not be unexpected. Increased circulation of MERS-CoV among dromedary camels during the cool season is consistent with the prevailing cooler ambient temperatures, which have been shown to enhance coronavirus survivability outside the host (*26*,*27*), and the cool season is the period of peak circulation of other respiratory viral pathogens of humans in Saudi Arabia (*28*–*30*). This period also corresponds with the peak calving season for dromedary camels in Saudi Arabia (*16*); higher rates of MERS-CoV infections among a greater proportion of young animals with higher virus loads may increase opportunities for virus spread (*5*,*16*).

Whereas the link between dromedary camels and MERS-CoV infection of humans is well established (*15*,*31*), the overall contribution of zoonotic infections to community-acquired MERS-CoV remains unclear. Serologic studies of animal handlers in Saudi Arabia who work

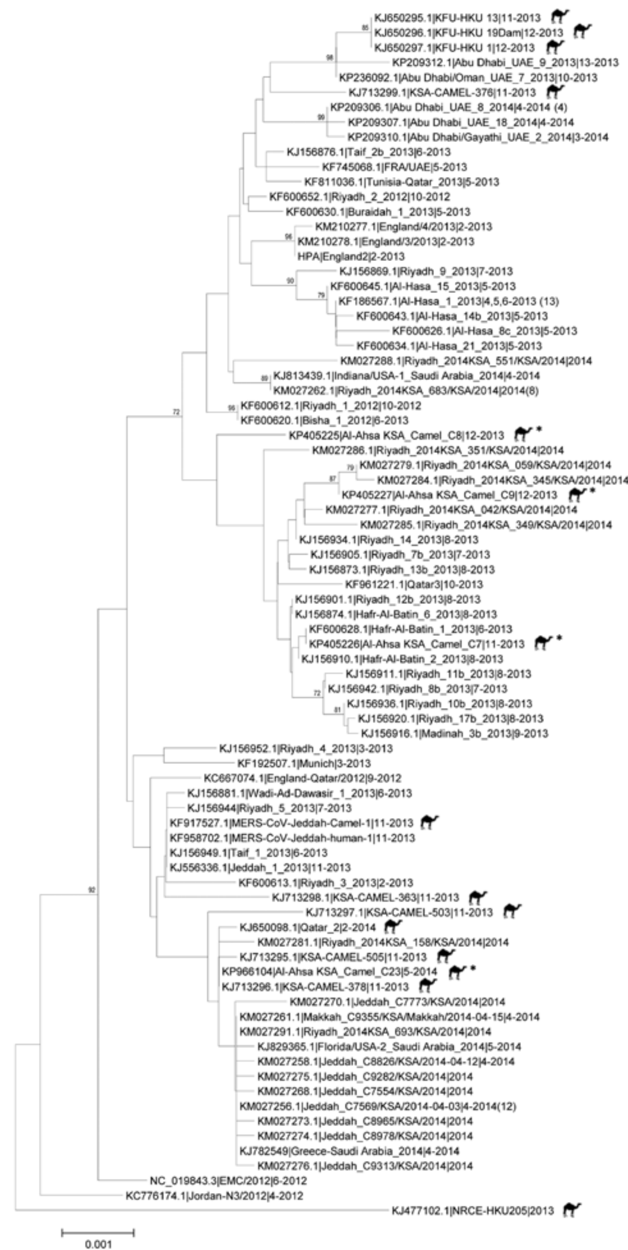

Figure 2. Midpoint-rooted phylogenetic tree of Middle East respiratory syndrome coronavirus spike gene open reading frame sequences of this virus obtained from camels and select humans (sequences available from GenBank). The estimated neighbor-joining tree was constructed from nucleotide alignments by using MEGA version 6.06 (http://www.megasoftware.net). Sequence names are derived from GenBank accession number | virus strain name | month-year of collection. Numbers in parentheses denote number of additional available identical spike gene sequences obtained from same identified region of the representative strains. Bootstrap support values (1,000 replicates) ≥70% are plotted at the indicated internal branch nodes. Scale bars indicate number of nucleotide substitutions per site. Sequences obtained from camels are designated by an icon; sequences obtained from camels in Al-Ahsa Province, Saudi Arabia, 2013–2014, are designated by an asterisk (*).

in close proximity to dromedary camels have shown limited evidence of MERS-CoV infection (*32–34*). Alghamdi et al. (*35*), who examined patterns of MERS-CoV infections among humans in Saudi Arabia between June 2013 and May 2014, did not find a concomitant temporal increase in human infections that corresponded with our findings in dromedary camels. Those authors observed a slight, temporary increase in cases among humans in June and September 2013 and few cases from October through February, after which cases and deaths sharply increased beginning in April 2014. The authors concluded that lower relative humidity and higher temperatures during these months might have contributed to the dramatic surge in reported cases. However, more recent data from the World Health Organization (*36*) show a sharp decline in MERS-CoV cases among humans in May 2014; low numbers of cases were reported from June through August 2014, when mean temperature was highest and relative humidity was lowest in Saudi Arabia (*34*). Moreover, a recent increase in numbers of MERS-CoV cases in humans from September 2014 through February 2015 corresponds more closely with the temporal pattern we found in dromedary camels the preceding year. Further studies conducted over multiple years are needed to better understand the ecology of MERS-CoV, which might help inform intervention strategies to reduce zoonotic infections.

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Assessment of Arbovirus Surveillance 13 Years after Introduction of West Nile Virus, United States[1]

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Before 1999, the United States had no appropriated funding for arboviral surveillance, and many states conducted no such surveillance. After emergence of West Nile virus (WNV), federal funding was distributed to state and selected local health departments to build WNV surveillance systems. The Council of State and Territorial Epidemiologists conducted assessments of surveillance capacity of resulting systems in 2004 and in 2012; the assessment in 2012 was conducted after a 61% decrease in federal funding. In 2004, nearly all states and assessed local health departments had well-developed animal, mosquito, and human surveillance systems to monitor WNV activity and anticipate outbreaks. In 2012, many health departments had decreased mosquito surveillance and laboratory testing capacity and had no systematic disease-based surveillance for other arboviruses. Arboviral surveillance in many states might no longer be sufficient to rapidly detect and provide information needed to fully respond to WNV outbreaks and other arboviral threats (e.g., dengue, chikungunya).

Before 1999, there was no appropriated funding in the United States for arboviral surveillance, and many states had no arboviral surveillance systems (*2*). After the emergence of West Nile virus (WNV) in New York, New York, in 1999 (*3*), Congress appropriated annual funding to support WNV surveillance activities in affected states and large cities; funds were awarded to these areas through epidemiology and laboratory capacity (ELC) cooperative agreements from the Centers for Disease Control and Prevention. CDC collaborated with state, local health, and academic partners to develop WNV detection, monitoring, and prevention guidance (*4,5*). By 2004, WNV had spread across the continental United States (*6*), and transmission to humans had been documented by multiple routes, including blood transfusions and organ transplantation (*7–10*). That year, CDC distributed nearly $24 million to all states and 6 large city/county health departments for WNV surveillance and prevention.

In 2000, CDC established ArboNET, a comprehensive national surveillance data capture platform to monitor WNV patterns. In 2003, CDC expanded ArboNET to include other arboviral diseases. ArboNET relies on a distributed surveillance system, whereby ELC-supported state and local health departments report data weekly on detection of arboviruses in humans, animals, and mosquitoes. CDC posts all data on the Internet with weekly updates (*11*). In 2004, the Council of State and Territorial Epidemiologists (CSTE) conducted a WNV surveillance capacity assessment and found that WNV surveillance programs were in place and well developed in jurisdictions receiving WNV surveillance funding (*12*). CSTE attributed the success of capacity development primarily to availability of federal funds and technical guidance from CDC.

Annual funding for WNV and other arbovirus surveillance distributed through the ELC cooperative agreements has steadily decreased since 2006 to 39% of its 2004 zenith, reaching lows of $9.3 million in 2012 and in 2013 (R.S. Nasci, unpub. data). Concomitantly in 2012, the nation experienced the highest incidence of confirmed WNV neuroinvasive disease since 2003 and the highest number of confirmed deaths (286) for any year thus far (*13*). In addition to the continued challenge of WNV to financially stressed arbovirus surveillance systems, there is the growing threat of other arboviral diseases, such as dengue (*14*), chikungunya (*15–17*), and Powassan virus encephalitis (*18*).

In August 2013, CSTE conducted another assessment of state and selected local health departments (LHDs) to

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[1]A shorter version of this report has been published previously (*1*).

measure their current surveillance and staffing capacity for WNV and other arboviruses and compare findings with those from the 2004 assessment (*19*). Its objectives were to describe 1) national capacities for surveillance for WNV and other arboviruses in the 50 states and 6 ELC-funded LHDs in 2012 and changes since 2004; 2) surveillance capacities of LHDs with historically high WNV burdens but no direct federal funding and how they compare with those in ELC-supported LHDs; and 3) the outstanding needs to bring US arbovirus surveillance to full capacity.

Methods

The assessment tool was developed by a working group that included representatives from CSTE, the Association of State and Territorial Health Officials, the National Association of County and City Health Officials, the Association of Public Health Laboratories, the CDC Division of Vector-Borne Diseases, and Emory University. The working group developed the 2013 survey by modifying the 2004 assessment tool and adding unique questions that reflected new WNV surveillance, prevention, and control guidance (*20*) and assessed specific staffing needs, presence of *Aedes aegypti* mosquitoes, and effect of federal WNV surveillance funding reductions on WNV surveillance activities over the past 5 years.

After pilot studies in 7 states and 4 LHDs, CSTE emailed the final state survey to the 50 state health departments and instructed key respondents to obtain relevant information from laboratory and mosquito surveillance and control staff, and complete the assessment online. The Epi Info Web Survey System was used to collect responses (*21*). CSTE used a similar process for distributing the assessment to 30 large city/county health departments that met at least 1 of 3 criteria: 1) receive supplemental WNV surveillance funding through the ELC grant (n = 6 [Washington, DC; New York, NY; Los Angeles County, CA; Chicago, IL; Houston, TX; and Philadelphia, PA); 2) had at least 100 cumulative reported cases of WNV neuroinvasive disease during 1999–2012 (n = 22, excluding 4 of the ELC-funded LHDs); or 3) had recent local dengue transmission (n = 2).

The 2 assessments were analyzed separately. Frequencies of response to each question were examined in aggregate and by groupings of state health departments on the basis of whether they reported a need for additional staff. LHDs were grouped by whether they received federal WNV surveillance funding, which was referred to as ELC-supported. Additional need to achieve full capacity was based on response to the question, “How many additional FTE (full-time equivalent) staff-persons are needed at the state level in your state to achieve full epidemiology and laboratory capacity to conduct WNV and other mosquito-borne disease surveillance?” Full capacity was defined as 1) ability to complete a standard case report form on every suspected/confirmed mosquito-borne arboviral disease case and report it to ArboNET; 2) ability to test for IgM for all relevant arboviruses (including dengue) on any cerebrospinal fluid (CSF) or serum specimen submitted to the state or city/county laboratory for a suspected case of arboviral disease); and 3) having an environmental surveillance system that includes mosquito surveillance to “routinely monitor arboviral activity in all parts of the jurisdiction in which there is the potential for human outbreaks of arboviral disease based on past experience.”

For staffing-related questions, nonresponses were coded as no staff needed. For all other questions, nonresponses were assumed to be missing responses. Differences of ≥10% between groups being compared were deemed functionally useful and are highlighted in the results. Data analysis was performed by using Microsoft Excel (Microsoft, Redmond, WA, USA) and Epi Info version 7 (CDC, Atlanta, GA, USA).

Results

All 50 states (100%), all 6 ELC-supported LHDs (100%), and 15 LHDs without ELC support (62.5%) responded. In 2012, nearly all states (98%) conducted surveillance for human WNV disease; fewer conducted WNV-related surveillance for equine disease (90%), mosquitoes (80%), and avian deaths (39%) (Table 1). Less than 60% of jurisdictions contacted medical specialists (neurologists, critical care, infectious disease) to encourage reporting of suspected WNV cases, and less than one third had an active surveillance component for human surveillance. Although only 80% of states conducted mosquito surveillance, 90% collected information about mosquito surveillance from LHDs in their state, including 86% by mosquito species. Overall, 46 (94%) states had at least some information on mosquito populations, either by collecting it themselves or from LHDs. It took a median of 6 days (range 1.5–17 days) from the date a WNV-positive human specimen was collected for data to be reported to the WNV surveillance program, and a median of 16.5 days (range 4–45 days) from date of onset to date reported to ArboNET.

Fewer jurisdictions in 2012 than in 2004 conducted WNV-related surveillance activities, particularly avian deaths (26 states, –59%), active human surveillance (9 states –18%), contact with infectious disease specialists (12 states, –24%), and state-level mosquito surveillance (8 states, –16%). In addition, the percentage of states responding that most LHDs in their state conducted adult mosquito surveillance decreased from 48% to 34% (Table 1). There was a slight improvement in timeliness of reporting, from a median of 7 days to 6 days.

Table 1. States conducting selected West Nile virus surveillance activities, United States, 2004 and 2012*

Surveillance activity	No. responding states (% with activity) 2012	2004	% Difference from 2004 to 2012
Human surveillance			
Formal surveillance system	50 (98)	49 (100)	–2
Active surveillance component	49 (29)	49 (47)	–18
Use official case definition	50 (88)	49 (88)	0
Require reporting of encephalitis of unknown etiology	50 (48)	49 (63)	–15
To encourage reporting and to suggest a high index of suspicion, did you contact			
Neurologists	48 (50)	48 (60)	–10
Critical care specialists	48 (48)	49 (57)	–9
Infectious disease specialists	48 (58)	49 (82)	–24
Equine surveillance			
Formal surveillance system	49 (90)	49 (94)	–4
Active surveillance component	44 (5)	46 (24)	–19
Designated public health veterinarian within the agency?			
Yes	50 (76)	49 (82)	–6
Avian surveillance			
Formal avian death surveillance	49 (39)	49 (98)	–59
Active component	19 (10)	48 (44)	–34
Sentinel chicken surveillance	50 (10)	–	NA
Adequate access to wildlife expertise within agency	50 (76)	49 (92)	–16
Mosquito surveillance			
Formal surveillance system	49 (80)	49 (96)	–16
Collect information about mosquito surveillance from LHDs in state? (states only)			
Yes	49 (90)	49 (94)	–4
By species?	43 (86)	45 (80)	+6
Do most LHDs in your state conduct surveillance for (states only)			
Adult mosquitoes	44 (34)	44 (48)	–14
Larval mosquitoes	44 (18)	44 (30)	–11
Adequate access to entomologist in agency or by contract	50 (64)	49 (71)	–7

*–, not asked; NA, not applicable; asked; LHDs, local health departments.

In 2012, 92% of states had some public health laboratory capacity for WNV testing to support human surveillance and 84% to support mosquito surveillance (Table 2). Most (93%) states tested human specimens for IgM and mosquito specimens by using PCR (72%) or culture (13%). Relatively few states tested human specimens by using PCR (13%) or culture (2%). When compared with 2004, many fewer laboratories conducted IgG, PCR and culture tests on human specimens in 2012. Testing methods for mosquitoes did not change greatly.

We also assessed state public health laboratory capacity to test for 10 arboviruses, in addition to WNV, in human serum or CSF specimens. St. Louis encephalitis (SLE) virus testing capacity was most common (34 laboratories), followed by testing for eastern equine encephalitis (EEE) (24), western equine encephalitis (WEE) (16), LaCrosse (16), dengue (9), Powassan (4), chikungunya (2), Colorado tick fever (2), yellow fever (2), and Japanese encephalitis (1) viruses. These laboratories reported performing 41,159 tests for arboviruses in 2012, of which 19,180 (46.6%) were for WNV. Of these tests, the highest percentage of positive test results was for dengue virus (137/328, 41.8%), followed by WNV (2,953/19,178, 15.4%), Powassan virus (62/1,257, 4.9%), LaCrosse virus (121/3,372, 3.6%), SLE virus (164/8,216, 2.0%), Colorado tick fever virus (2/139, 1.4%), and WEE virus (12/3,888, 0.03%).

Although many laboratories had the capability to test for arboviruses other than WNV, not all routinely did so. Overall, 26 (60%) of 43 responding laboratories reported routinely testing human CSF specimens submitted for WNV for at least 1 other arbovirus. Of these 26 laboratories, 24 routinely tested for SLE virus, 12 for EEE virus, 6 for WEE virus, 5 for LaCrosse virus, and 2 for Powassan viruse. Among laboratories serving the 45 states that either test mosquitoes or use another laboratory, 24 reported routinely testing mosquito pools for SLE virus, 22 for EEE virus, and 13 for the California serogroup. To manage federal WNV surveillance funding reductions over the past 5 years, 57% of states reported eliminating avian death surveillance, 58% decreased mosquito trapping, 68% decreased mosquito testing, and 46% decreased the number of human specimens tested for WNV.

The responses from the 6 LHDs with ELC WNV surveillance support to each surveillance capacity were similar to those from the 50 states in 2004 and 2012, except for laboratory capacity. Currently, only 4 ELC-supported LHDs do some of their own WNV testing.

Arboviral Surveillance in LHDs without Federal WNV Surveillance Support

The 15 LHDs without ELC grants included 13 with high WNV burden and 2 with recent dengue transmission. These LHDs were generally less likely to take an active role in

Table 2. States with laboratory capacity to support WNV and other arboviral surveillance activities, United States, 2004 and 2012*

Laboratory capacity	No. responding states (% with activity) 2012	2004	% Difference from 2004 to 2012
Overall			
Have some in-state capacity for WNV testing	50 (92)	–	NA
Human surveillance			
Test for IgG	46 (48)	47 (72)	–24
Test for IgM	46 (93)	47 (100)	–7
Test by culture	46 (2)	47 (19)	–17
Test by PCR	46 (13)	47 (49)	–36
Test by PRNT	46 (22)	47 (21)	+1
Test all CSF specimens submitted for WNV for ≥1 other arbovirus	43 (60)	–	NA
Avian surveillance			
Test by culture	46 (4)	47 (13)	–9
Test by PCR	46 (39)	47 (77)	–38
Test IgG or IgM	46 (11)	47 (9)	+2
Test by any of above methods	46 (43)	47 (77)	–34
Mosquito surveillance			
In-state capacity to test mosquitoes (state or local level)	50 (84)	–	NA
Testing for >1 other arbovirus	42 (81)	–	NA
Culture or PCR	42 (81)	47 (81)	0
Vec Test or RAMP	42 (19)	47 (21)	–2

*WNV, West Nile virus; –, not asked; NA, not applicable; PRNT, plaque reduction neutralization test; CSF, cerebrospinal fluid; Vec Test, vector test; RAMP, rapid analyte measurement platform.

surveillance for human disease or avian deaths than the 6 LHDs with ELC WNV surveillance support (Table 3). Furthermore, they were less likely to conduct their own mosquito surveillance (67% vs. 100%); 5 LHDs did not conduct any mosquito surveillance. LHDs that conducted mosquito surveillance tended to more consistently conduct larval surveillance and identify trapped mosquitoes to species.

Few of these 15 LHDs had their own laboratory capacity to support either testing of human specimens (n = 1) or mosquitoes (n = 3) for WNV. Most were dependent on their state health department for this function.

Staffing Levels and Need for Additional Staffing

A total of 503 persons worked on arbovirus surveillance in state health departments in 2012. Of these, 206 worked at least half-time on it and 297 worked less than half-time. Overall, 40% of those working at least half-time were CDC funded. When converted to FTEs, there were 208.9 FTEs working on arbovirus surveillance in state health departments in 2012; 17% were epidemiologists, 31% laboratory workers, 27% mosquito surveillance staff, and 25% support staff (Table 4).

In the 21 LHDs, 187 persons worked on arbovirus surveillance in 2012; a total of 104 worked at least half-time

Table 3. Local health departments conducting selected WNV surveillance activities, by whether they received federal WNV surveillance funding (ELC) support, United States, 2012*

Surveillance activity	No. responding LHDs (% with activity) No ELC support	ELC support	% Difference between no ELC and some ELC support
Human surveillance			
Formal local-level surveillance system	15 (0)	6 (100)	–100
To encourage reporting and suggest a high index of suspicion, did you contact			
Neurologists	15 (33)	6 (83)	–50
Critical care specialists	15 (47)	6 (83)	–36
Infectious disease specialists	15 (47)	6 (100)	–53
Emergency departments	15 (53)	6 (100)	–47
Equine surveillance			
Formal surveillance system	15 (33)	55 (39)	–6
Designated public health veterinarian within the agency?			
Yes	15 (33)	6 (50)	–17
Avian surveillance			
Formal avian death surveillance	15 (20)	6 (67)	–47
Mosquito surveillance			
Formal surveillance system	15 (67)	6 (100)	–33
For larval mosquitoes?	10 (90)	3 (67)	+23
For adult mosquitoes?	10 (100)	6 (100)	0
Identify trapped mosquitoes to species?	10 (90)	6 (83)	+7
Calculate minimal mosquito infection rates?	10 (50)	6 (83)	–33
Adequate access to entomologist in agency or by contract	14 (31)	6 (50)	–19

*WNV, West Nile virus: ELC, epidemiology and laboratory capacity (received specific WNV surveillance funding through the Epidemiology and Laboratory Capacity Cooperative Agreement); LHDs, local health departments.

Table 4. FTE positions for arbovirus surveillance in 2012 and additional FTEs needed by functional job category, 50 states and 21 local health departments, United States*

Characteristic	FTE epidemiologists	FTE laboratory staff	FTE mosquito surveillance staff	FTE support and administrative staff	Total FTEs
State					
2012	34.6 (16.6)	64.6 (30.9)	57.2 (27.4)	52.5 (25.1)	208.9
No. needed	25.1 (20.5)	26.4 (21.5)	53.6 (43.7)	17.5 (14.3)	122.6
Total	59.7	91.0	110.8	70.0	331.5
Local					
2012	32.8 (19.3)	7.4 (4.4)	93.9 (55.6)	34.8 (20.6)	168.9
No. needed	6.2 (9.7)	7.5 (11.7)	36.3 (56.5)	14.2 (22.1)	64.2
Total	39.0	14.9	130.2	49.0	233.1

*Values are no. (%). FTE, full-time equivalent.

and 83 worked less than half-time on it. Similar to state health departments, only 35% of the at least half-time time staff were CDC funded (either directly or through the state). These persons accounted for 168.9 FTEs: 19% were epidemiologists, 4% laboratory workers, 56% mosquito surveillance staff, and 21% support staff (Table 4). LHDs had the same proportions of FTEs involved in mosquito surveillance (56%), regardless of whether they were ELC-supported.

Staffing Changes from 2004 to 2012 and Additional Needs

In states and the 6 LHDs with ELC grants for WNV surveillance, the overall numbers of persons working in arbovirus surveillance and the numbers of those working at least half-time on it decreased from 2004 to 2012. In states, the decreases were 28% (from 702 to 503) and 41% (from 348 to 206), respectively (Figure 1). In LHDs, these decreases were 18% (from 228 to 187) and 5% (from 109 to 104), respectively.

Regarding staffing needs, 40 (80%) states reported needing 122.6 additional FTEs, a 59% increase over current capacity: 27 needed epidemiologists, 30 laboratory staff, 28 mosquito surveillance staff, and 19 support staff. Of the 122.6 needed FTEs, the single largest category was mosquito surveillance staff, which accounted for 44% of additional need, followed by laboratorians (22%). For LHDs, 64.2 additional FTEs were needed, a 38% increase, and most (57%) needed positions in mosquito surveillance staff (Table 4).

Association of Staffing Needs with Level of Arbovirus Surveillance

States needing more staff were less likely to conduct WNV and other arbovirus surveillance activities than those with no need. States needing more epidemiologists were less likely to have conducted outreach to encourage medical specialists to report WNV cases (Figure 2, panel A). These states were also less likely to have performed year-end catch-up surveillance by contacting hospital or commercial laboratories (0% vs. 16%). States reporting a need for laboratorians were less likely to have at least some WNV testing capacity, perform testing on mosquito pools in 2012, and test WNV-positive specimens for other mosquito-borne viruses and were more likely to report a reduction in mosquito pool testing capacity since 2008 (Figure 2, panel B). States needing additional mosquito surveillance staff were less likely to test mosquito pools and to have identified any *Ae. aegypti* mosquitoes in the past 5 years and were more likely to have decreased the numbers of mosquito trap-nights and mosquito pools tested and report that their mosquito testing capacity had decreased since 2008 (Figure 2, panel C).

Discussion

There are several critical objectives of arbovirus-related surveillance at each level of government: 1) monitor for and detect early signs of an outbreak threat to enable a timely response and prevent human illness and death; 2) monitor for arboviruses of human health concern and their vector populations; 3) detect changes in arbovirus disease burden over time and space; and 4) inform the public of the risks and how they can decrease them. Several findings from this assessment highlight the current capacity to meet these objectives and help to inform federal, state, and local public health and preparedness officials interested in evaluating their current arbovirus surveillance capacity.

First, current surveillance capacities at the national and state levels are far greater now than those in 1999, before the introduction of WNV. Almost all states are conducting surveillance for human WNV disease, and most are monitoring mosquito populations for WNV and have some

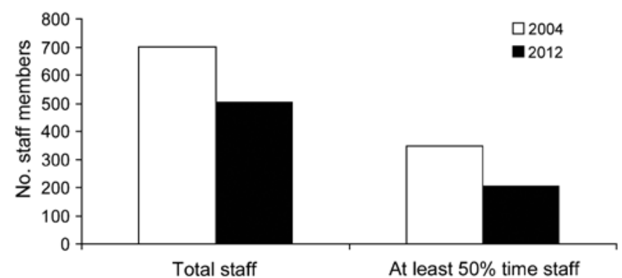

Figure 1. Total and at least 50% time staff performing West Nile virus surveillance in state health departments, United States, 2004 and 2012.

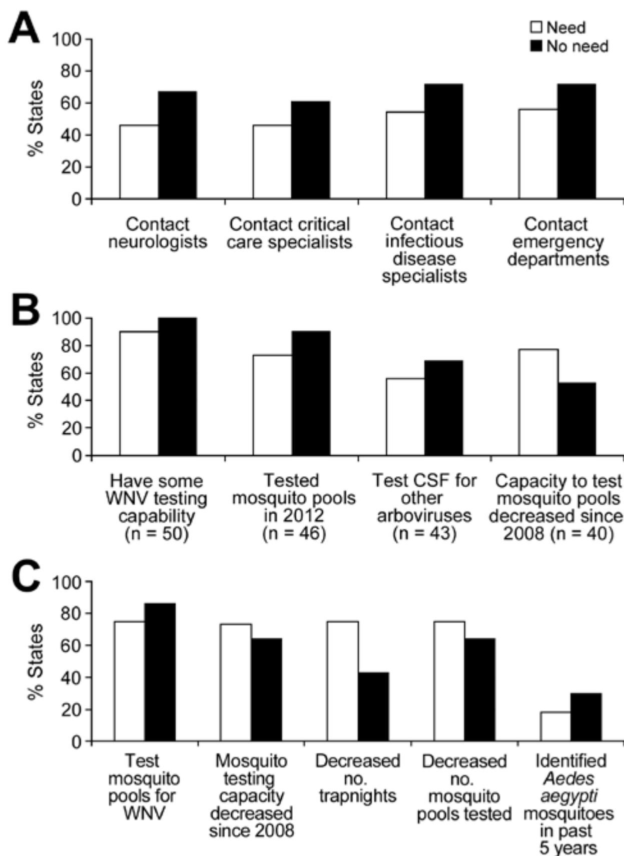

Figure 2. Comparison of surveillance indices in states reporting need for additional staff with those not reporting a need by type of staff needed, United States, 2012. A) Epidemiologists; B) Laboratory staff; C) Mosquito surveillance staff. WNV, West Nile virus; CSF, cerebrospinal fluid. Values in parentheses are number of states.

WNV testing capacity. All are reporting WNV and other arbovirus activity to ArboNET.

Second, the ability to detect the early signs of an outbreak of WNV and other arboviruses that can threaten large human populations has been compromised since 2004. Endemic arboviruses that have caused outbreaks of severe illness and death in densely populated areas continue to pose annual threats, and emerging diseases, such as dengue and chikungunya, pose new ones (*14,15–17,22*). Knowledge of local vector mosquito populations and early detection of arbovirus activity in these vectors, animals, and humans are essential to guide public health action ranging from health advisories to mosquito control. Many fewer states now conduct any form of active surveillance that enables rapid detection of the first sentinel human cases of arbovirus disease. Most states have cut back on support for mosquito surveillance. Some states and large metropolitan areas, including some with previously large WNV outbreaks, lack the necessary mosquito surveillance information to anticipate a surge in WNV infection. Most lack the resources to map the distribution and size of either *Ae. aegypti* or *Ae. albopictus* mosquito populations to enable risk evaluation or to mount an effective response to identification of local transmission of dengue or chikungunya viruses.

Third, in addition to the decreased ability to monitor vector mosquito populations, testing for arboviruses other than WNV, SLE virus, and EEE virus is patchy and inadequate to detect or monitor their presence in many states. Some endemic arboviruses that cause either encephalitis or acute systemic or febrile disease (e.g., Powassan, LaCrosse, Colorado tick fever, and Heartland viruses) have not been included in systematic public health surveillance, and their ecology and epidemiology might be changing.

For example, Powassan virus spreads to humans from animal reservoirs by the same tick genus (*Ixodes*) that transmits Lyme disease and babesiosis. Although Lyme disease and babesiosis have increased dramatically in incidence and geographic distribution in the United States in the past decades, there is still a poor understanding of Powassan virus epidemiology >50 years after its discovery. Most state health department laboratories do not test for Powassan virus when they test for WNV or other arboviruses, and few clinicians order commercial tests specifically for this virus. Powassan, LaCrosse, and Colorado tick fever viruses were tested for in only 8%, 32%, and 4% of state laboratories, respectively, in 2012. However, a higher percentage of specimens were positive for Powassan and LaCrosse virus infections than for SLE and for Colorado tick fever than EEE or WEE. These results support surveillance for these viruses in jurisdictions with relevant vectors when routinely testing for WNV. If their epidemiology were better understood, estimates of their disease burden could be improved, and the public could be better informed of the risk for infection.

Fourth, state laboratory capacity is essential to enable LHDs to monitor virus activity through mosquito, avian death, or sentinel-chicken surveillance. The ability of ArboNET to synthesize and report useful surveillance information is possible only because of efforts made at each state and local health department to conduct the nationally recommended level of surveillance to meet surveillance objectives. This assessment documents, that as resources have decreased, LHDs dependent on state laboratories to conduct testing for them have reduced or eliminated mosquito-based surveillance to the point where 15% of states no longer provide support for LHDs and one third of responding LHDs in areas with a high incidence of WNV no longer conduct mosquito-based surveillance.

In 2004, all states approached full capacity for 2 of the 3 criteria for full arbovirus surveillance capacity used in this report: ability to complete a standard case report

form on every suspected/confirmed mosquito-borne arboviral disease case and report it to ArboNET and having an environmental surveillance system that includes mosquito surveillance "to routinely monitor arboviral activity in all parts of the jurisdiction in which there is the potential for human outbreaks of arboviral disease based on past experience." In 2012, although the first criterion continued to be met, the second criteria was no longer met. Although the 2004 assessment did not measure the ability to test for IgM for all relevant arboviruses (including dengue viruses) on any CSF or serum specimen submitted to the state or city/county laboratory on a suspected case of arboviral disease, this assessment found that many states are not meeting this remaining criterion.

This assessment has several major limitations. First, not all jurisdictions answered all questions. Second, additional personnel needs were based on state and local health department self-assessment and are subjective. In addition, because of the way the assessment was worded and responded to, we assumed states not specifying a need for additional personnel had no need. Thus, results showing that states that identified a need also performed far fewer surveillance activities than states with no reported additional need are subject to possible inaccuracies in this assumption. Third, the relative role of different surveillance methods shifted between 2004 and 2012. Whereas needs for human surveillance and laboratory testing capability and capacity are largely unchanged, the need for avian death and equine surveillance data in many jurisdictions has decreased, but the need for mosquito surveillance data has increased. Jurisdictions have adjusted resources to accommodate these changes. This adjustment may explain, in part, the generally high US WNV surveillance capacity, despite federal funding cuts of more than 50%. Fourth, measures of workload and staffing need may be difficult to compare among years because they depend, in part, on levels of WNV activity. The human WNV burden in 2012 was more than double that in 2004, which may have influenced estimates of need. Finally, the 2012 assessment did not solicit information on funding or unmet needs for anything other than staff. For example, limited fiscal resources might preclude purchase of updated laboratory equipment and testing reagents, thereby limiting laboratory testing of mosquito pools and testing of human and nonhuman specimens for arboviruses other than WNV. Unmet nonpersonnel needs might have contributed to loss of arbovirus surveillance capacity and would need to be addressed in any effort to maintain or improve it.

In summary, WNV emergence in the United States stimulated building of a robust national arbovirus surveillance system with human and vector early detection components and laboratory services. This system, although still highly functional, has become less robust and might be near a large-scale tipping point, especially in areas of vector surveillance and laboratory support for human diagnostic and mosquito testing. Already, arboviral surveillance is inadequate in many states to rapidly detect and control outbreaks and to give the public the critical information it needs for prevention.

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Results from the National *Legionella* Outbreak Detection Program, the Netherlands, 2002–2012

Jeroen W. Den Boer, Sjoerd M. Euser, Petra Brandsema, Linda Reijnen, Jacob P. Bruin

In 2002, the National *Legionella* Outbreak Detection Program was implemented in the Netherlands to detect and eliminate potential sources of organisms that cause Legionnaires' disease (LD). During 2002–2012, a total of 1,991 patients with LD were reported, and 1,484 source investigations were performed. Of those sources investigated, 24.7% were positive for *Legionella* spp. For 266 patients with LD, 105 cluster locations were identified. A genotype match was made between a strain detected in 41 patients and a strain from a source location. Despite the systematic approach used by the program, most sources of LD infections during 2002–2012 remained undiscovered. Explorative studies are needed to identify yet undiscovered reservoirs and transmission routes for *Legionella* bacteria, and improved laboratory techniques are needed to detect *Legionella* spp. in samples with a high background of microbial flora such as soil.

Legionnaires' disease (LD) is an acute pneumonia characterized by clinical symptoms and signs (e.g., cough, fever, lung infiltration observed on a chest radiograph) similar to those of pneumonias resulting from other pathogens. LD is caused by infection with *Legionella* spp. bacteria, which are most often transmitted to persons through inhalation of bacteria disseminated into the air as an aerosol from natural or man-made sources of water (*1*). The incubation period is 2–14 days. LD is thought to account for 2%–20% of all community-acquired pneumonias (*2*) and is fatal in ≈6%–11% of cases (*3,4*).

After a large outbreak of LD at a flower show in Bovenkarspel, the Netherlands, in 1999 (*5*), prevention and control of *Legionella* spp. infections became a national concern in the Netherlands, and legislation to prevent *Legionella* spp. in drinking water systems was introduced (*6,7*). This legislation obligated owners of aerosol-producing devices (e.g., shower heads and whirlpools), if third parties may be exposed to them, to conduct a risk analysis, develop a control plan, keep logs of control measures, and perform regular sampling for *Legionella* spp. contamination. In addition, in 2002, a National *Legionella* Outbreak Detection Program (NLODP) was implemented (*8*) on the basis of a report that LD outbreaks are often preceded and followed by small clusters of solitary cases (*9*). The aims of NLODP are early detection of small clusters of cases, identification of sources of infection, and implementation of early control measures to prevent additional LD cases or an outbreak. For evaluation of transmission pathways, infection sources are sampled, and genotypes of *Legionella* strains found in these samples are compared with those of clinical isolate(s) from the patient(s) associated with that source. To evaluate the findings of the NLODP during 2002–2012, we analyzed data to determine whether extensive investigation efforts could detect *Legionella* spp. in collected samples and conclusively identify environmental sources.

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Methods

Patients

LD has been notifiable in the Netherlands since 1987. A case of LD is defined as laboratory-confirmed infection in a person having symptoms compatible with pneumonia or radiologic signs of infiltration. Laboratory evidence may be ≥1 of the following: isolation of *Legionella* spp. from respiratory secretions or lung tissue, detection of *L. pneumophila* antigen in urine, seroconversion or a ≥4-fold rise in antibody titers to *L. pneumophila* in paired acute- and convalescent-phase serum samples, a high antibody titer to *L. pneumophila* in a single serum sample, and direct fluorescent antibody staining of the organism or detection of *Legionella* DNA by PCR in respiratory secretions or lung tissue. In the Netherlands, microbiologic laboratories involved in the diagnosis and treatment of patients with pneumonia are requested to send available clinical isolates of *Legionella* spp. to the *Legionella* Source Identification Unit (LSIU), a part of the NLODP. LD cases in persons who had been outside the country for ≥5 of 9 days before disease onset were defined as nondomestic cases and excluded from

the analyses. Cases in persons who stayed in a hospital or other health care setting (e.g., nursing home or rehabilitation center) for ≥1 day during the 2–14 days before symptom onset were defined as nosocomial cases.

Source Identification and Cluster Detection

Potential sources of infection were identified by Municipal Health Services (MHS) public health physicians and nurses, who used a standardized questionnaire to interview patients or relatives (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/21/7/14-1130-Techapp1.pdf). The interview focused on tracking each patient's exposure to potential sources of infection during the 2–14 days before symptom onset. All potential sources of infection were recorded in a database by the LSIU and used to identify clusters of LD cases by location and date. Each new LD case in this database was examined to determine if reported potential sources were linked to other LD cases. Because outbreaks of Legionnaires' disease are often preceded and followed by small clusters of solitary cases (*9*), an arbitrary cluster definition was constructed that defined 2 types of clusters: location and geographic. A location cluster, which may represent a local contamination, was defined as cases reported within 2 years of each other in ≥2 persons who were reported to have been exposed to the same potential source of infection during the 2–14 days before symptom onset. A geographic cluster was defined as cases in >3 persons who lived <1 km apart and whose infections were reported within 6 months of each other. The concept of a geographic cluster was constructed to identify sources that patients were exposed to but unaware of (e.g., cooling towers). Patients could belong to >1 cluster. Data from the location cluster of the LD outbreak in Amsterdam in 2006 (*7*) were excluded from our analyses.

Sampling Procedure

As part of the NLODP, the LSIU is available to each MHS to collect samples from potential sources of *Legionella* infection for reported domestic LD cases. During 2002–2006, all identified potential sources of infection were investigated. However, because of budgetary reasons, after June 1, 2006, potential sources were investigated only if ≥1 of 4 sampling criteria was met: 1) a patient-derived isolate of *Legionella* spp. (from respiratory secretions or lung tissue) was available; 2) a location cluster was identified; 3) a geographic cluster was identified; or 4) the patient had stayed in a hospital or other health care setting during the incubation period. For geographic clusters, efforts were focused on identifying yet undiscovered potential sources (e.g., cooling towers near patients' residences). If ≥1 of the 4 sampling criteria was met, trained LSIU laboratory staff collected water and swab samples from identified potential sources when possible. For each location, sampling points were selected by LSIU staff in cooperation with the facility's technical team (when a team is available), and a comprehensive collection of water and swab samples was obtained from that location for further analysis.

Laboratory Investigations

Samples collected during the source investigation were analyzed for the presence of *Legionella* spp. (for an extensive description, see online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/21/7/14-1130-Techapp2.pdf). All *L. pneumophila* serogroup 1 (SG1) strains (clinical and environmental) were subsequently genotyped by sequence-based typing, as recommended by the ESCMID Study Group for Legionella Infections (*10–12*), and further determined by using the Dresden panel of monoclonal antibodies (*13*). The sequence-based typing profiles of the patient isolates were compared with those of the environmental strains found in samples of potential sources.

Statistical Analyses

Comparisons were made by using independent samples *t*-tests, nonparametric Mann-Whitney U-test, 2-tailed χ^2 tests (proportions), and linear regression analyses (trends over time). All analyses were performed with PASW Statistics 8.0 (SPSS Inc., Chicago, IL, USA).

Results

Patients

During August 2002–August 2012, a total of 2,796 LD cases were reported in the Netherlands, 805 (28.8%) of which were nondomestic (Figure). These travel-associated cases were excluded from the analyses, resulting in 1,991 reported possible domestic LD cases (mean of 193 [SD 76] cases annually); 119 (6.0%) of these were characterized as nosocomial cases. Most patients (72%) for this period were male (Table 1). The median age of reported case-patients

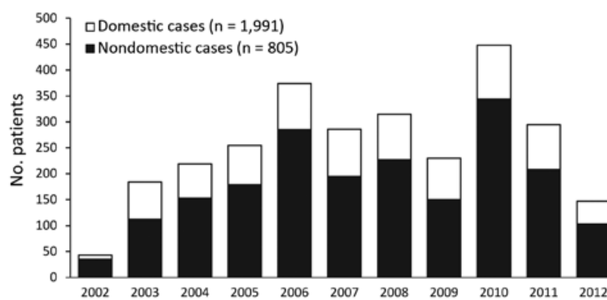

Figure. Legionnaires' disease cases reported in the Netherlands, August 1, 2002–August 1, 2012. A total of 2,796 cases were reported; LD cases in persons who had been outside the country for ≥5 of 9 days before disease onset were defined as nondomestic cases and excluded from analyses. All other cases were classified as domestic.

Table 1. Number and demographic characteristics of patients with domestically acquired cases of Legionnaires' disease, the Netherlands, 2002–2012*

Year	No. patients	Median age, y (range)	Male patients, no. (%)
2002	35	55.3 (26.4–78.3)	24 (68.6)
2003	112	57.4 (4.8–87.9)	73 (65.2)
2004	153	57.3 (21.3–88.1)	113 (73.9)
2005	179	59.1 (28.2–94.2)	132 (73.7)
2006	285	60.2 (17.0–90.1)	193 (67.7)
2007	195	59.1 (19.6–93.1)	153 (78.5)
2008	227	60.2 (11.0–98.1)	158 (69.6)
2009	150	61.3 (14.6–94.8)	106 (70.7)
2010	344	61.5 (23.2–94.1)	249 (72.4)
2011	208	62.2 (24.3–93.0)	155 (74.5)
2012	103	62.5 (27.0–91.6)	73 (70.9)
2002–2012	1,991	60.2 (4.8–98.1)	1,429 (71.8)

*Study period was August 1, 2002–August 1, 2012. Patients who had been out of the country for ≥5 of the 9 days before disease onset (n = 805) were excluded.

increased from 55.3 (range 26.4–78.3) years in 2002 to 62.5 (range 27.0–91.6) years in 2012 (Table 1; linear regression, p trend <0.001).

Diagnostic Tests

The 1,991 LD cases were ascertained by 2,541 diagnostic tests (Table 2). Most cases were diagnosed by using urinary antigen tests (83.2%) or cultures (23.1%). Nosocomial LD cases (n = 119) were more often diagnosed by culture compared with community-acquired cases (37.0% vs. 22.2%; Pearson χ^2 test, p<0.001). Nosocomial cases were more evenly distributed among male and female patients than were community-acquired cases (52.9% vs. 73.1% of cases in male patients, respectively; Pearson χ^2 test, p<0.001).

Source Investigation

A total of 3,035 potential sources were identified for the 1,991 reported LD cases (mean of 1.5 [SD 1.0] potential sources per patient). Online Technical Appendix 2 Table 1 shows the distribution of the different types of reported sources. Using the NLODP sampling criteria, the LSIU sampled 1,418 unique potential sources (47% of 3,035 reported sources). Some sources were sampled >1 time, resulting in 1,484 source investigations performed during the study period. In 367 (24.7%) of these investigations, *Legionella* spp. were identified in ≥1 sample, but large variations were seen among the different source types (Table 3). In 30 investigations, >1 *Legionella* spp. was found, identified as *L. pneumophila* SG1 or *L. pneumophila* non-SG1 if no *L. pneumophila* SG1 was found (Table 3). The proportions before and after introduction of the 4 criteria for sampling on June 1, 2006, were similar: 24.6% vs. 25.2%, respectively.

L. pneumophila SG1 was found in 97 (6.5%) investigations, *L. pneumophila* non-SG1 in 76 (5.1%), and *Legionella* spp. other than *L. pneumophila* in 194 (13.1%) (Table 3). The proportion of investigations in which *L. pneumophila* SG1 was found showed large variations among source types (Table 3). For instance, *L. pneumophila* SG1 was often detected in wellness centers (i.e., facilities offering spas, saunas, fitness equipment, massages, etc.) (40.5%); hospitals and health care settings (25.6%); and cooling towers (20.9%). However, *L. pneumophila* SG1 was not detected in investigated campsites, car wash or gasoline stations, or decorative water fountains and was detected in only a small proportion of investigated garden centers (1.2%). Residences were the most frequently sampled sources (51.3% of investigations); *L. pneumophila* SG1 was found in 21 (2.8%) of the 762 investigated residences (Table 3). Exclusion of source investigation data for the 119 nosocomial cases did not markedly change these results (online Technical Appendix 2 Table 2).

Clusters

The cluster definition used by NLODP resulted in 105 identified clusters, of which 98 (93.3%) were location clusters and 7 (6.7%) were geographic clusters. These clusters involved 266 patients with LD (Table 4; online Technical Appendix 2 Figure). An average of 2.9 (range 2–11) patients with LD were associated with each cluster (some patients were part of multiple clusters). In 50 clusters (47.6%), patients from ≥1 MHS were involved. Garden centers were the most frequently identified cluster site (27 [25.7%] clusters), followed by hospitals and health care settings (17 [16.2%]

Table 2. Characteristics of and test results for patients with domestically acquired Legionnaires' disease, the Netherlands, 2002–2012*

Characteristic	Total, N = 1,991	Community acquired, n = 1,872	Nosocomial, n = 119	p value†
Patient demographics				
Age, y (SD)	60.2 (4.8–98.1)	60.0 (4.8–98.1)	68.9 (11.2–94.8)	<0.001
Male sex, no. (%)	1,429 (71.8)	1,366 (73.1)	63 (52.9)	<0.001
Diagnostic tests, no. (%)‡	2,541	2,380	162	NA
Culture‡	460 (23.1)	416 (22.2)	44 (37.0)	<0.001
Urinary antigen‡	1,656 (83.2)	1,567 (83.7)	89 (74.8)	0.012
Seroconversion‡	109 (5.5)	102 (5.4)	7 (5.9)	0.840
Direct immunofluorescence‡	3 (0.2)	3 (0.2)	0	NA
PCR‡	156 (7.8)	145 (7.7)	12 (10.1)	0.346
Single high titer‡	157 (7.9)	147 (7.9)	10 (8.4)	0.829

*Study period was August 1, 2002–August 1, 2012. For 1,499 patients, >1 diagnostic test was available. NA, not possible to calculate.
†p value reflects the difference between patients with community-acquired Legionnaires' disease and those with nosocomial Legionnaires' disease.
‡Percentages reflect the proportion of patients for whom a diagnostic test result was available.

Table 3. Sampling results (N = 1,484) by potential sources of infection for patients with Legionnaires' disease, the Netherlands, 2002–2012*

Source type (no. samples)	Samples positive for *Legionella* spp., no. (%) Total	*L. pneumophila* non-SG1	*L. pneumophila* SG1	*L. non-pneumophila*	Samples negative for *Legionella* spp., no. (%)
Wellness center (37)†	27 (73.0)	4 (10.8)	15 (40.5)	8 (21.6)	10 (27.0)
Hospital/health care setting (90)	46 (51.1)	5 (5.6)	23 (25.6)	18 (20.0)	44 (48.9)
Cooling tower (43)	19 (44.2)	8 (18.6)	9 (20.9)	2 (4.7)	24 (55.8)
Sports facility (29)	10 (34.5)	2 (6.9)	5 (17.2)	3 (10.3)	19 (65.5)
Swimming pool (40)	13 (32.5)	2 (5.0)	6 (15.0)	5 (12.5)	27 (67.5)
Hotel (20)	8 (40.0)	3 (15.0)	3 (15.0)	2 (10.0)	12 (60.0)
Holiday park (23)	5 (21.7)	1(4.3)	2 (8.7)	2 (8.7)	18 (78.3)
Residence (762)	155 (20.3)	30 (3.9)	21 (2.8)	104 (13.6)	607 (79.7)
Workplace (92)	19 (20.7)	8 (8.7)	2 (2.2)	9 (9.8)	73 (79.3)
Car wash/gasoline station (44)	6 (13.6)	1 (2.3)	NA	5 (11.4)	38 (86.4)
Garden center (86)	8 (9.3)	1 (1.2)	1 (1.2)	6 (7.0)	78 (90.7)
Campsite (28)	2 (7.1)	1 (3.6)	NA	1 (3.6)	26 (92.9)
Decorative fountain (23)	1 (4.3)	NA	NA	1 (4.3)	22 (95.7)
Other (167)	48 (28.7)	10 (6.)	10 (6.0)	28 (16.8)	119 (71.3)
Total (1,484)	367 (24.7)	76 (5.1)	97 (6.5)	194 (13.1)	1,117 (75.2)

*Study period was August 1, 2002–August 1, 2012. SG1, serogroup 1; NA, not possible to calculate.
†Recreational facility offering spas, saunas, fitness equipment, massages, etc.

clusters), residences (10 [9.5%] clusters), wellness centers (9 [8.6%] clusters), and hotels (7 [6.7%] clusters) (Table 5). For the 98 location clusters, 142 source investigations were performed (23 cluster locations were investigated ≥1 time during the study period). *Legionella* spp. were found in 56 (39.4%) of investigations. *L. pneumophila* SG1 was found in 28 (19.7%) investigations, *L. pneumophila* non-SG1 in 6 (4.2%), and *Legionella* spp. other than *L. pneumophila* in 22 (15.5%).

Strain Characteristics

For the 1,991 reported patients with LD, 392 clinical isolates of *Legionella* spp. (85% of 460 reported patients diagnosed by culture) were sent to LSIU by the participating microbiologic laboratories in the Netherlands. All *L. pneumophila* SG1 clinical isolates and environmental strains were genotyped by using sequence-based typing (*10–12*), and monoclonal antibody determination was performed (*13*) (online Technical Appendix 2 Tables 3, 4).

Matches

For the 392 patients with LD for whom a clinical isolate was available, 704 unique potential sources of investigation were identified (mean 1.8 [SD 1.2] sources per patient). For these sources, 478 investigations were performed, and *Legionella* spp. were found in a sample from 120 (25.1%) investigations.

Environmental strains were compared with the clinical isolate(s) from the patients associated with the sampled potential sources. During August 2002–August 2012, a total of 38 genotype matches were found for 41 patients with LD (3 matches involved 2 clinical isolates, and 35 matches involved 1 clinical isolate). For each patient with an isolate that was part of a genotype match, a mean of 1.9 (SD 1.6) potential sources of infection was identified. This mean was significantly higher than the mean 1.5 (SD 1.0) sources identified for patients whose clinical isolate could not be matched with an environmental strain (independent samples *t*-test, p<0.01). Table 6 shows the different types of sources from which the matching environmental strains were isolated. Most matches (15 [39%]) were with strains from hospitals or other health care settings, followed by those from residences (7 [18%]). A genotype match was found for 38 (31.7%) of 120 available clinical isolates that could be compared with an environmental strain (online Technical Appendix Table 5). For the 266 patients who were part of a cluster, 24 had clinical isolates that could be genotypically compared with environmental strains, and a genotype match occurred for 19 (79.2%) of these 24 patients.

Table 4. Characteristics of 105 clusters reported for patients with Legionnaires' disease (n = 266), the Netherlands, 2002–2012*

Characteristic	Value
Location clusters (%)†	98 (93.3)
Geographic clusters (%)‡	7 (6.7)
Mean no. patients per cluster (range)	2.9 (2–11)
No. multiple municipal health services involved (%)	50 (47.6)
Mean no. municipal health services involved (range)	1.7 (1–5)

*Study period was August 1, 2002–August 1, 2012.
†A location cluster is defined as cases reported within 2 years of each other in ≥2 persons who were reported to have been exposed to the same potential source of infection during the 2–14 days before symptom onset.
‡A geographic cluster is defined as cases in >3 persons who lived <1 km apart and whose infections were reported within 6 months of each other.

Table 5. Cluster locations reported for 266 Legionnaires' disease patients, the Netherlands, 2002–2012*

Reported cluster location	No. (%) clusters	Cluster type, no. (%)	
		Location†	Geographic‡
Garden center	27 (25.7)	27 (27.6)	0
Hospital/health care setting	17 (16.2)	17 (17.3)	0
Residence	10 (9.5)	4 (4.1)	6 (85.7)
Wellness center§	9 (8.6)	9 (9.2)	0
Hotel	7 (6.7)	7 (7.1)	0
Cooling tower	5 (4.8)	5 (5.1)	0
Holiday park	5 (4.8)	5 (5.1)	0
Swimming pool	4 (3.8)	4 (4.1)	0
Industrial complex	3 (2.9)	2 (2.0)	1 (14.3)
Car wash installation	3 (2.9)	3 (3.0)	0
Sports facility	2 (1.9)	2 (2.0)	0
Cruise ship	2 (1.9)	2 (2.0)	0
Other	11 (10.5)	11 (11.2)	0
Total	105 (100.0)	98 (100.0)	7 (100.0)

*Study period was August 1, 2002–August 1, 2012.
†A location cluster is defined as cases reported within 2 years of each other in ≥2 persons who were reported to have been exposed to the same potential source of infection during the 2–14 days before symptom onset.
‡A geographic cluster is defined as cases in >3 persons who lived <1 km apart and whose infections were reported within 6 months of each other.
§Recreational facility offering spas, saunas, fitness equipment, massages, etc.

Discussion

During 2002–2012, a total of 1,991 patients with LD were reported in the Netherlands, and 1,484 source investigations were performed; 367 (24.7%) of the sources investigated were positive for *Legionella* spp. A total of 105 clusters were identified among 266 patients with LD. For 41 patients, a genotype match was found between the patient isolate and an environmental strain.

More than half of all source investigations were performed in residences, but only 20% of these investigations were positive for *Legionella* spp.; residences ranked tenth on the list of source types. A total of 43 cooling towers were investigated, ranking them third on the list of source types; >40% of those investigations were positive for *Legionella* spp. This well-known source of LD outbreaks should be considered often during source identification and investigation efforts performed by the MHS and LSIU.

For each patient, a mean of 1.5 potential sources of infection were reported, and about half of the reported sources were sampled. Although several attributes are being used by the MHS to improve source investigation (e.g., an elaborate questionnaire and a geographic information system implemented in 2009 [https://lpgis.geoxplore.nl/webify/?app=lpgis_ggd]), the number of sources being sampled could be increased. When the genotypic matches were analyzed, the mean number of sources identified and investigated for the patients involved was considerably higher (1.9 sources per patient), suggesting that identification and investigation of more potential sources of infection by the MHS may increase the proportion of patients with LD for whom a likely source of infection can be established.

Garden centers ranked third (after residences and workplaces) on the list of the most frequently reported potential sources of LD infection; 26% of identified clusters were associated with a garden center, indicating that this source type is often visited by patients with LD during the 2–14 days before symptom onset. However, only 8 of 86 investigated garden centers were found positive for *Legionella* spp. during source investigations. Several studies have shown the

Table 6. Genotypic matches (n = 38) from available isolates (n = 41) by source type reported for patients with Legionnaires' disease, the Netherlands, 2002–2012*

Source type	No. (%) matches	No. (%) available isolates
Hospital/health care setting	15 (39.5)	17 (41.5)
Residence	7 (18.4)	8 (19.5)
Industrial complex	3 (7.9)	3 (7.3)
Swimming pool	2 (5.3)	2 (4.9)
Wellness center†	3 (7.9)	3 (7.3)
Hotel	2 (5.3)	2 (4.9)
Travel trailer	1 (2.6)	1 (2.4)
Whirlpool	2 (5.3)	2 (4.9)
Sports facility‡	1 (2.6)	1 (2.4)
Potting soil	1 (2.6)	1 (2.4)
Car wash installation	1 (2.6)	1 (2.4)
Total	38 (100.0)	41 (100.0)

*Study period was August 1, 2002–August 1, 2012. Data from the LD outbreak in Amsterdam in 2006 (7) are excluded from these data.
†Recreational facility offering spas, saunas, fitness equipment, massages, etc.
‡This genotypic match was made with a clinical isolate collected during 2000 and an environmental strain collected in 2005.

presence of *Legionella* spp. in potting soil samples (*14–16*), and the use of amebal coculture techniques has shown promising results in recovering *L. pneumophila* SG1 sequence type (ST) 46 (the third most frequently found ST in clinical isolates) from samples with a high likelihood of microbial flora (*17*). At this time, potting soil samples collected by NLODP are not regularly being investigated by the amebal coculture technique. These findings suggest that potting soil samples from garden centers identified as potential sources of infection for patients with LD should be examined closely.

Notwithstanding the extensive efforts by NLODP collaborators, the number of *L. pneumophila* SG1 strains that could be derived from investigated potential sources was relatively low (114 strains over 10 years). Despite systematic methods of source identification by using a standardized questionnaire covering >20 source types, a source could not be confirmed in most cases. Although the questionnaire is regularly evaluated and adjusted on the basis of new insights concerning reported sources of infection, it primarily covers sources identified from the literature, possibly explaining the low success rate; actual sources of infection may not be captured in the questionnaire. This hypothesis is supported by the differences in genotype variation between clinical isolates and environmental strains: one third of all culture-positive patients with LD were infected by *L. pneumophila* SG1 ST47, a rare finding in environmental samples.

The experiences of NLODP show the importance of organizing a multidisciplinary collaboration in which MHSs, treating physicians, and microbiologic laboratories are represented and aware of the importance of different aspects of surveillance and source investigation for patients with LD. Our findings show the necessity of increasing awareness among various groups: physicians for diagnosis of LD, MHSs for extensive source identification, and laboratories for performance of adequate diagnostics and collection of clinical and environmental isolates. During 2002–2012, the number of reported patients with LD and the number of identified clusters of patients did not change dramatically, which may suggest the limited effects of NLODP. However, one could argue that this relatively stable number of patients with LD could have resulted from the program. Despite the rational, systematic approach used by NLODP during this decade, most sources of LD infections went undiscovered, stressing the need for evaluating other, yet unknown, potential sources of infection. Also, a need exists for further investment in improving laboratory techniques for detection of *Legionella* spp. in clinical samples with a high background of microbial flora such as soil.

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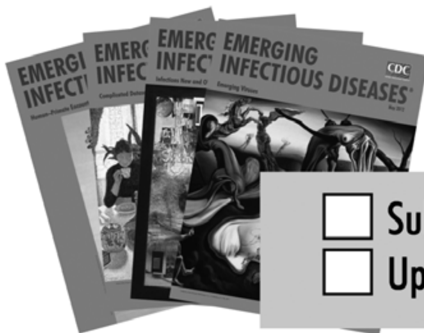

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Seroprevalence for Hepatitis E and Other Viral Hepatitides among Diverse Populations, Malawi

Taha E. Taha, Laura K. Rusie, Alain Labrique, Mulinda Nyirenda, Dean Soko, Melvin Kamanga, Johnstone Kumwenda, Homayoon Farazadegan, Kenrad Nelson, Newton Kumwenda

Data on prevalence of hepatitis E virus (HEV) in Malawi is limited. We tested blood samples from HIV-uninfected and -infected populations of women and men enrolled in research studies in Malawi during 1989–2008 to determine the seroprevalence of HEV, hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV). Samples were tested for IgG against HEV, total antibodies against HAV and HCV, and presence of HBV surface antigens. Of 800 samples tested, 16.5% were positive for HEV IgG, 99.6% were positive for HAV antibodies, 7.5% were positive for HBV surface antigen, and 7.1% were positive for HCV antibodies. No clear trends over time were observed in the seroprevalence of HEV, and HIV status was not associated with hepatitis seroprevalence. These preliminary data suggest that the seroprevalence of HEV is high in Malawi; the clinical effects may be unrecognized or routinely misclassified.

Hepatitis E virus (HEV) is primarily a waterborne virus that is transmitted by the fecal–oral route. First recognized in the early 1980s, it is now acknowledged to be the primary cause of enterically transmitted non-A, non-B hepatitis (*1*). HEV has 1 serotype and 4 genotypes (*1*). Clinical characterization of HEV infection is similar to that of other viral hepatitis infections, ranging from asymptomatic infection to fulminant hepatitis (*2*). Although illness caused by HEV most often tends to be mild and self-limiting, high rates of illness and death among pregnant women is a unique complication and key epidemiologic feature of HEV infection. Additionally, chronic infection leading to fibrosis and cirrhosis of the liver can occur in the immunosuppressed (*3*).

Currently, no data on HEV seroprevalence are available for Malawi. However, outbreaks of HEV infection have been documented in several countries in the southern and eastern regions of Africa. In Zambia, the overall seroprevalence of HEV was 42% among 106 adults who participated in a community study in 1999; among children who were included in a prospective study of the same community in 2011, the seroprevalence of HEV was 8% in age group 1–4 years (n = 96), 16% in age group 5–9 years (n = 62), and 36% in age group 10–14 years (n = 36) (*4*). In northern Uganda, surveillance of health care facilities during 2010–2012 showed that 42% of 347 persons with reported acute jaundice syndrome cases had hepatitis E, 14% had hepatitis B, and 5% had hepatitis C (*5*). During 2012 in a refugee camp in eastern Kenya, 77.1% of 170 samples from persons with acute jaundice syndrome were positive for HEV IgM, RNA, or both (*6*). Data from earlier studies in Tanzania suggested either lack of exposure or low levels of HEV among women (*7,8*). A review of the epidemiology of HEV in Africa by Kim et al. (*9*) provides a listing of seroprevalence of HEV antibodies in various African countries.

Similar to HEV, HAV is transmitted by the fecal–oral route, although the epidemiology of the viruses is substantially different. Infection with HAV is considered a childhood disease in developing countries; nearly all children are infected at an early age. Disease tends to be mild in children and does not result in chronic infection (*10*). Unlike HAV and HEV, hepatitis B and C viruses (HBV and HCV) are transmitted through contact with infectious body fluids and can cause acute or chronic infection. Acute infection with HBV or HCV can manifest with a wide range of mild to severe symptoms. Chronic HBV and HCV infection can lead to serious outcomes such as cirrhosis, cancer, and failure of the liver (*11,12*). High HBV and HCV prevalence have been reported in southern Africa, where HIV prevalence is also high (*13*). HCV prevalence in Africa varies by country; estimates range from 1% to 10% (*14*). However, it is unclear whether HCV seroprevalence on the basis of antibody testing alone represents a true estimate because a high number of false-reactive results (compared to those for HCV RNA) have been reported in several HIV-prevalent populations

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in Africa (*15,16*). The rate of chronic HBV carriers in sub-Saharan Africa is estimated to be >8% (*17*).

Previous studies have shown that HBV and HCV are prevalent in Malawi. Among patients in hospitals in Malawi, 17.5% tested positive for hepatitis B surface antigen (HBsAg), and samples from 4.5% were HCV antibody–positive (*18*). Among male sugar estate workers in Malawi, 14.9% tested positive for HBsAg, and samples from 10.6% were HCV antibody–positive (*19*).

Co-infection of HIV and HBV or HCV leads to accelerated progression of liver disease (*13*). The interaction of HEV with HIV has not yet been confirmed but is conceivable, and a strong association has been reported among adults in a retrospective study in Zambia in which 28% of HIV-seronegative and 71% of HIV-seropositive adults were found to be HEV seropositive (*4*). Superinfection with >1 type of hepatitis has been shown to cause severe disease. Children with simultaneous infection of HAV and HEV may experience accelerated disease progression. A study among persons who chronically carried the HBV surface antigen showed rapid clinical deterioration when co-infected with HEV (*20*). The high prevalence of HIV in Malawi, combined with the severe implications of co-infections with hepatitis viruses, necessitate clarification of the levels of multiple hepatitis virus infections within the same population. The primary aim of this study, conducted in 2012, was to determine the seroprevalence of HEV, as well as HAV, HBV, and HCV, in samples collected during 1989–2008 from diverse adult populations in Malawi.

Methods

Study Design and Populations

In a cross-sectional study, we analyzed serum and plasma samples collected during 6 studies in Malawi (Table 1) (*21–26*). These studies were conducted during 1989–2008 and represent diverse populations of HIV-infected and HIV-uninfected adult men and women from rural and urban settings in Malawi. Almost all HIV-infected persons in these studies were antiretroviral naive. Selection of samples stored at –80°C for laboratory testing was aimed to include approximately equal numbers of HIV-negative and HIV-positive samples from each year, on the basis of availability of sufficient sample volume, to test for all hepatitides. Inclusion of the samples was not based on previous knowledge of demographic factors of the study populations. In addition to collecting samples, we used structured case report forms to document demographic and clinical data.

All participants signed written informed consent and agreed to provide study samples. All studies were approved by appropriate institutional review boards in the United States and Malawi.

Laboratory Tests for Hepatitis Seroprevalence

HEV Testing

Serum or plasma samples were tested for the presence of antibodies against HEV by using Wantai HEV-IgG ELISA kits (Wantai Biologic Pharmacy Enterprise Co., Ltd., Beijing, China). The Wantai assay uses a recombinant capsid protein (E2) encoded by the conserved region of open reading frame 2 of HEV. Compared with the Genelabs HEV IgG EIA (Genelabs, Inc., Singapore), the Wantai assay has been shown to be more sensitive (*27*). The HEV assays were performed manually following the manufacturer's instructions in the serology laboratory of the Department of Epidemiology at Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland, USA.

Testing for Other Hepatitides

We used the same samples tested for HEV to additionally test for total antibodies (IgG and IgM) against HAV and HCV and for the presence of HBsAg by using commercially available kits from Bio-Rad Laboratories (Hercules, CA, USA). The HAV kits detect both acute and past infection; the HCV assays detect acute, past, and chronic infections; and the HBsAg assay detects acute and chronic infection with HBV. These tests were performed by using the automated EVOLIS microplate system from Bio-Rad at the Johns Hopkins Bloomberg School of Public Health in Baltimore. The Johns Hopkins School of Public Health serology laboratory is Clinical Laboratory Improvement Amendments–certified to perform virologic and serologic testing.

Because of operational constraints, all samples were tested once for each type of hepatitis and no duplicate testing was done. Quality control was performed with the inclusion of positive and negative controls in each assay. Results were considered positive when the optical density of a well was equal to or greater than the cutoff value calculated for each run of each assay as recommended by the manufacturer.

Statistical Analysis

Overall seropositivity for each virus was estimated and stratified by study year, HIV status, sex, and age category. Associations of the 4 hepatitides with selected exposure variables were assessed by using logistic regression. The covariates considered were education level, rural or urban residence, marital status, employment, running water in house, electricity in house, parity, multiple sexual partners, reported history of sexually transmitted infections, and condom use. Variables with a statistically significant association with a hepatitis seroprevalence in univariate analysis as well as variables considered epidemiologically important were included in the multivariate logistic

Table 1. Description of studies included in analysis of hepatitis virus seroprevalence, Malawi, 1989–2008*

Study name, period (reference)	Study site/setting	Study type	Study population	HIV status	Aims of study
ICAR,1989–1994 (*21*,*22*)	Queen Elizabeth Central Hospital, Blantyre/urban	Observational cohort	Pregnant and postpartum women	+/–	Assess adverse pregnancy outcomes and rates of mother-to-child transmission of HIV
SUCOMA, 1998 (*23*)	Nchalo District, sugar estate/rural, occupational	Observational survey	Adult men	+/–	Identify epidemiologic and biologic determinants of HIV infection in an occupational male cohort
NVAZ, 2000–2004 (*24*)	Queen Elizabeth Central Hospital, Blantyre/urban	Randomized clinical trial	Pregnant women	+	Assess efficacy of short antiretroviral post-exposure prophylaxis to prevent mother to child transmission of HIV
MWANZA, 2001 (T.E. Taha, unpub. data)	Mwanza District/rural	Observational survey	Adult men and women	+/–	Assess risk factors associated with prevalent HIV infection in a rural area adjacent to the borders with Mozambique
METRO, 2003–2005 (*25*)	Queen Elizabeth Central Hospital; health centers in Blantyre/urban	Randomized clinical trial	Adult women (nonpregnant)	+/–	Assess the efficacy of intermittent intravaginal metronidazole gel use in reducing bacterial vaginosis infection
PEPI, 2004–2008 (*26*)	Queen Elizabeth Central Hospital; health centers in Blantyre/urban	Randomized clinical trial	Pregnant women	+	Assess efficacy of extended antiretroviral postexposure prophylaxis to prevent mother to child transmission of HIV

*ICAR, International Collaborations on AIDS Research; SUCOMA, a study of HIV prevalence among male workers for the Sugar Company of Malawi; NVAZ, a study of efficacy of nevirapine/zidovudinel postexposure prophylaxis to prevent mother-to-child transmission of HIV; MWANZA, a study of risk factors associated with prevalent HIV infection in the rural town of Malawi; METRO, a study in which efficacy of intravaginal metronidazole gel in reducing bacterial vaginosis was assessed; PEPI, a study of antiretroviral postexposure prophylaxis to prevent mother to child transmission of HIV.

regression to estimate adjusted odds ratios. Because of differences in follow-up periods and inconsistencies in availability of comparable data, we used baseline data for all covariates, regardless of whether an enrollment or follow-up sample was used for the hepatitis testing. A p value of ≤0.05 was considered statistically significant. Analysis was performed by using Stata version 11.2 (StataCorp LLC, College Station, TX, USA).

Previous data on the prevalence of hepatitis E in Malawi was not available. To estimate an appropriate sample size for this analysis, a conservative prediction of 2% prevalence was used. A sample size of 800 was determined to enable detection of a prevalence of 2% with a precision of 1% (*28*).

Results

Table 2 shows the distribution of hepatitis seropositivity overall and across main exposure variables. Overall, 132 (16.5%) of 800 samples tested positive for HEV antibodies. The overall seroprevalence of HAV antibodies, HBsAg and HCV antibodies in samples collected from different populations in Malawi was 99.6%, 7.5%, and 7.1%, respectively (Table 2). HEV seroprevalence was higher among HIV-uninfected (20.2%) than among HIV-infected (12.9%) persons, and HCV seroprevalence was higher among male (10.2%) than female (5.6%) (p<0.05 by Fisher exact test) participants. Seroprevalence varied by study year and age group; seroprevalence of enteric HEV was 20.8% for a male occupational cohort in the 1994–1999 Sugar Company of Mulawi (SUCOMA) study(*23*), and 26.4% for an female urban cohort in the 2003–2005 metronidazole gel (METRO) study (*25*) in which efficacy of intravaginal metronidazole gel in reducing bacterial vaginosis was assessed. These overall differences by study year were statistically significant (p<0.001 by χ^2 test).

Unlike HEV seroprevalence, the seroprevalence of the other enterically transmitted infection (HAV) did not show variation by year of study and was ≈100% in all studies. The prevalence of HBsAg and HCV antibodies was low (0% HBsAg; 2.9% HCV antibodies) in the 1989–1995 cohort of urban pregnant and postpartum women of the International Collaborations on AIDS Research (ICAR) study (*21,22*). In subsequent years, the reported prevalence of HBsAg and HCV antibodies increased and was approximately comparable in all studies reviewed for this study; exceptions are the high seroprevalence of both hepatitides in the men of the occupational cohort of SUCOMA and the high seroprevalence of HBsAg among women in the postexposure prophylaxis of infants (PEPI) cohort (2004–2009) (*26*). Stratification by age group (excluding those 60–69 years of age because of limited numbers) showed comparable HEV seroprevalence among persons <50 years of age (or trend of decrease with increase in age) and a seroprevalence of 33% among persons 50–59 years of age. The seroprevalence of HAV, however, was uniformly high and was ≈100% in all age groups. Both HBsAg and HCV seroprevalence were stable and comparable in age groups <50 (no. persons in older age groups was small). Among 757 persons with laboratory results available for both HBsAg and HCV, 7 were dually seropositive for HBsAg and HCV (0.9%; 95% CI 0.2–1.6; p = 0.11 by χ^2 test); all 7 were HIV-uninfected.

Table 3 shows univariate (crude) and multivariate (adjusted) odds ratios (OR) of being seropositive for HEV. In the univariate analysis, participants in the SUCOMA (1994–1999) and METRO (2003–2005) studies were

Table 2. Prevalence of anti-HEV IgG, total anti-HAV Ig, HBsAg, and total anti-HCV Ig in Malawi*

Characteristic	HEV		HAV		HBsAg		HCV	
	No. positive/no. tested	% Positive (95% CI)	No. positive/no. tested	% Positive (95% CI)	No. positive/no. tested	% Positive (95% CI)	No. positive/no. tested	% Positive (95% CI)
Total cohort	132/800	16.5 (13.9–19.1)	777/780	99.6 (99.2–100.0)	58/773	7.5 (5.6–9.4)	55/779	7.1 (5.3–8.9)
HIV status								
Negative	80/397	20.2 (16.2–24.1)	390/391	99.7 (99.2–100.0)	32/388	8.2 (5.5–11.0)	29/394	7.4 (4.8–10.0)
Positive	52/403	12.9 (9.6–16.2)	387/389	99.5 (98.8–100.0)	26/385	6.8 (4.2–9.3)	26/385	6.8 (4.2–9.3)
Sex								
M	45/246	18.3 (13.4–23.2)	244/245	99.6 (98.8–100.0)	25/245	10.2 (6.4–14.0)	25/245	10.2 (6.4–14.0)
F	87/554	15.7 (12.7–18.7)	533/535	99.6 (99.1–100.0)	33/528	6.3 (4.2–8.3)	30/534	5.6 (3.7–7.6)
Study, period								
ICAR, 1989–1995	6/70	8.6 (1.8–15.3)	70/70	100.0	0/70	0.0	2/70	2.9 (–1.1–6.9)
SUCOMA, 1994–1999	37/178	20.8 (14.8–26.8)	176/177	99.4 (98.3–100.0)	21/178	11.8 (7.0–16.6)	19/178	10.7 (6.1–15.3)
NVAZ, 2000–2003	19/165	11.5 (6.6–16.4)	157/157	100.0	6/156	3.9 (0.8–6.9)	8/148	5.4 (1.7–9.1)
MWANZA, 2001	13/100	13.0 (6.3–19.7)	99/99	100.0	7/98	7.1 (2.0–12.3)	9/99	9.1 (3.3–14.9)
METRO, 2003–2005	39/148	26.4 (19.2–33.5)	141/142	99.3 (97.9–100.0)	8/137	5.8 (1.9–9.8)	9/146	6.2 (2.2–10.1)
PEPI, 2004–2009	18/139	12.9 (7.3–18.6)	134/135	99.3 (97.8–100.0)	16/134	11.9 (6.4–17.5)	8/138	5.8 (1.8–9.7)
Age range, y								
15–19	15/77	19.5 (10.4–28.5)	75/75	100.0	5/76	6.6 (0.9–12.3)	6/75	8.0 (1.7–14.3)
20–29	72/438	16.4 (13.0–19.9)	424/426	99.5 (98.9–100.0)	33/420	7.9 (5.3–10.4)	30/424	7.1 (4.6–9.5)
30–39	28/191	14.7 (9.6–19.7)	185/186	99.5 (98.4–100.0)	12/184	6.5 (2.9–10.1)	12/186	6.5 (2.9–10.0)
40–49	7/60	11.7 (3.3–20.0)	59/59	100.0	3/59	5.1 (–0.7 to 10.9)	3/60	5.0 (–0.7 to 10.7)
50–59	6/18	33.3 (9.2–57.5)	18/18	100.0	2/18	11.1 (–5.0 to 27.2)	2/18	11.1 (–5.0 to 27.2)
60–69	2/4	50.0 (–41.9 to 141.9)	4/4	100.0	4/4	100.0	1/4	25.0 (–54.6 to 104.6)

*HEV, hepatitis E virus; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; ICAR, International Collaborations on AIDS Research; SUCOMA, a study of HIV prevalence among male workers for the Sugar Company of Malawi; NVAZ, a study of efficacy of nevirapine/zidovudinel postexposure prophylaxis to prevent mother-to-child transmission of HIV; MWANZA, a study of risk factors associated with prevalent HIV infection in the rural town of Malawi; METRO, a study in which efficacy of intravaginal metronidazole gel in reducing bacterial vaginosis was assessed; PEPI, a study of antiretroviral postexposure prophylaxis to prevent mother to child transmission of HIV.

significantly more likely to be positive for HEV antibodies compared with participants in the ICAR earlier study (1989–1994) (OR 2.80 and 3.82, p = 0.03 and 0.004, respectively). In the multivariate analysis, only the METRO study year (2003–2005) remained statistically significant after adjusting for other variables included in Table 3 (p = 0.01 for METRO study). Although not statistically significant in the multivariate analysis, participants who tested HIV-positive and those who reported having running water in the house were less likely to be seropositive for HEV antibodies in the univariate analyses (HIV-positive OR 0.59, p = 0.01; have running water OR 0.61, p = 0.06).

HAV seroprevalence was high, and the distribution was comparable across all variables and strata of each covariate; therefore, no associations of HAV with covariates included in this study were observed in both univariate and multivariate analyses. Table 4 shows the univariate, unadjusted ORs for HBsAg and HCV. Participants in the Nevirapine-Zidovudine (NVAZ) study were less likely to be positive for HBsAg than participants in the ICAR study (OR 0.30, p = 0.01 [*21,22*]). Residents of urban areas also had lower odds for testing positive for HBsAg compared with residents of rural areas (OR 0.57, p = 0.04), and female participants were less likely to test positive than male participants (OR 0.59, p = 0.05). A statistically nonsignificant trend of increasing odds of HBsAg over time on the basis of enrollment calendar year in the parent study is also shown in Table 4 (OR 1.07, p = 0.06). The univariate analysis of HCV association with the risk factors listed in Table 3 showed that female participants had statistically significantly lower odds of being seropositive for HCV compared with male

Table 3. Crude and adjusted odds ratios for enterically transmitted hepatitis E virus, Malawi*

Characteristics	Crude OR	p value	Adjusted OR (95% CI)
Study, period			
ICAR, 1989–1995	Reference	NA	Reference
SUCOMA, 1994–1999	2.80	0.03	3.54 (0.75–16.76)
NVAZ, 2000–2003	1.39	0.51	1.59 (0.55–4.62)
MWANZA, 2001	1.59	0.37	1.85 (0.51–6.70)
METRO, 2003–2005	3.82	0.004	3.44 (1.37–8.67)
PEPI, 2004–2009	1.59	0.35	1.87 (0.64–5.49)
Enrollment year†	1.02	0.38	NA
HIV status			
HIV-negative	Reference	NA	Reference
HIV-positive	0.59	0.01	0.72 (0.38–1.36)
Age, y†	1.01	0.56	1.00 (0.97–1.02)
Age range, y			
15–19	Reference	NA	NA
20–29	0.81	0.51	NA
30–39	0.71	0.33	NA
40–49	0.55	0.22	NA
50–59	2.07	0.21	NA
60–69	4.13	0.17	NA
Sex			
M	Reference	NA	1.00
F	0.83	0.36	1.40 (0.41–4.76)
Setting			
Rural	Reference	NA	NA
Urban	0.85	0.41	NA
Running water in house			
No	Reference	NA	1.00
Yes	0.61	0.06	1.40 (0.41–4.76)
Electricity in house			
No	Reference	NA	NA
Yes	0.82	0.40	NA
Education			
None	Reference	NA	NA
Any	0.92	0.78	NA
Marital status			
Married	Reference	NA	NA
Single	0.60	0.14	NA
Employment status			
Unemployed	Reference	NA	NA
Employed	1.18	0.41	NA
Parity†	0.90	0.61	NA

*OR, odds ratio; ICAR, International Collaborations on AIDS Research; SUCOMA, a study of HIV prevalence among male workers for the Sugar Company of Malawi; NVAZ, a study of efficacy of nevirapine/zidovudinel post-exposure prophylaxis to prevent mother-to-child transmission of HIV; MWANZA, a study of risk factors associated with prevalent HIV infection in the rural town of Malawi; METRO, a study in which efficacy of intravaginal metronidazole gel in reducing bacterial vaginosis was assessed; PEPI, a study of antiretroviral postexposure prophylaxis to prevent mother-to-child transmission of HIV; NA: not applicable.
†Continuous variable.

participants, and urban residents were less likely to test positive than rural residents (OR 0.52 and 0.51, p = 0.02 and 0.02, respectively). Although not statistically significant (p = 0.06), the ORs for participation in the SUCOMA study and having electricity in the house (an indicator of higher socioeconomic status) were 4.06 (95% CI 0.92–17.93) and 0.46 (95% CI 0.20–1.03), respectively. In the multivariate logistic regression analyses for the association of HBsAg and HCV with various covariates (study enrollment, HIV status, age, sex, whether there were multiple lifetime sexual partners, history of sexually transmitted diseases, and history of condom use), only enrollment in the NVAZ study (2000–2003) remained significantly associated with lower likelihood to test positive for HBsAg (adjusted OR 0.24, p = 0.006). No other variables were found to be associated with HBV or HCV seroprevalence.

Discussion

This study provides preliminary estimates of HEV seroprevalence in Malawi, which has a population of ≈12 million, is mostly rural, and has a limited safe water supply and constrained health care services (*29*). Overall, 16.5% (95% CI 13.9%–19.1%) of the samples were positive for HEV IgG. In addition to examining seroprevalence of HEV in Malawi, this study has several other notable features: 1) samples from 6 epidemiologic studies conducted by the same research team among adult men and women in urban and rural Malawi during 1989–2008 were included, representing diverse

Table 4. Crude odds ratios for the association of HBV and HCV with selected exposures in studies of hepatitis prevalence, Malawi, 1989–2009*

	HBsAg		HCV	
Characteristics	OR	p value	OR	p value
Study, period				
ICAR, 1989–1995	Reference	NA	Reference	NA
SUCOMA, 1994–1999	0.99	0.97	4.06	0.06
NVAZ, 2000–2003	0.30	0.01	1.94	0.41
MWANZA, 2001	0.57	0.23	3.40	0.13
METRO, 2003–2005	0.46	0.08	2.23	0.31
PEPI, 2004–2009	ND	ND	2.09	0.36
Enrollment year, continuous	1.07	0.06	1.00	0.96
HIV status				
HIV-negative	Reference	NA	Reference	NA
HIV-positive	0.81	0.43	0.91	0.74
Age, continuous, years	0.99	0.68	1.00	0.79
Age range, y				
15–19	Reference	NA	Reference	NA
20–29	1.21	0.70	0.88	0.78
30–39	0.99	0.99	0.79	0.66
40–49	0.76	0.72	0.61	0.49
50–59	1.78	0.52	1.44	0.67
60–69	ND	ND	3.83	0.28
Sex				
M	Reference	NA	Reference	NA
F	0.59	0.05	0.52	0.02
Setting				
Rural	Reference	NA	Reference	NA
Urban	0.57	0.04	0.51	0.02
Running water in house				
No	Reference	NA	Reference	NA
Yes	1.00	1.00	0.74	0.43
Electricity in house				
No	Reference	NA	Reference	NA
Yes	1.17	0.61	0.46	0.06
Education				
None	Reference	NA	Reference	NA
Any	0.72	0.50	0.93	0.88
Marital status				
Married	Reference	NA	Reference	NA
Single	1.90	0.07	0.42	0.15
Employment				
Unemployed	Reference	NA	Reference	NA
Employed	1.61	0.09	1.45	0.19
Parity (continuous)	0.86	0.20	0.80	0.09
Lifetime sex partners				
≤1	Reference	NA	Reference	NA
>1	1.29	0.37	1.19	0.54
History of STIs				
Never	Reference	NA	Reference	NA
Ever	0.63	0.29	0.79	0.57
Condom use				
Never	Reference	NA	Reference	NA
Ever	0.75	0.38	0.95	0.88

*HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; OR, odds ratio; ICAR, International Collaborations on AIDS Research; SUCOMA, a study of HIV prevalence among male workers for the Sugar Company of Malawi; NVAZ, a study of efficacy of nevirapine/zidovudinel post-exposure prophylaxis to prevent mother-to-child transmission of HIV; MWANZA, a study of risk factors associated with prevalent HIV infection in the rural town of Malawi; METRO, a study in which efficacy of intravaginal metronidazole gel in reducing bacterial vaginosis was assessed; PEPI, a study of antiretroviral postexposure prophylaxis to prevent mother to child transmission of HIV; NA, not applicable; ND; no data; STI, sexually transmitted infection.

populations and time periods; 2) analyses of viral hepatitides seroprevalence included both HIV-negative and HIV-positive persons (the prevalence of HIV remains high in southern Africa); and 3) these samples were also tested for total antibodies (IgG and IgM) against HAV and HCV and for the presence of HBsAg, leading to a comprehensive seroprevalence profile of all viral hepatitides in these populations.

Although some statistically significant differences were observed in univariate analyses for the association of selected risk factors with seroprevalence of HEV, none of these remained significant in the multivariate logistic regression analyses, including HIV status, age category, sex, or having access to running water in the household. Recent studies have also found no differences in infection

rates by gender or HIV status (*1,30*). The current study did not include children, which likely explains why previously identified trends of increasing infection levels with age were not detected. We also did not find a trend of seroprevalence over time. The only risk factor substantially associated with HEV seroprevalence was the METRO study period, 2003–2005, as compared to the ICAR study, which was conducted during 1989–1995. Both studies were conducted among women in Blantyre, Malawi.

It is unclear what may have caused the METRO study participants to have higher levels of HEV seroprevalence. We did not collect data on seasonality and source of water and food; over the years Malawi had fluctuating bouts of drought and food scarcity. We tested diverse study populations from rural and urban settings recruited over a period of nearly 20 years, but no clear major trends were observed.

As expected, HAV seroprevalence was nearly universal in this sample of adults, with an overall prevalence of ≈100%. In low-resource settings such as Malawi, HAV is a childhood infection, and nearly all persons are infected within the first few years of life. Though children were not included in our study, it should be considered that they can be exposed to HEV because of the high prevalence among adults we observed. Although HAV and HEV infection tend to cause mild disease independently, concurrent infections with these pathogens in children may lead to accelerated disease progression (*20*). The changing epidemiology of HAV in some settings related to rapid industrialization and urban migration in developing countries may result in some children not being exposed to HAV (*10*).

An overall prevalence of 7.5% (95% CI 5.6%–9.4%) was found for HBsAg and 7.1% (95% CI 5.3%–8.9%) for HCV. The results of the HBsAg and HCV seroprevalence tests are consistent with previously published data from sub-Saharan Africa. Specifically in Malawi, a study published in 2002 reported a prevalence of 14.9% for HBsAg and 10.6% for HCV among the SUCOMA participants (*23*). In our study, we found a prevalence of 11.8% (95% CI 7.0%–16.6%) and 10.7% (95% CI 6.1%–15.3%) for HBsAg and HCV, respectively, in the SUCOMA study samples. Women who enrolled in NVAZ had lower odds of testing positive for HBsAg compared to those in ICAR. The only distinct difference between these studies was the HIV status of the women (some women in the ICAR study were HIV negative), but because this was included in the multivariate logistic regression, results should not be confounded by the HIV status. No covariates were found to be associated with HCV seroprevalence in this study.

HEV epidemiology is evolving, and circulating genotypes and modes of transmission appear to be complex in both developing and industrialized countries (*1*). We do not know in Malawi if only waterborne HEV genotypes are the source of potential infections or if other less virulent zoonotic HEV genotypes coexist. High seroprevalence of HEV antibodies does not imply clinical infection or increased association with clinical complications. Nonetheless, some misclassification of acute viral hepatitis is likely in Malawi where screening for HEV antibodies is not performed. Because the seroprevalence of HEV in this study was twice that of HBV or HCV, cases of acute hepatitis may frequently be caused by HEV as opposed to HBV or HCV. Notably, 15.7% (95% CI 12.7%–18.7%) of women in this study cohort had antibodies against HEV, which can pose serious health risks for pregnant women (*3*).

Although no association was found that HIV-positive persons are at higher risk for anti-HEV than are HIV-negative persons, the finding that 12.9% (95% CI 9.6%–16.2%) of samples from HIV-positive persons were HEV positive is of concern. Malawi and other countries in sub-Saharan Africa have high levels of HIV infection, and co-infection with HEV may lead to chronic HEV infection and accelerated disease progression.

The findings from this study should be regarded as preliminary and require confirmation. Therefore, additional epidemiologic and virological studies should be conducted in this region. As with all cross-sectional data, inferences regarding associations should be interpreted with caution. A related limitation is that the samples used for serologic testing in this study included both enrollment and follow-up samples (in cohort studies) to maximize availability of samples, whereas the covariate data used for all participants was baseline enrollment data. Despite these limitations, we suspect that many of the covariates used, such as having running water in the house, did not substantially change over time. Very few risk factors associated with HEV, HBV, or HCV seroprevalence were found. This result may be because of differences in population characteristics or definitions used in multiple studies. For example, participants in the SUCOMA study were all men working in a rural sugar estate occupational setting and may be considered to be at high risk (*23*). We also did not have data on some behaviors associated with HCV seroprevalence, such as intravenous drug use, although the practice is very rare in Malawi. The lack of association between viral hepatitides and various risk factors conventionally collected in these studies suggests that better data collection tools to evaluate potential risk factors and different study designs targeting at risk populations may need to be considered in future studies.

Confirmation of the hepatitis testing results, particularly HEV seroprevalence, reported in this study will be critical in subsequent studies because each sample was tested once. Retesting of a subset of positive and negative samples should ideally be done with the same Wantai assay used in this study as well as with other assays. The hepatitis

A, B, and C assays used were commercially available in the United States and well validated, but it is recommended that samples be tested in duplicate for these assays. The consistency of our results with findings from previous seroprevalence studies in Malawi and other countries suggests misclassification may be minimal (taking into account the controversy regarding HCV testing). Molecular analysis to determine what genotypes of HEV are prevalent in Malawi may also be useful and would provide further insight into the epidemiology of this virus.

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Chronic Q Fever Diagnosis—Consensus Guideline versus Expert Opinion

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Chronic Q fever, caused by *Coxiella burnetii*, has high mortality and morbidity rates if left untreated. Controversy about the diagnosis of this complex disease has emerged recently. We applied the guideline from the Dutch Q Fever Consensus Group and a set of diagnostic criteria proposed by Didier Raoult to all 284 chronic Q fever patients included in the Dutch National Chronic Q Fever Database during 2006–2012. Of the patients who had proven cases of chronic Q fever by the Dutch guideline, 46 (30.5%) would not have received a diagnosis by the alternative criteria designed by Raoult, and 14 (4.9%) would have been considered to have possible chronic Q fever. Six patients with proven chronic Q fever died of related causes. Until results from future studies are available, by which current guidelines can be modified, we believe that the Dutch literature-based consensus guideline is more sensitive and easier to use in clinical practice.

Coxiella burnetii is the causative agent of Q fever, a zoonosis occurring worldwide (*1*). Recently, a large epidemic occurred in the Netherlands with >4,000 cases of acute Q fever notified from 2007 through 2010 (*2,3*). Chronic Q fever develops in an estimated 1%–5% of all infected humans and can become manifest even years after primary infection (*1,4*). Endocarditis and infection in aneurysms or vascular prostheses are the most common manifestations (*1,5,6*). Untreated chronic Q fever has a poor prognosis, with a reported mortality rate of up to 60% (*1,7*). Adequate antibiotic treatment reduces the mortality rate for Q fever endocarditis to <5% (*7*). Treatment preferably consists of a combination of doxycycline and hydroxychloroquine for at least 18 months (nonprosthetic infection) to 24 months (prosthetic infection) and is recommended to be continued in case of unfavorable clinical or serologic response (*7,8*). Antibiotic guidelines for vascular chronic Q fever are not yet available, but antibiotic regimes for Q fever endocarditis have been applied to this disease entity as well. Early surgical intervention, with removal of infected material, might improve the prognosis of vascular chronic Q fever (*6,9*).

In the early course of chronic Q fever, most patients are asymptomatic or experience nonspecific symptoms such as low-grade fever, night sweats, and weight loss (*1,4,6,7*). In the case of endocarditis, findings on echocardiograph are often nonspecific or absent, which makes the diagnosis of chronic Q fever challenging (*7*). A PCR positive for *C. burnetii* or culture of the organism in blood or tissue, in the absence of acute Q fever, is a strong indicator for chronic Q fever. However, sensitivity on blood samples is only 50%–60% for both PCR and culture in patients with chronic Q fever (*10,11*). Therefore, serologic testing is also valuable for the diagnosis of chronic Q fever. A phase I IgG cutoff titer of 1:800, which is based on an in-house–developed immunofluorescence assay (IFA), has been internationally accepted for the diagnosis of chronic Q fever and is included in the modified Duke criteria for diagnosis of endocarditis (*12,13*). In the Netherlands, a commercial IFA (Focus Diagnostics, Inc., Cypress, CA, USA) is primarily used, with a proposed IgG cutoff value of 1:1,024 for chronic Q fever (*14*). Yet, recent studies show that serology results alone are not sufficient for the diagnosis of chronic Q fever, but that they should be combined with clinical data (*15*).

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Dutch Consensus Guideline

Faced with a large Q fever outbreak in the Netherlands and a rising number of (presumed) chronic Q fever patients, we were not able to find answers to all our questions about this complex disease in the literature. Moreover, randomized trials on diagnosis and treatment of this disease were lacking, and available data were not all applicable to the Dutch situation. For example, we found far more vascular localizations of chronic Q fever, with often severe complications, than had been described previously. Therefore, the Dutch Q Fever Consensus Group was initiated in 2010, in which diagnosis and subsequent treatment consequences for suspected chronic Q fever were discussed. We performed a thorough literature review and constructed a new guideline for the diagnosis of chronic Q fever, differentiating between proven, probable, and possible chronic Q fever (Table 1). We added advice for treatment and follow-up regimes for these 3 groups of patients. Antibiotic treatment and, if indicated, surgical treatment are recommended for all patients with proven chronic Q fever. The decision to start antibiotic treatment in patients with probable chronic Q fever depends on clinical characteristics and the condition of the patient, and should be determined by a multidisciplinary team. For possible chronic Q fever patients, antibiotic treatment should not be initiated, but follow-up is indicated.

After the Dutch consensus guideline was reported (*14*), a reaction by French researcher Didier Raoult was published; he did not agree with this proposed guideline and formulated alternative diagnostic criteria on the basis of his expert opinion (Table 2) (*16*). Professor Raoult is the undisputed leading authority on Q fever, and his opinion and the scientific publications from his research group should be considered by anyone working in the field of Q fever. Here, we attempt to resolve these differences of opinion by applying both criteria to cases from the Dutch National Chronic Q Fever Database.

Dutch Consensus Guideline versus Expert Opinion Guideline

A critical difference in the diagnostic criteria proposed by Raoult and those of the Dutch Q Fever Consensus Group is the diagnostic value attributed to *C. burnetii* PCR positivity of blood samples. Because we are unaware of clinical entities, other than acute and chronic Q fever, for which a PCR positive for *C. burnetii* in blood would be exhibited, we believe that positive blood PCR findings, in the absence of acute Q fever, prove chronic Q fever. The alternative criteria, on the other hand, state that a positive PCR finding in blood should be accompanied by a clear endocarditis focus shown on echocardiograph, a clear vascular focus on imaging studies, or at least 2 or 3 "minor criteria" (Table 2). Moreover, the alternative criteria attribute great value to the phase I IgG titer, proposing a phase I IgG ≥1:6,400 as a major criterion for Q fever endocarditis and Q fever vascular infection, in contrast to a phase I IgG ≥1:800 and <1:6,400 proposed as a minor criterion. This proposal contradicts the internationally accepted modified Duke criteria, which state that a phase I IgG ≥1:800 is a major criterion for infective (Q fever) endocarditis (*13*).

The alternative criteria also generally oppose the term chronic Q fever but makes a distinction in 2 manifestations: Q fever endocarditis and Q fever vascular infection. More

Table 1. Dutch consensus guideline on chronic Q fever diagnostics*

Proven chronic Q fever	Probable chronic Q fever	Possible chronic Q fever
1. Positive *Coxiella burnetii* PCR of blood or tissue† OR 2. IFA ≥1:800 or 1:1,024 for *C. burnetii* phase I IgG† AND Definite endocarditis according to the modified Duke criteria (*13*) OR Proven large vessel or prosthetic infection by imaging studies (FDG-PET, CT, MRI, or AUS)	IFA ≥1:1,024 for *C. burnetii* phase I IgG‡ AND any of the following: Valvulopathy not meeting the major criteria of the modified Duke criteria (*13*) Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection by means of TEE/ TTE, FDG-PET, CT, MRI, or AUS Suspected osteomyelitis or hepatitis as manifestation of chronic Q fever Pregnancy Symptoms and signs of chronic infection, such as fever, weight loss and night sweats, hepato-splenomegaly, persistent raised ESR and CRP Granulomatous tissue inflammation, proven by histological examination Immunocompromised state	IFA ≥ 1:1,024 for *C. burnetii* phase I IgG‡ without manifestations meeting the criteria for proven or probable chronic Q fever

*Source: (*14*). IFA, immunofluorescence assay; TEE, transesophageal echocardiography; TTE, transthoracic echocardiography; FDG-PET, fluorodeoxyglucose positron emission tomography; CT, computed tomography; MRI, magnetic resonance imaging; AUS, abdominal ultrasound; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
†In the absence of acute infection.
‡Cut-off depends on the IFA technique used, whether in-house developed or commercial.

Table 2. Diagnostic guideline for chronic Q fever proposed by Raoult*

Q fever endocarditis
A. Definite criteria
Positive culture, PCR, or immunochemistry of a cardiac valve
B. Major criteria
Microbiology: positive culture or PCR of the blood or an emboli or serology with IgG I antibodies ≥6,400
Evidence of endocardial involvement:
Echocardiogram positive for IE: oscillating intra-cardiac mass on valve or supporting structure, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation; or abscess; or new partial dehiscence of prosthetic vale; or new valvular regurgitation (worsening or changing of pre-existent murmur not sufficient)
PET scan showing a specific valve fixation and mycotic aneurysm
C. Minor criteria
Predisposing heart condition (known or found on echocardiograph)
Fever, temperature >38°C
Vascular phenomena, major arterial emboli, septic pulmonary infarcts, mycotic aneurysm (see at PET scan), intracranial hemorrhage, conjunctival hemorrhages, and Janeway lesions
Immunologic phenomena: glomerulonephritis, Osle nodes, Roth spots, or rheumatoid factor
Serologic evidence: IgG I antibodies ≥800 <6,400
Diagnosis definite
1. 1A criterion
2. 2B criterion
3. 1B, and 3C criterion
Diagnosis possible
1. 1B criterion, 2C criteria (including microbiology evidence, and cardiac predisposition)
2. 3C criteria (including positive serology, and cardiac predisposition)
Q fever vascular infection
A. Definite criteria
Positive culture, PCR, or immunochemistry of an arterial sample (prosthesis or aneurysm) or a periarterial abscess or a spondylodiscitis linked to aorta
B. Major criteria
Microbiology: positive culture or PCR of the blood or an emboli or serology with IgG I antibodies ≥6,400
Evidence of vascular involvement
CT scan: aneurysm or vascular prosthesis + periarterial abscess, fistula, or spondylodiscitis
PET scan: specific fixation on an aneurysm or vascular prosthesis
C. Minor criteria
Serological IgG I ≥800 <6,400
Fever, temperature >38°C
Emboli
Underlying vascular predisposition (aneurysm or vascular prosthesis)
Diagnosis definite
1. 1A criterion
2. 2B criterion
3. 1B and 2C criterion (including microbiology findings and vascular predisposition)
Diagnosis possible
Vascular predisposition, serological evidence and fever or emboli

*Source: (*16*). IE, infective endocarditis; PET, positron emission tomography; IFA, immunofluorescence assay; CT, computed tomography.

rare manifestations, such as pericarditis, hepatitis, and osteomyelitis, are left undefined, however.

After the recent outbreak of Q fever in the Netherlands, we initiated the Dutch National Chronic Q Fever Database, a joint effort by multiple hospitals in areas affected by Q fever, to monitor all chronic Q fever cases in the Netherlands. All hospitals with chronic Q fever patients, also outside the notified Q fever–epidemic areas, were actively approached. Design of the database and use of the collected information for analysis and scientific publications were approved by the Medical Research Ethics Committee of the University Medical Center Utrecht in Utrecht, the Netherlands. Part of these data had previously been published in a report discussing serologic profiles of patients with chronic Q fever (*15*).

Until the end of May 2012, a total of 284 patients had been included in our database (although the epidemic started in 2009, all patients from 2006 on are included): 151 patients (53.2%) had proven chronic Q fever, 64 patients (22.5%) had probable chronic Q fever, and 69 patients (24.3%) had possible chronic Q fever, according to the Dutch consensus guideline. We reevaluated these chronic Q fever cases with the alternative diagnostic criteria (Table 3). Of the case-patients with proven chronic Q fever according to the Dutch guideline, 46 (30.5%) would have been left undiagnosed with the alternative criteria. For cases of probable chronic Q fever, 58 cases (90.6%) would have been undiagnosed, and for possible cases of chronic Q fever, all 69 cases.

The conditions of 8 patients with proven chronic Q fever (based on PCR positivity for *C. burnetii* in blood and suspicion of endocarditis) would have been diagnosed with possible Q fever endocarditis only by the alternative criteria (Table 4). Eighteen patients with proven chronic Q fever would not have been diagnosed with Q fever endocarditis at all, because echocardiography results did not match any major clinical Duke criterion, as is often observed in cases of Q fever endocarditis (*7*). Of the 8 patients with proven chronic Q fever endocarditis (by Dutch consensus guideline) who had been given a diagnosis of possible endocarditis according to the alternative criteria, 2 patients would have been considered to have definite endocarditis by the modified Duke criteria (*13*).

Twenty-four patients with a vascular *C. burnetii* infection (Dutch consensus guideline) would not have been diagnosed with chronic Q fever by using the alternative criteria (Table 4). Seventeen of these patients had a positive vascular lesion on fluorodeoxyglucose–positron emission tomography/computed tomography (FDG-PET/CT) with phase I IgG $\geq$1:800 and <1:6,400. Seven patients had a PCR of blood positive for *C. burnetii*, in combination with an aneurysm or vascular prosthesis but no signs of infection on FDG-PET/CT. According to the Dutch consensus

Table 3. Comparison of chronic Q fever diagnosis according to the Dutch consensus guideline* and the alternative criteria†

Alternative criteria	Dutch consensus chronic Q fever guideline		
	Proven, no. (%), n =151	Probable, no. (%), n = 64	Possible, no (%), n = 69
Definite Q fever endocarditis	21 (13.9)	0	0
Possible Q fever endocarditis	8 (5.3)	4 (6.3)	0
Definite Q fever vascular infection	76 (50.3)	0	0
Possible Q fever vascular infection	0	2 (3.1)	0
No diagnosis of chronic Q fever	46 (30.5)	58 (90.6)	69 (100.0)

*Source: (*14*).
†Source: (*16*).

guideline, there were 5 patients with proven chronic Q fever with no known focus and 2 patients with Q fever with a focus other than endocarditis or vascular infection who would have been missed by using the alternative criteria. Five (repeatedly) had a positive *C. burnetii* PCR of blood but no clear infectious focus on echocardiograph and FDG-PET/CT scan. One patient had a positive PCR in blood with clinical pericarditis, and 1 patient had a positive PCR in blood during pregnancy with phase I IgG >1:1,024 and a positive PCR of placental tissue.

Notably, 10 patients with cases of proven chronic Q fever that were not diagnosed as definite chronic Q fever by the alternative criteria died (2 with possible chronic Q fever and 8 without chronic Q fever according to the alternative guideline). Six of these patients died due to clear chronic Q fever–related manifestations (2 with possible chronic Q fever and 4 without chronic Q fever according to the alternative guideline, Table 4). The 2 patients with possible chronic Q fever died of complications caused by endocarditis, one had a double-pathogen endocarditis with *Staphylococcus aureus*. Two of the 4 patients without chronic Q fever according to the alternative guideline died due to aortoduodenal fistula, both with a phase I IgG >1:1024, but <1:6400, negative PCR on blood, and a clear FDG-positive vascular focus on PET/CT. In 1 of these 2 patients, Q fever vascular infection was confirmed postmortem with a positive PCR of the abdominal aortic aneurysm. No autopsy was performed on the other patient, unfortunately. The third patient, who had a history of a biologic heart valve replacement, an FDG-PET/CT negative aortic aneurysm, and a positive *C. burnetii* PCR of blood, eventually died of heart failure. Postmortem analysis demonstrated that PCR of the heart valve confirmed *C. burnetii* infection and thus Q fever endocarditis. Another chronic Q fever patient with positive PCR results of blood and minor valve lesions, according to the Duke criteria, died of gastrointestinal bleeding, probably due to aorto-intestinal fistula.

Conclusions

Several major differences exists between the Dutch consensus guideline for the diagnosis of chronic Q fever and the alternative criteria. These alternative criteria define only Q fever endocarditis and Q fever vascular infection and oppose the term chronic Q fever. However, this distinction

Table 4. Characteristics and outcome of patients diagnosed with chronic Q fever using the Dutch consensus guideline* but without (definite) chronic Q fever according to alternative criteria†

Dutch consensus guideline	Alternative criteria	
	Possible Q fever endocarditis or vascular infection, no. (%), n =14	No diagnosis, no. (%), n = 173
Proven Q fever	8 (57.1)	46 (26.6)
Endocarditis	8 (57.1)	18 (10.4)
PCR positive for *Coxiella burnetii* in blood	6 (42.9)	18 (10.4)
Evidence of endocardial involvement	2 (14.3)	0
Vascular infection	0	24 (13.9)‡
PCR positive in blood	0	7 (4.0)
Vascular focus on imaging	0	17 (9.8)
Other or no focus§	0	7 (4.1)
Deceased	2 (14.3)	8 (4.6)
Death probably due to Q fever	2 (14.3)	4 (2.3)¶
Probable Q fever	6 (42.9)	58 (33.5)
Endocarditis	4 (28.6)	22 (12.7)
Vascular infection	2 (14.3)	16 (9.3)
Other or no focus	0	20 (11.6)
Deceased	2 (14.3)	4 (2.3)
Death probably due to Q fever	1 (7.1)	0
Possible Q fever	0	69 (39.9)

*Source: (*14*).
†Source: (*16*).
‡In 3 patients with proven chronic Q fever, imaging studies showed that the focus of infection was in both the heart valves and the vascular structures.
§All were PCR positive.
¶For 2 patients, PCR of vascular and heart valve tissue obtained at autopsy was positive for *C. burnetii*.

is not accompanied by therapeutic consequences for each of these manifestations, which we believe makes these guidelines less practical.

It must be acknowledged that, because all patients included in our study met the Dutch criteria for proven, probable, or possible chronic Q fever, other guidelines can only perform with less accuracy in comparison. Nevertheless, sensitivity of the Dutch guideline is markedly higher than with the alternative criteria: ≈31% of proven chronic Q fever case-patients would have been missed as well as almost all patients with probable and possible cases, including at least 4 patients who eventually died of chronic Q fever related causes. Specificity of the Dutch consensus guideline is probably lower than that of the alternative criteria, but because mortality and morbidity rates are high when chronic Q fever cases are untreated, we believe sensitivity is of greater importance in clinical practice. Our data illustrate that, when proven cases of chronic Q fever are missed, and patients are therefore not adequately treated, these patients are at high risk for severe complications and death.

As stated before, the most critical difference between the criteria of the Dutch guideline and those of the alternative guideline is the acknowledgment of a positive *C. burnetii* PCR as a marker of proven chronic Q fever in the absence of acute Q fever. Of course, this difference should be interpreted with care. In our opinion, patients without endocarditis or vascular infection on imaging studies but with a positive PCR in blood should also be treated for chronic Q fever, as they may suffer from not yet clinically visible endocarditis or vascular infection, which was confirmed by the postmortem results of 2 of our patients described above. A single positive *C. burnetii* PCR of blood is highly suggestive for chronic Q fever when acute Q fever is excluded. A PCR test will not be performed in patients without symptoms and without any risk factors for chronic Q fever, so cases in whom a positive PCR is the only factor indicating chronic Q fever is a theoretical consideration. We have observed few patients, in the absence of signs of acute Q fever, with elevated phase I IgG titers not fulfilling the serologic criteria of chronic Q fever (phase I IgG ≥1:800 or 1,024) but with a positive *C. burnetii* PCR of blood or tissue. In these cases, we are convinced that PCR-positivity proves chronic Q fever. No patients in our chronic Q fever database who had a positive PCR on blood or tissue had a phase I IgG titer of ≤1:256.

We agree with the statement that proven chronic Q fever will not develop in some patients with probable chronic Q fever and in most patients with possible chronic Q fever. We therefore do not advocate treating all of these patients with long-term antibacterial drugs. Nevertheless, we do think that these patients should all be examined for a chronic Q fever focus and should continue to be monitored closely, at least until further research offers more clarity regarding the prognosis of these patients. If these patients do not receive a diagnosis of possible or probable chronic Q fever, they might not receive such close follow-up. Moreover, the Dutch consensus guideline is easier to use, adds treatment advice, and also applies to patients with chronic Q fever manifestations that are rarer than endocarditis and vascular infection.

We hope that, with the future results from the Dutch National Chronic Q Fever Database and joint efforts of international researchers and experts in the field of Q fever, these guidelines can be modified to provide definite evidence-based criteria for diagnosis and treatment of this complex disease. In the meantime, the Dutch consensus guideline created on the basis of the scarce available literature is, in our opinion, safer and easier to use in clinical practice than the alternative expert-based criteria.

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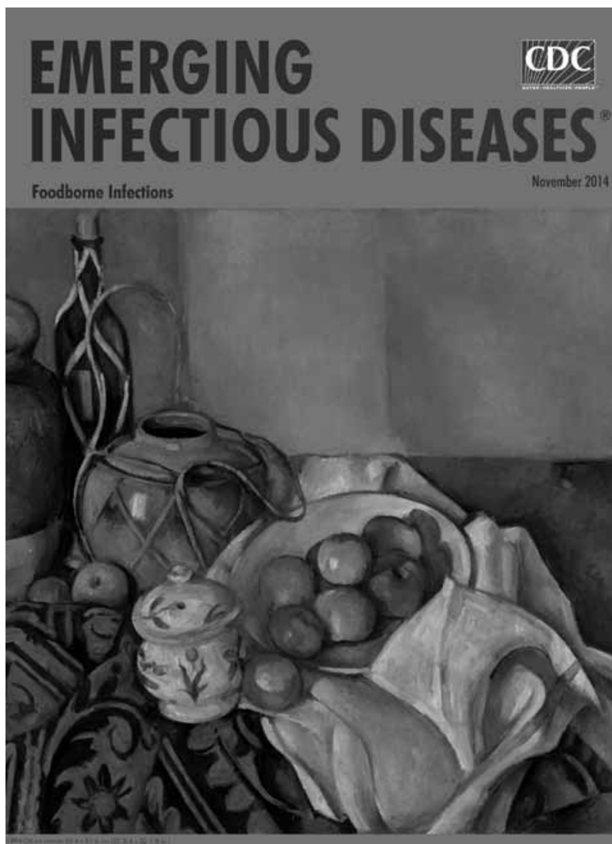
November 2014: **Foodborne Infections**



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- Novel *Chlamydia trachomatis* Strains in Heterosexual Sex Partners, Indianapolis, Indiana, USA

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Swine Influenza A(H3N2) Virus Infection in Immunocompromised Man, Italy, 2014

Antonio Piralla, Ana Moreno, Maria Ester Orlandi, Elena Percivalle, Chiara Chiapponi, Fausto Vezzoli, Fausto Baldanti, and the Influenza Surveillance Study Group[1]

Because swine influenza virus infection is seldom diagnosed in humans, its frequency might be underestimated. We report a immunocompromised hematologic patient with swine influenza A(H3N2) virus in 2014 in Italy. Local pigs were the source of this human infection.

Pigs are considered the "mixing vessel" in which avian, human, and swine influenza genetic material can be exchanged and result in new influenza viruses (*1*). Zoonotic influenza A infections in humans caused by swine influenza viruses (SIVs) have been infrequently reported in Europe (*1*,*2*), even though at least 19% of occupationally exposed humans, such as pig farmers, slaughterers, and veterinarians, have SIV antibodies (*3*). However, because the infection is clinically mild in most cases, its frequency might be underdiagnosed in humans (*4*).

Three influenza A subtypes (H1N1, H1N2, and H3N2) circulate in swine herds in Italy (*1*). We report a European swine A(H3N2) influenza virus that occurred in an immunocompromised man in Italy in 2014.

The Study

On January 14, 2014, a 67-year-old man with multiple myeloma underwent the eighth cycle of chemotherapy at the Hematology Unit of the Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo in Pavia, Italy. The patient had mild upper respiratory syndrome (fever, cough, and cold). A nasal swab sample was tested by real-time reverse transcription PCR (RT-PCR) and PCR with a panel for 17 respiratory viruses (*5*,*6*). The clinical specimen was positive for influenza A (6×10^6 RNA copies/mL). However, attempts to subtype the strain by using real-time RT-PCR specific for human influenza subtypes H1 and H3, as well as avian influenza subtype H7N9, were unsuccessful.

The clinical sample was inoculated onto a mixed-cell (Mv1Lu and A549 cells) monolayer. After 48 h incubation, it scored positive using a monoclonal antibody specific for influenza A/H3 antigen (Millipore, Billerica, MA, USA).

On January 24, 2014, the influenza virus strain A/Pavia/07/2014 was recovered from the supernatant propagated in MDCK cell culture. An RT-PCR that amplifies all 8 segments of the influenza A genome was then conducted (*7*). The purified amplicons were sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a 3130xl Genetic Analyzer (Applied Biosystems). We BLAST searched the sequences obtained for closely related sequences in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial nucleotide sequences of polymerase, nucleoprotein, hemagglutinin (HA), neuraminidase (NA), matrix, and nonstructural genes showed swine influenza A(H3N2) virus with an internal gene belonging to the European SIV lineage.

At a second control visit, on January 29, 2014, the patient's nasal swab sample was negative for respiratory viruses, but cough and cold persisted. No vaccination or antiviral treatment was administered to the patient before or during the influenza episode. During January 2014, he had spent several weeks visiting his relatives on a pig farm in the province of Lodi (northern Italy) and reported contact with pigs, as well as with his grandson. No respiratory symptoms had developed in any of his family members (owners of the pig farm) or in farm co-workers.

On February 6, 2014, the A/Pavia/07/2014 strain was propagated in embryonated specific pathogen–free chicken eggs at the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna (Brescia, Italy). The sequences of complete genome segments were obtained with the MiSeq platform (Illumina, San Diego, CA, USA) as previously described (*8*). The data were de novo assembled on BaseSpace Cloud (Illumina) with the DNAStar application and analyzed with the Lasergene package software (version 10.1.2). We conducted phylogenetic analysis online using PhyML v.3.0 (*9*) and MEGA5 software (*10*). A phylogenetic tree of the HA and NA genes confirmed that the A/Pavia/07/2014 strain was closely related to European A(H3N2) SIV (Figure 1). In addition, phylogenetic trees constructed with sequences of the polymerase base

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[1]Members of the Influenza Surveillance Study Group who contributed data are listed at the end of this article.

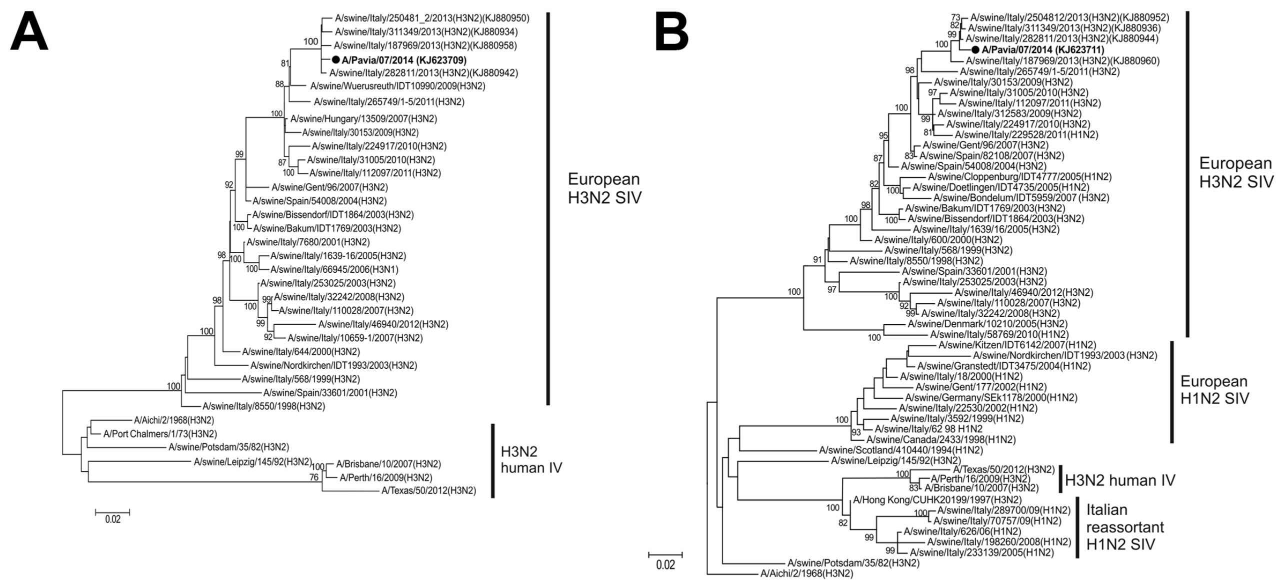

Figure 1. Phylogenetic trees of the hemagglutinin (A) and neuraminidase (B) genes of swine influenza viruses (SIVs). The 8 genome segment sequences of the A/Pavia/07/2014 strain (black dot, in bold) were submitted to GenBank under accession nos. KJ623706–KJ623713. Scale bars indicate nucleotide substitutions per site.

1, polymerase base 2, polymerase, nucleoprotein, matrix, and nonstructural genes showed that the A/Pavia/07/2014 strain clustered within the European avian-like SIVs, including H1N1, H1N2 and H3N2 subtypes (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/7/14-0981-Techapp1.pdf).

The HA gene of the A/Pavia/07/2014 strain is 567 aa long and has antigenic sites identical to those of SIV A(H3N2) strains that circulated in swine in Italy during 2013 (Figure 2, http://wwwnc.cdc.gov/EID/article/21/7/14-0981/F2.htm). In addition, the pattern of A/Pavia/07/2014 glycosylation sites in the HA is identical to that in the A/swine/Italy/282811/2013 HA sequence and different from the human influenza A/Brisbane/10/2007 strain (online Technical Appendix Figure 2).

In December 2013, respiratory symptoms were observed mainly in piglets and weaning pigs on the farm that the patient visited, a farrow-to-finish pig farm where 400 sows were reared. The various production phases (mating, gestation, farrowing, nursery, and growing/finishing) were located in separated buildings. Forty-two nasal swab samples were collected at the end of January 2014 from pigs at all production phases. Considering that clinical signs were observed in piglets and weaning pigs, most samples (30 samples) were collected from weaning pigs. Influenza A real-time RT-PCR yielded negative results, probably because samples were collected ≈2 months after clinical signs appeared. Serologic investigations were performed on 29 serum samples from sows of different ages in mid-May that were collected within a national monitoring plan for Aujeszky disease. Samples were tested by hemagglutination-inhibition test according to standard procedures (*12*) by using A/Pavia/07/2014 and the reference SIVs A/swine/CA/3633/84 H3N2, A/swine/Italy/1521/98 H1N2, and A/swine/Finistere/2899/82 as antigens. Two-fold serum dilutions were tested starting at 1:20. All animals showed antibodies against H3 (137.16 geometric mean vs. A/Pavia/07/2014 and 84.2 vs. A/swine/CA/3633/84), whereas 15 of 29 serum samples that originated from the oldest animals also yielded positive results for A(H1N1) SIV. No antibodies against A(H1N2) SIV were detected.

Conclusions

The swine influenza A(H3N2) viruses present in Europe since 1984 resulted from a genomic reassortment between human-like swine H3N2 viruses and avian-like swine H1N1 viruses (*13*). Until 2011, only 3 episodes of SIV H3N2 infection had been reported in the Netherlands and Switzerland (*1*,*2*). Recently, in 2011 the emergence of a new SIV H3N2 variant was reported in the United States that had limited person-to-person transmission (*14*). Here we report a case of SIV H3N2 infection in a human host in January 2014 in Italy. In this patient, the presence of an uncommon influenza strain was suspected after the failure of molecular typing in the presence of high influenza load. The SIV strain identified here correlated with strains circulating in pigs during the 2013–14 influenza season in Italy. In addition, serologic results on pig serum collected from a farm close to the patient's home suggested a recent exposure with an H3N2 strain similar to the A/Pavia/07/2014 isolate. These virologic and serologic data suggest that local pigs were the source of human infection.

In agreement with previous observations (*1*,*2*), the European H3N2 swine viruses seem to cause a benign disease with mild influenza-like symptoms in humans. In addition, the SIV strain we identified was found only in an immunocompromised patient. The uncomplicated clinical course might not be uncommon in immunocompromised patients. Indeed, in 2 epidemiologically correlated immunocompromised patients, the emergence of human influenza H3N2-resistant strains was associated with opposite clinical outcomes: 1 patient had mild upper respiratory syndrome; the other died of severe acute respiratory distress syndrome (*15*). On the other hand, on the basis of the observation that none of the patient's family members and co-workers showed respiratory infection, we can hypothesize that immune impairment of the patient could have favored the zoonotic transmission of the SIV strain. Surveillance of circulating SIVs and monitoring of occupationally exposed workers are 2 important tools to prevent spread of potential pandemic viruses.

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Severe Pediatric Adenovirus 7 Disease in Singapore Linked to Recent Outbreaks across Asia

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During November 2012–July 2013, a marked increase of adenovirus type 7 (Ad7) infections associated with severe disease was documented among pediatric patients in Singapore. Phylogenetic analysis revealed close genetic links with severe Ad7 outbreaks in China, Taiwan, and other parts of Asia.

Human adenoviruses (HAdVs) are classified into >50 types and are associated with clinical manifestations that include respiratory, gastrointestinal, ocular, genitourinary, and neurologic disease (*1*). HAdV infections have been estimated to cause 5%–10% of acute respiratory illnesses in children <5 years of age. Although most infections are subclinical or result in mild upper respiratory tract illnesses, HAdVs can also cause severe pneumonia. Among the HAdV types, type 7 (Ad7) has most often been associated with severe respiratory disease (*2*).

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Recent reports have noted increased incidence of severe Ad7 disease in Asia: among the general population and pediatric inpatients in Taiwan; among persons in a military training camp in Shaanxi, China; and among those in a police training center in Kuala Lumpur, Malaysia (*2*–*4*). During January–June 2013, physicians in Singapore noted an increase in HAdV pediatric inpatients. Here we characterize the clinical and molecular epidemiology of this outbreak by reviewing data from government hospitals, the military, and a nationwide influenza-like illness (ILI) laboratory surveillance network in Singapore.

The Study

We retrospectively reviewed demographic and clinical information of adenovirus infections reported in Singapore during January 2011–July 2013 in 3 populations: 1) pediatric inpatients at KK Women's and Children's Hospital (formerly known as Kandang Kerbau Hospital) and National University Hospital, which are the only government hospitals in Singapore that have pediatric departments; 2) military personnel; and 3) outpatients reported to the nationwide ILI surveillance network housed in the National Public Health Laboratories (NPHL). Institutional review boards of the participating hospitals approved this study.

Cases of HAdV infection among military personnel were detected by a sentinel surveillance program in 5 military camps in which occurrences of febrile respiratory illnesses, defined as presence of acute respiratory symptoms (cough, sore throat, or both) and fever (oral temperature ≥37.5°C) (*5*), are monitored. All male citizens of Singapore undergo 2 years of conscripted military service upon turning 18–19 years of age; new personnel continuously enter the camps. To identify cases among the civilian population, the NPHL ILI laboratory surveillance network processes upper respiratory tract samples from patients with acute onset of fever (oral temperature ≥38°C) and respiratory symptoms referred by physicians at 23 sentinel clinics (*6*).

HAdV infection cases were defined by the detection of adenovirus by PCR assay, immunofluorescence, viral culture, or antigen detection in clinical samples (respiratory specimens, including nasal wash, bronchoalveolar lavage, endotracheal tube aspirate, oropharyngeal, nasopharyngeal, throat, and nasal swab; or urine or eye swab specimens). HAdV typing was performed by sequencing

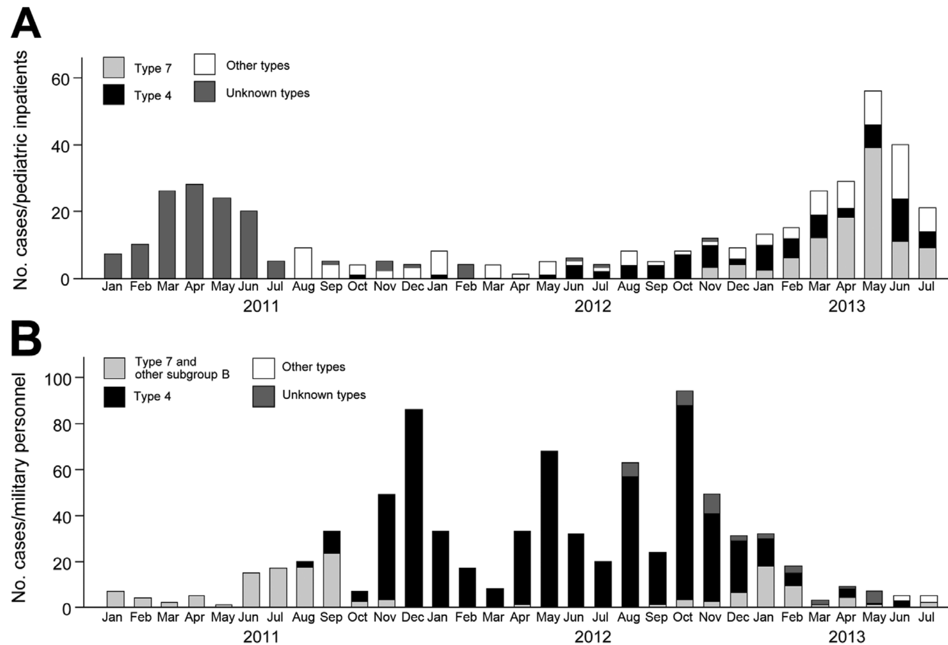

Figure 1. Number of adenovirus cases by month, January 2011–July 2013, Singapore. A) The first confirmed case of adenovirus type 7 was reported in November 2012 in KK Women's and Children's Hospital. The number of human adenovirus cases among the pediatric inpatient population increased and peaked in May 2013. B) A retrospective examination of the military surveillance data revealed the first appearance of adenovirus type 7 among military personnel in September 2012 and a small increase and decline over the next few months.

of HAdV hexon gene hypervariable regions 1–6 ($HVR_{1–6}$) (Ad7 reference Gomen AY594255 hexon gene nt 324–1123) (*7*). To assess whether Ad7 was associated with severe disease, diagnoses of inpatients were dichotomised as invasive (pneumonia, gastroenteritis, disseminated disease, or hemorrhagic cystitis) and noninvasive (upper respiratory tract infection, acute laryngotracheobronchitis, bronchitis, bronchiolitis, tonsillitis, otitis media, or conjunctivitis) on the basis of clinical syndromes identified by physicians.

During January 2011–July 2013, samples from 421 pediatric inpatients, 752 military personnel, and 85 pediatric outpatients from the NPHL ILI surveillance network were positive for adenovirus. During August 2011–July 2013, a total of 289 (96.0%) pediatric inpatient cases were genotyped. The number of pediatric inpatients increased from 32 during January–July 2012 to 200 cases during January–July 2013 (Figure 1). This increase was predominantly related to Ad7 infections, which were first detected in November 2012 and represented 48.5% (n = 97) of all genotyped adenovirus cases in the first 7 months of 2013. The increase in Ad7 pediatric inpatients was accompanied by a smaller increase in detection of subgroup B HAdV infections (n = 47) among military personnel, from September 2012 to July 2013; all patients recovered with outpatient treatment. Of the samples from military personnel, 35 (74.5%) were genotyped; all were Ad7. During September 2012–July 2013, of 19 HAdV cases among pediatric patients (<16 years of age) detected and genotyped by the community ILI surveillance, none were Ad7; of 17 HAdV cases identified among adults, 3 (17.6%) were Ad7.

Clinical information was available for 188 HAdV-positive pediatric inpatients (<16 years of age) admitted during January–September 2013 (Table 1). A total of 54 patients had invasive infections and 134 had noninvasive infections (Table 2). More patients (n = 21, 38.9%) who had invasive infections had comorbid conditions than did patients who had noninvasive infections (n = 14, 10.5%; p<0.001). Ad7 was more frequently identified among patients who had invasive infection (57.4% vs. 41.0%; p = 0.002). In univariate analysis, invasive infection was significantly associated with presence of comorbid conditions (crude odds ratio [OR] 5.45, 95% CI 2.50–11.88) and Ad7 infection (crude OR 6.95, 95% CI 1.98–24.41; p<0.001). After adjusting for age and gender, presence of comorbid conditions (adjusted OR 6.78, 95% CI 2.59–17.72) and Ad7 infection (adjusted OR 9.00, 95% CI 2.34–34.59) remained significantly associated with invasive infection (p<0.001).

We used the maximum-likelihood method to compare the phylogenetic relationships among representative Ad7 partial hexon gene sequences from pediatric inpatients (n = 9, November 2012–June 2013); the nationwide ILI laboratory surveillance network (n = 1 adult sample, January–June 2013); and military personnel (n = 34, September 2012–May 2013) by using reference Ad7 sequences (GenBank accession nos. KP729815–KP729824) (Figure 2) (*8*,*9*). All Singapore Ad7 isolates except KK341 and KK342 had 100% nucleotide identity with strains reported from a 2011 adenovirus community outbreak in Taiwan (JX174430), severe disease in infants in Shaanxi in 2009 (GU230898), and a military training camp outbreak in Shaanxi in 2012 (KC689913) (*2*,*3*,*10*).

Table 1. Demographics and clinical features of 188 hospitalized children with adenovirus, by age group, Singapore*

Characteristics	No. (%)
Median age, y (range)	3.0 (0.2–15.7)
Male sex	120 (63.8)
Positive contact history	
Yes	79 (42.0)
Unknown	12 (6.4)
Contact with confirmed adenovirus case	
Yes	8 (10.1)
Unknown	1 (1.3)
Comorbid conditions†	35 (18.6)
Symptom days before care sought, median (range)	5 (0–21)
Diagnostic method	
PCR	51 (27.1)
Immunofluorescence	137 (72.9)
Type	
4	40 (21.3)
7	86 (45.7)
Others (1, 2, 3, 5, 11)	33 (17.6)
Unknown	29 (15.4)
Syndrome	
Upper respiratory tract infection	107 (56.9)
Acute laryngotracheobronchitis	1 (0.5)
Bronchiolitis/bronchitis	13 (6.9)
Tonsillitis	11 (5.9)
Otitis media	2 (1.1)
Conjunctivitis	0
Pneumonia	35 (18.6)
Gastroenteritis	15 (8.0)
Disseminated adenovirus	1 (0.5)
Hemorrhagic cystitis	3 (1.6)
Mechanical ventilation	13 (6.9)
Hospitalization days, median (range)	4 (1–247)
Died	6 (3.2)

*Values are no. (%) patients except as indicated.
†Comorbid conditions included primary and secondary immunodeficiencies, congenital heart disorders, chronic lung diseases, congenital malformations, inborn errors of metabolism and Down syndrome.

Conclusions

An abrupt increase in severe Ad7disease in pediatric inpatients in Singapore occurred during November 2012–July 2013. A corresponding rise was noted among military personnel during October 2012–April 2013, but no statistically significant increase in Ad7 infections was detected by the NPHL community ILI surveillance program. Partial hexon gene sequences of the Singapore isolates had 100% nucleotide identity with sequences reported from outbreaks in Taiwan and China (*2*,*3*,*10*).

Ad7 has been reported to cause outbreaks in 3 main patterns: 1) severe disease among young children, especially during winter in temperate countries; 2) less severe disease in nonseasonal community outbreaks; and 3) outbreaks among military personnel (*11*). The outbreak we report was marked by severe disease among young children and mild disease among military personnel. In the 2 government-owned hospitals, 1–2 cases among pediatric patients were identified per month by using community ILI surveillance. Failure to detect an increase in Ad7 remains unexplained but might be related to the low number of samples collected and tested.

Ad7 can be subclassified by restriction enzyme analysis (*12*). The available Singapore partial hexon gene sequences were most closely related to Ad7d and Ad7d2 genome types, which have been associated with outbreaks of acute respiratory illness in Asia. Ad7d, the predominant Ad7 circulating virus in China since the early 1980s, was the cause of outbreaks in South Korea during 1995–1997; during a community and pediatric outbreak in Taiwan in 2011, Ad7d replaced Ad7b as the main Ad7 strain (*2*,*13*). The closely related Ad7d2 was described in Israel in 1992 and has caused outbreaks in the United States and Japan (*14*,*15*). Systematic HAdV typing in Singapore was initiated in late 2011, so it remains unknown if Ad7 substrain replacement, specifically the circulating pediatric HAdV strain in early 2011, was a factor in the outbreak we report.

Our findings indicate a need for improved vigilance for detection and surveillance of severe Ad7 disease in Asia, as well as whole-genome sequencing and seroprevalence studies to perform accurate typing of outbreak strains and to identify correlates of pathogenicity. These practices could facilitate effective, early deployment of vaccine prevention and antiviral therapy.

Acknowledgments

We thank the staff of all participating institutions who contributed to patient care and data collection for this analysis.

Table 2. Number and percentages of 188 hospitalized pediatric adenovirus case-patients with noninvasive and invasive infection, by key characteristics, and risk factors associated with invasive infection, Singapore*

Characteristics	Noninvasive infection, n = 134	Invasive infection, n = 54	Univariate analysis		Multivariate analysis†	
			Crude OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Median age, y (range)	3.0 (0.2–15.7)	3.1 (0.3–15.5)	1.03 (0.95–1.12)	0.988	1.04 (0.92–1.17)	0.568
Male sex	88 (65.7)	32 (59.3)	0.76 (0.40–1.46)	0.331	0.53 (0.23–1.20)	0.126
Comorbid conditions†	14 (10.5)	21 (38.9)	5.45 (2.50–11.88)	<0.001	6.78 (2.59–17.72)	<0.001
Adenovirus type†						
4	37 (27.6)	3 (5.6)	1.00		1.00	
7	55 (41.0)	31 (57.4)	6.95 (1.98–24.41)	0.002	9.00 (2.34–34.59)	0.001
Others (1, 2, 3, 5, 11)	24 (17.9)	9 (16.7)	4.63 (1.14–18.83)	0.033	5.33 (1.20–23.68)	0.028
Unknown	18 (13.4)	11 (20.4)	NA	NA	NA	NA

*Values are no. (%) patients except as indicated; OR, odds ratio; NA, not applicable.
†For multivariate analysis, variables selected in the best-fit model are shown; the model including all covariates did not alter the independent predictors.

Figure 2. Phylogenetic analysis of adenovirus type 7 (Ad7) sequences from this study based on sequenced Ad7 hexon gene hypervariable regions 1–6 (Ad7 reference Gomen AY594255 hexon gene nt 324 to 1123). The phylogenetic relationships between Ad7 isolates in this study were inferred by using the maximum-likelihood method based on the Tamura-Nei model (*8*). Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated by using the maximum-composite likelihood approach. Tree is drawn to scale; branch lengths are measured in the number of substitutions per site, the rate of which was assumed to be uniformly distributed. The analysis involved 63 nt sequences. Green indicates isolates from military personnel, dark blue indicates isolates from patients in National University Hospital, pink indicates isolates from patients in KK Women's and Children's Hospital, and light blue indicates isolates from patients in Changi General Hospital. The sequence labeled as "AY594255_7_Gomen (USA 1954)" represents 1 of the published Gomen sequences (AY594255). "_7" is added to GenBank accession numbers to denote that these are Ad7 sequences. All other strains shown are published Ad7 reference isolates. Phylogenetic analyses were conducted by using MEGA6 (*9*). KK341 and KK342 had a single thymine-to-cytosine mutation (Ad7 reference Gomen AY594255 hexon gene nt 780). The remaining 41 isolates from Singapore had 100% nucleotide identity.

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Hemagglutinin Receptor Binding of a Human Isolate of Influenza A(H10N8) Virus

Irene Ramos, Mena Mansour, Teddy J. Wohlbold, Megan E. Ermler, Ariana Hirsh, Jonathan A. Runstadler, Ana Fernandez-Sesma, Florian Krammer

Three cases of influenza A(H10N8) virus infection in humans have been reported; 2 of these infected persons died. Characterization of the receptor binding pattern of H10 hemagglutinin from avian and human isolates showed that both interact weakly with human-like receptors and maintain strong affinity for avian-like receptors.

Human infections with avian influenza A(H10N8) virus were reported in China during the 2013–14 winter influenza season. The first patient, a 73-year old woman, became ill in November 2013 a few days after visiting a live poultry market in Jiangxi Province (*1*). Two additional patients, a 55-year-old woman and a 75-year-old man, were admitted to hospitals in the same province in January 2014 (*2*). Severe pneumonia and subsequent acute respiratory distress syndrome developed in all 3 patients; 2 of the patients died, 5 and 6 days after admission (*2*).

Epithelial cells of the human upper respiratory tract contain mostly α2,6-linked sialic acids (SAα2,6) and low levels of α2,3-linked sialic acids (SAα2,3) (*3*). Hemagglutinin (HA) of avian influenza virus strains shows preferential binding to SAα2,3 receptors, which partially accounts for the reduced ability of avian influenza strains to establish infections in humans (*3*). Interaction with SAα2,6 receptors is one of the requirements for efficient replication in the human upper respiratory tract. In addition, reduced binding to SAα2,3 facilitates respiratory droplet-based transmission in ferrets (*4*). Therefore, emerging avian influenza viruses with increased binding to SAα2,6 and reduced binding to SAα2,3 pose a major pandemic threat, and active research and surveillance to detect animal viruses with modified receptor binding are warranted.

The Study

We analyzed the amino acid sequence of the receptor binding site of HA from the isolate A/Jiangxi-Donghu/346-1/2013 (H10-JD346; Global Initiative on Sharing Avian Influenza Data [GISAID, http://www.gisaid.org)] accession no. EPI530526) from the first patient infected by influenza A(H10N8) virus. In addition, several human and avian influenza viruses (sequences from GISAID or the National Center for Biotechnology Information website) and a recent harbor seal isolate (5) were compared with H10-JD346 (Table). We observed that residues involved in receptor binding for H10 subtype influenza viruses suggest avian-like receptor specificity. However, we identified 2 amino acids in avian and human H10, T135 and S186, that are common in circulating human influenza viruses and were associated with changes in receptor binding in other avian influenza A virus subtypes (6,7). In accordance with this finding, Vachieri et al. found substantial levels of binding of an avian H10 HA to SAα2,6 that retained the ability to interact with SAα2,3 (8).

Given the role of receptor binding specificity of emerging influenza viruses, we analyzed the interaction of HA of the human H10-JD346 influenza A(H10N8) virus isolate in comparison with that of an avian H10N7 subtype virus. First, we used a solid-phase binding assay (*9*,*10*) and the following biotinylated glycans conjugated with a polyacrylamide (PAA) support (provided by the Consortium of Functional Glycomics [CFG]): Neu5Acα2,6Galβ1–4GlcNAcβ-PAA (6′ SLN-PAA); Neu5Ac±2 6(Gal²1 4GlcNAc²1 3)2β-PA A (6’sDi-LN-PAA); Neu5Acα2,3Galβ1–4GlcNAcβ-PAA (3′ SLN-PAA); Neu5Ac±2 3(Gal²1 4GlcNAc²1 3)2β-PA A (3′sDi-LN-PAA); and Neu5Acα2–3(Galβ1–4GlcNAcβ-sp)3β-PAA (3′sTri-LN-PAA). We also analyzed recombinant hexahistidine-tagged HAs (*11*) from H10-JD346, an avian H10N7 subtype strain from North America (A/mallard/Interior Alaska/10BM01929/2010; H10-mallard), a human H3N2 subtype seasonal influenza A virus (A/Panama/2007/1999; H3-P99), and an H5N1 subtype avian influenza virus from a fatal human case (A/Vietnam/1203/2004; H5-Viet).

As expected, H3-P99 bound strongly to the SAα2,6 tested, and H5 showed higher levels of binding to SAα2,3 than to SAα2,6 (Figure 1, panel A). When we analyzed H10-mallard and H10-JD346, we found a similar binding profile, which is consistent with the presence of similar amino acids affecting the receptor binding specificity (Table). Although both H10 proteins had a prevalent avian-like binding profile, low levels of binding to SAα2,6 were also observed.

To confirm this data, we used a flow cytometry–based assay and the same synthetic glycans (*9*,*10*). We infected

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Table. Alignment of residues involved receptor binding of hemagglutinin of influenza A viruses*

		Amino acid position (H3 numbering)														
Origin/subtype	Isolate name	131	135	137	138	152	186	190	193	200	222	224	225	226	227	228
Human/H3N2	A/Panama/2007/1999	A	**T**	S	A	N	**S**	D	S	G	W	R	G	V	S	S
Human/H3N2	A/Texas/50/2012	T	**T**	S	A	N	G	D	F	G	R	R	N	I	P	S
Human/H3N2	A/Brisbane/10/2007	T	**T**	S	A	N	V	N	F	G	R	R	N	I	P	S
Human/H1N1	A/California/04/2009	D	V	A	A	I	**S**	D	S	T	K	R	D	Q	E	G
Human/H1N1	A/Texas/36/1991	V	V	T	S	L	**S**	D	A	A	K	R	G	Q	E	G
Human/H1N1	A/Brisbane/59/2007	T	V	A	S	L	P	D	A	A	K	R	D	Q	E	G
Avian/H1N1	A/duck/Alberta/1976	T	V	A	A	L	P	E	S	A	E	R	G	Q	A	G
Avian/H7N1	A/rhea/North Carolina/39482/1993	R	A	S	A	K	G	E	K	T	F	S	G	R	I	D
Avian/H6N1	A/mallard/Sweden/81/2002	D	V	K	A	L	P	E	T	R	A	N	G	Q	R	G
Avian (human isolate)/H5N1	A/Vietnam/1203/2004	A	V	S	A	V	N	E	K	T	K	N	G	Q	S	G
Avian (human isolate)/H7N9	A/Anhui/1/2013	R	A	S	A	K	V	E	K	K	Q	N	G	L	S	G
Avian/H10N7	A/shorebird/Delaware Bay/10/2004	N	**T**	R	A	K	**S**	E	D	L	Q	N	G	Q	S	G
Avian/H10N7	A/mallard/Interior Alaska/10BM01929/2010	N	**T**	K	A	K	**S**	E	D	L	Q	N	G	Q	S	G
Avian (seal isolate)/H10N7	A/harbor seal/Germany/1/2014	N	**T**	K	A	K	**S**	E	D	L	Q	N	G	Q	S	G
Avian (human isolate)/H10N8	A/Jiangxi-Donghu/346–1/2013	N	**T**	R	A	K	**S**	E	D	L	Q	N	G	Q	S	G

*Residues found in human H1 or H3 and in H10 hemagglutinin but not in other avian hemagglutinin sequences are shown in bold.

MDCK epithelial cells with H10-JD346 virus (6:2 reassortant with the backbone of laboratory strain A/Puerto Rico/8/1934 [PR8], which was generated as described) (*9*,*10*); H10-mallard (wild-type); human isolate H3-P99 (wild-type); and H5-Viet 6:2 (low pathogenicity reassortant with the backbone of PR8) (*9*,*10*) at a multiplicity of infection of 1. Cells were harvested 24-h postinfection and incubated with antibody against matrix protein 2 (E10), which was detected by using an antibody against IgG (Alexa 647 antibody; Invitrogen, Carlsbad, CA, USA) as a control of infection and with the sialyl-glycans (detected with streptavidin–fluorescein isothiocyanate; Jackson Laboratories, Bar Harbor, ME, USA). We determined the percentage of infected cells in each sample and gated the infected population to determine the SA binding profile (Figure 1, panel B).

H3-P99 showed high levels of binding to SAα2,6 and H5-Viet bound more efficiently to SAα2,3 than to SAα2,6, which is similar to observations with recombinant HAs in the solid-phase binding assay. H10-mallard and H10-JD346 showed similar binding profiles with preferential binding for SAα2,3 and binding to SAα2,6 slightly higher than that for the negative control.

Analysis of receptor binding of H10-JD346 and of H10-mallard with 2 independent assays indicated that the H10 subtype influenza virus interacts slightly with human-like receptors and maintains preferential binding to avian-like receptors. Consequently, these data suggest that H10 subtype influenza virus might have the ability to interact with the upper human respiratory tract, which is rich in SAα2,6 (*3*).

To test this hypothesis, we precomplexed H3-P99 and H10-JD346 with primary antibody (mouse anti-His tag) and secondary fluorescent antibody, then incubated the complex with 2 human tracheal samples (*12*). As expected, H3-P99 HA bound to the surface of respiratory epithelia (Figure 2). Recombinant H10-JD346 HA also interacted with respiratory epithelia (Figure 2), which suggested that the virus might be able to attach and replicate in the human upper respiratory tract. However, the 6:2 reassortant virus H10-JD346 virus showed markedly decreased replication compared with that of an H3N2 subtype virus (PR8 6:2 reassortant) in a human lung epithelial cell line (Figure 3, http://wwwnc.cdc.gov/EID/article/21/7/14-1755-F3.htm).

Conclusions

HA of novel influenza A(H10N8) virus interacts with SAα2,3 and slightly with SAα2,6, at levels similar to that for an avian H10 subtype HA, and binds to cells in the human upper respiratory tract. Our findings are consistent with those of Vachiery et al. (*8*) but show some differences from those of Yang et al. (*13*) and Wang et al. (*14*), who did not detect interaction with SAα2,6 or human trachea. Variations in the experimental settings and protocols (e.g., concentration of HA or glycans used) might account for these dissimilarities.

Only 3 cases of human infections with influenza A(H10N8) viruses have been reported. However, H10N7 subtype viruses have caused conjunctivitis or mild respiratory symptoms in humans. An epidemic among seals

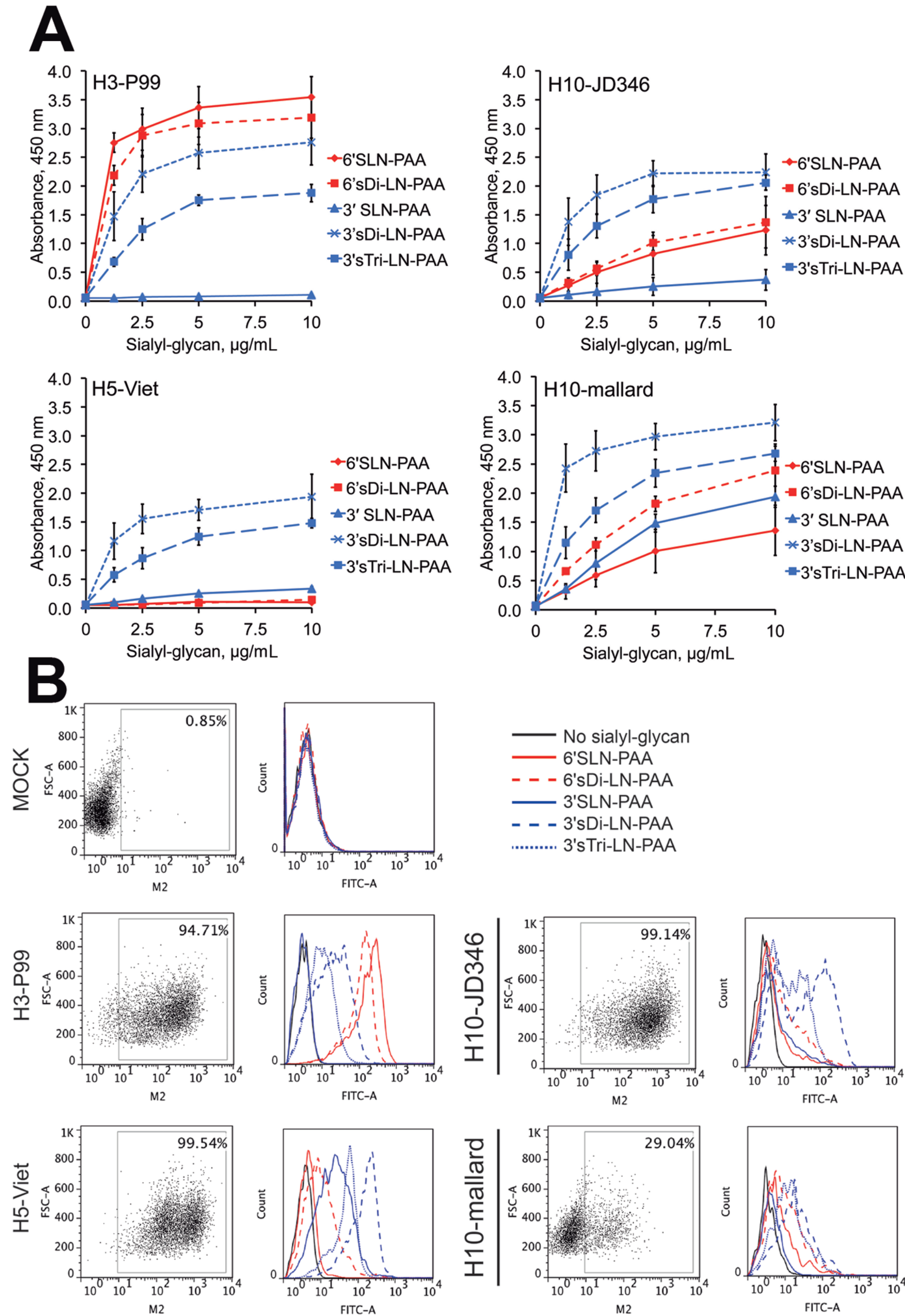

Figure 1. Receptor binding specificity of hemagglutinin of influenza A(H10N8) virus H10-JD346. A) Binding of recombinant hemagglutinins to glycans in a solid-phase binding assay. Results are means ± SEM of triplicate samples. PAA, polyacrylamide. B) Flow cytometry–based assay. H3-P99 (human), H5-Viet (avian origin isolated from a human case), and H10-mallard (avian) viruses were included in the analysis for comparison and as controls. Values at the top right of the dot plots indicate percentage of cells expressing matrix protein 2 (M2). FSC, forward-scattered light; FITC, fluorescein isothiocyanate.

caused by this virus subtype is currently ongoing in Europe (*5*). A study by Beare and Webster showed that ≈50% of volunteers experimentally infected with influenza A(H10N7) virus shed virus (*15*), which our data suggests might be caused by initial attachment to the upper respiratory tract. Immune responses were not detected in these volunteers, and mild, if any, symptoms developed, which indicated limited virus replication.

The low incidence of H10 influenza virus indicates a limited pandemic potential of H10N7 and H10N8 viruses. Therefore, further changes in receptor binding, as well as acquisition of genomic segments from other avian influenza virus strains through co-infection, would be required to increase fitness and transmissibility in mammals. Isolate H10-JD346 amino acid sequence had a mixture of E and K in position 627 of basic polymerase protein 2; the K627

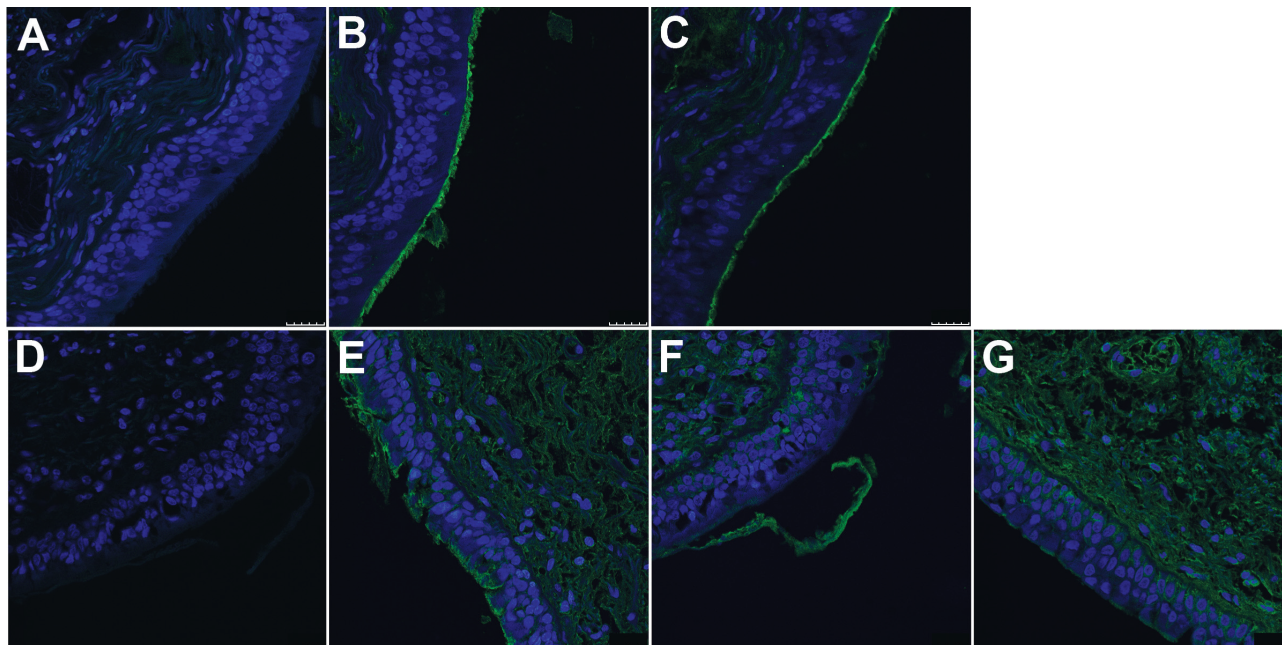

Figure 2. Interaction of hemagglutinin (HA) of H3-P99 (panels B and E) and H10-JD346 (panels C, F, and G) isolates of influenza A(H10N8) viruses with human trachea. Sections from 2 persons are shown (A–C and D–G). A and D, negative control staining (secondary antibody without HA). Blue indicates nuclei stained with 4',6-diamidino-2-phenylindole; green indicates HA binding. Scale bars indicate 25 μm.

mutation is associated with mammal adaptation (*1*). This finding highlights the need for an efficient surveillance network to track and identify possible changes, as well as extensive research to identify them and understand their functional consequences.

Acknowledgments

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Schmallenberg Virus Recurrence, Germany, 2014

Kerstin Wernike, Bernd Hoffmann, Franz J. Conraths, Martin Beer

Schmallenberg virus (SBV) emerged in Germany in 2011, spread rapidly across Europe, and almost disappeared in 2013. However, since late summer 2014, new cases have occurred in adult cattle. Full-genome analysis revealed some amino acid substitution differences from the first SBV sample. Viremia developed in experimentally infected sheep and cattle for 4–6 days.

Schmallenberg virus (SBV), an insect-transmitted orthobunyavirus with a negative-stranded tripartite RNA genome, causes no or only mild nonspecific clinical signs for a few days in adult ruminants (*1*). However, infection of immunologically naive animals during a vulnerable period of pregnancy can cause premature birth, stillbirth, or severe malformations in the offspring (*2*).

SBV was first detected in autumn 2011 in the blood of an acutely infected cow in North Rhine-Westphalia, Germany (*1*). In early 2012, a large number of malformed lambs and calves, which tested positive for SBV, were born in central Europe (*2*). The malformations resulted from infection of the dams in summer or autumn 2011 and transplacental transmission. Within this first vector season, the virus spread rapidly within and between the animal holdings. In the center of the epidemic in northwestern Germany, the Netherlands, and Belgium, >90% of tested cattle became seropositive (*3*). During the following year, SBV circulated again in Germany (*4*) but at a much lower level and predominantly at the margin of the initially most affected area because susceptible animals had remained there.

During the 2013 vector season and the following winter, SBV cases were detected only sporadically (only SBV genome detections without successful virus isolation) (*5*). For example, only 7 cases of viral genome detection were reported to the German Animal Disease Reporting System during January 1–March 24, 2014; these cases resulted from infection of the respective dams during summer or autumn 2013. The high seroprevalence of the ruminant population and the marked decline of births with SBV-associated malformations in newborns raised hopes that SBV had disappeared after the first epidemic, as occurred with the transient appearance of bluetongue virus serotype 8 in the same region of Europe (*6*).

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The Study

Surprisingly, during summer and autumn 2014, SBV reappeared in Germany to a greater extent. Viral genome was repeatedly detected in acutely infected cattle. Several samples from various federal states were submitted to the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Insel Riems, Germany), to confirm an SBV infection (Table) and to further characterize these reemerging viruses.

To evaluate sequence variations among SBV variants circulating in Germany since 2011, the original SBV isolate (BH80/11) and viruses isolated in 2012 from the blood of viremic sheep (BH619/12) or cattle (D495/12-1 and BH652/12) were compared with 3 genomes obtained from the viruses in acutely infected adult cattle in 2014 (BH119/14-1/2, BH119/14-3/4, BH132/14). RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations, and the open reading frames of all 3 genome segments (large [L], medium [M], and small [S] segment; primer sequences available on request) were sequenced as described (*7*). Sequences generated in the current study were submitted to GenBank (accession nos. KP731865–KP731882). Sequence alignments and translation in amino acids were supported by Geneious version 7.1 (Biomatters, Auckland, New Zealand). We generated a maximum-likelihood tree (Hasegawa-Kishino-Yano model, 1,000 bootstrap replicates) using MEGA5 (*8*). For the phylogenetic analysis of the M segment, sequences previously obtained from organ samples from newborns malformed because of SBV infection also were integrated (*7*).

For the S segment (830-nt long), which encodes the nucleocapsid protein (N) and a nonstructural protein (NSs), sequence analysis revealed very high stability. The samples obtained from acutely infected animals in 2014, BH619/12 and BH652/12, were 100% identical to the original SBV strain BH80/11 from 2011. In the sample D495/12-1 from 2012, a single nucleotide was substituted (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/7/15-0180-Techapp1.pdf). The viral RNA-dependent RNA polymerase encoding L segment (6864 nt) also showed high stability, and only a few nucleotide substitutions compared with BH80/11 were found in the latest samples (D495/12-1 and BH652/12: 6 nt; BH619/12: 10 nt; BH119/14-1/2, BH119/14-3/4. and BH132/14: 18 nt). Overall, sequence identity was 99.7%–99.9% (online Technical Appendix Figure).

Table. Origin of Schmallenberg virus real-time reverse transcription PCR–positive samples submitted to the Friedrich-Loeffler-Institut, Germany, 2014

Submission date	Federal state	No. samples	Quantification cycle value
Sep 25	Lower Saxony	3	25.1–28.1
Sep 29	Lower Saxony	1	26.2
Oct 13	North Rhine-Westphalia	1	30.1
Oct 21	Lower Saxony	4	24.1–29.9
Oct 22	North Rhine-Westphalia	2	29.7–30.6
Oct 22	North Rhine-Westphalia	1	31.1
Oct 22	Saxony-Anhalt	1	22.8
Oct 23	Saxony	14	24.1–29.1
Nov 4	Rhineland-Palatinate	4	27.7–38.3
Nov 11	Lower Saxony	1	26.1

Nonsynonymous substitutions ranged from 1 aa to 4 aa (online Technical Appendix Figure).

The M segment (4415-nt long) encodes 2 glycoproteins (Gn and Gc) and a nonstructural protein (NSm). It is the most variable genome segment of SBV and related viruses (*7*,*9*,*10*). Despite the identification of a highly variable region within the Gc-coding sequence in viruses in malformed newborns (*7*,*9*), there was a high sequence stability of the viruses detected in the blood of acutely infected adult animals; all sequences clustered closely (Figure 1). In contrast to the maximum of 77 nt and 43 aa substitutions or 12 aa deletions or 2 aa insertions found in organ samples of lambs or calves (*7*), we detected only 6–12 nt and 2 aa (BH619/12), 3 aa (D495/12-1, BH119/14-3/4, BH132/14) or 4 aa (BH652/12, BH119/14-1/2) substitutions (online Technical Appendix Figure). Because Gn and Gc are major immunogens of orthobunyaviruses (*10*), the mutation hot spot was supposed to be involved in immune evasion mechanisms and/or adaption of the cell tropism within the individual host (*7*,*9*). However, insect-transmitted viruses such as SBV have to adapt to 2 hosts and undergo replication cycles in both the arthropod vector and the mammalian host. Thus, the high sequence stability of virus strains detectable in viremic animals might be necessary for transmission to the vector. Notably, in comparison with the original SBV isolate, K→E substitutions at aa 746 and 1340 of the M segment were found in all other samples. Because these mutations have now been consistently present for at least 2 years, they might have occurred during the adaptation to European ruminants or insects and could provide a growth advantage within the individual host or might be beneficial for transmission between host and vector.

To investigate whether the detected sequence variations correlated with any change in pathogenicity, 5 female sheep and 1 female calf were subcutaneously inoculated with pools of up to 5 serum or whole blood samples from 1 of the holdings with new cases confirmed in 2014 (sheep 1 and 3 and the calf with samples from Lower Saxony; sheep 2, 4, and 5 with samples from Saxony [permit no. LALLF-M-V/TSD/7221.3–1.1–004/12]). None of the animals showed fever or any other clinical sign. Blood samples were taken daily for the first 2 weeks after inoculation and analyzed by real-time reverse transcription PCR (*11*). Thereafter, serum samples were taken in weekly intervals and tested in a microneutralization assay (*12*) and a commercially available SBV antibody ELISA (ID Screen Schmallenberg Virus Competition; IDvet, Grabels, France). The calf and sheep 2–5 became infected; viral genome was detectable in their blood for 4–6 days (Figure 2), which agrees with the short-lived viremia previously observed after experimental infection of cattle and sheep with the original SBV sample or the first cell culture isolate (*1*,*13*). Antibodies were first detected on day 7 after infection (sheep 2, sheep 5) or day 14 after infection (calf, sheep 3, sheep 4) in both tests.

Conclusions

SBV, first detected in 2011, circulated again in Germany in 2014. Virus genome with a high sequence identity to the first SBV sample was repeatedly detected in the blood of acutely infected adult cattle, and in experimentally infected animals, viremia developed that was identical to the original SBV isolate. The renewed virus circulation during the 2014 vector season was observed primarily in an area less affected in the 2 previous years. The missing or markedly reduced virus circulation led to a decline in herd seroprevalence caused by a missing infection of the young stock

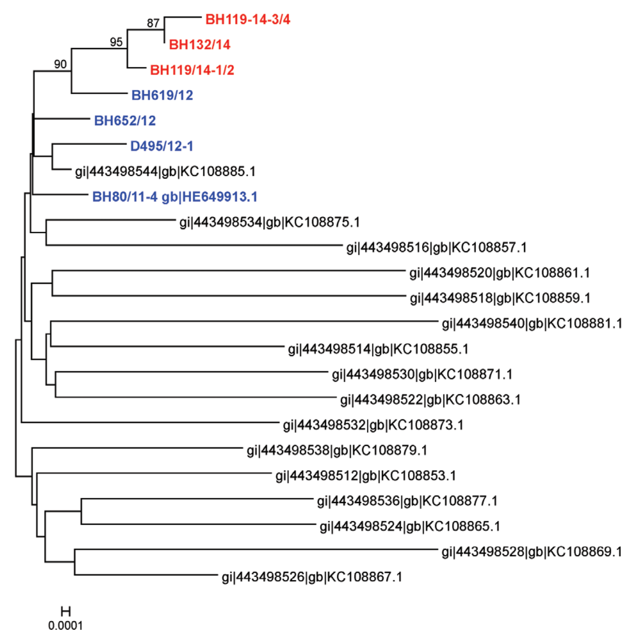

Figure 1. Phylogenetic analysis based on nucleotide sequences of the medium segment of Schmallenberg virus samples isolated from blood of acutely infected animals in 2011 or 2012 (blue) or sequenced directly from the blood of viremic cattle in 2014 (red) and from organ samples of malformed newborns (black) (*7*). Scale bar indicates nucleotide substitutions per site.

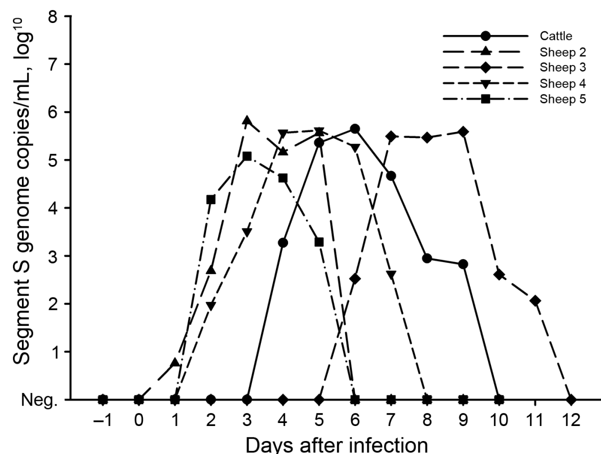

Figure 2. Detection of Schmallenberg virus genome in the blood of experimentally infected cattle and sheep, Germany, 2014.

(*14*,*15*); further reasons for the unexpected recurrence of SBV could be persistence within the insect vectors. As a consequence, the infection of naive animals in autumn 2014 resulted in an increasing frequency of the birth of malformed offspring in the following winter.

Acknowledgments

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Detection of Circovirus in Foxes with Meningoencephalitis, United Kingdom, 2009–2013

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A fox circovirus was identified in serum samples from foxes with unexplained neurologic signs by using viral metagenomics. Fox circovirus nucleic acid was localized in histological lesions of the cerebrum by in situ hybridization. Viruses from the family *Circoviridae* may have neurologic tropism more commonly than previously anticipated.

Circoviruses (family *Circoviridae*) are nonenveloped, single-stranded, circular DNA (≈2 kb) viruses (*1*). Two genera, *Circovirus* and *Gyrovirus*, are recognized, and an additional genus, *Cyclovirus*, has been proposed (*1*,*2*). Circoviruses have an ambisense genome organization with 2 major inversely arranged open reading frames encoding the rolling circle replication initiator protein gene (Rep) and a capsid protein gene (Cap) (*1*). A conserved stem–loop structure, required for viral replication, is located between the 5′ ends of the 2 main open reading frames. Circoviruses are thought to exhibit host species specificity and have been detected in various species, including birds, pigs, and dogs (*1*,*3*,*4*). These viruses have been associated with a variety of diseases, including respiratory and enteric disease, dermatitis, and reproductive problems (*1*,*3*–*5*). Recently, many small circular DNA genomes have been described from different hosts by using different methods, including high-throughput sequencing (*6*). Here we describe the identification, characterization, and prevalence of a newly discovered fox circovirus that was present in serum and brain samples from foxes with unexplained meningoencephalitis in the United Kingdom.

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The Study

During 2009–2013, a total of 31 adult foxes with signs of a neurologic disorder were brought to the RSPCA Norfolk Wildlife Hospital in East Winch, United Kingdom. The foxes exhibited abnormal behavior, lack of fear, reduced alertness, aimless wandering, circling, facial muscle twitching, hind limb paresis, and visual abnormalities. Cases were only detected when free-living foxes became debilitated and were taken to the wildlife rescue center. Once in captivity, diseased foxes had good appetite and generally survived with no substantial disease progression or death, but they showed no evidence of natural recovery. After a few weeks, the foxes were usually euthanized because they did not respond to (nonspecific) medical treatment. All procedures were performed in compliance with relevant laws and institutional guidelines. Following euthanasia, necropsies were performed according to standard procedures. Samples were stored in 10% neutral buffered formalin and embedded in paraffin, and 4 µm–thick sections were stained with hematoxylin and eosin and evaluated for the presence of histologic lesions.

All foxes had similar histologic findings consisting of chronic multifocal or diffuse lymphoplasmacytic meningoencephalitis oriented on the forebrain with a predilection for cortical gray matter (Figure 1; Table 1; online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/7/15-0228-Techapp1.pdf). Characteristic histopathologic features were nonspecific perivascular cuffing, rod cell proliferation, spongiosis, neuronal necrosis, moderate to severe gliosis, neuronal satellitosis, and neurophagia. Substantial pathologic changes were restricted to the central nervous system. Histopathologic changes suggested viral, protozoal, microsporidial, immune-mediated, or idiopathic disease. Immunohistochemistry of brain samples was negative for canine distemper virus, canine adenovirus, Borna disease virus, *Toxoplasma gondii*, and *Neospora canium* (data not shown). Serologic test results for canine distemper virus, rabies virus, *N. canium*, and tickborne encephalitis virus were negative, and Ziehl-Neelsen and Giemsa staining results for microsporidia were negative. Minor white matter involvement, the duration of animal survival, and the current absence of documented rabies cases in the United Kingdom eliminated rabies virus as the cause of the neurologic disorder.

Serum samples from 6 of the foxes (VS7100001–6) were available for virus discovery studies. To perform the

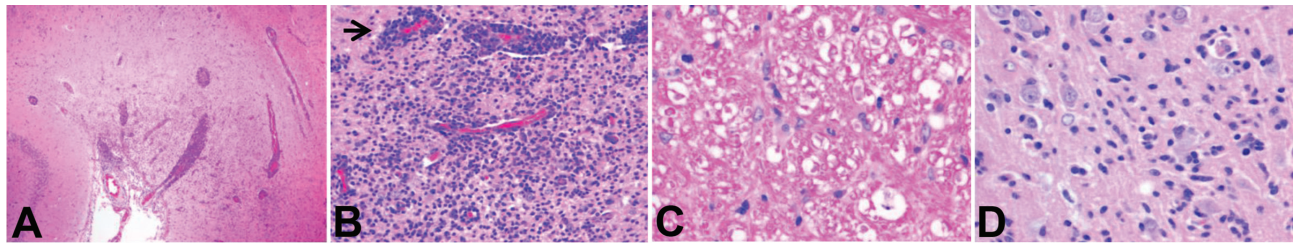

Figure 1. Histopathologic features of brain tissue from foxes with possible virus-induced neurologic disease. A) Multifocal, randomly distributed areas of severe encephalitis and meningitis in the cerebrum (original magnification ×40). B) Detail of encephalitis in the cerebrum (original magnification ×200). Gray and, to a lesser extent, white matter of the cerebrum showed randomly dispersed areas of astrocytosis, gliosis, and infiltration with lymphocytes and plasma cells. Blood vessels in affected areas show perivascular cuffing with distention of Virchow-Robin spaces with up to 10 layers of lymphocytes and plasma cells (arrow). C) Detail of white matter in the cerebellum (original magnification ×400). Axons in affected white matter showed degeneration, characterized by formation of spheroids, shrinkage, and fragmentation; axon sheaths containing microglia or macrophages; and presence of gitter cells in surrounding neuropil. Cerebellum was mildly affected, and meninges, especially of the cerebrum, were frequently distended with lymphocytes and plasma cells. D) Detail of gray matter of cerebrum (original magnification ×400). Individual neuronal cell bodies were frequently surrounded by up to 5 glial cells (i.e., sattelitosis) and showed margination of Nissl substance, hyperchromasia, degeneration, and necrosis. Tissue sections were subjected to conventional hematoxylin and eosin staining.

studies, we used a viral metagenomics approach with the 454 sequence platform (GS Junior; Roche, Basel, Switzerland) as described previously (*7–10*) (Table 1). More than 22,000 reads were analyzed as described previously (*10–12*) (online Technical Appendix Figure 2). The complete genome sequences of circoviruses from 3 foxes were obtained; the sequences were 99% identical at the nucleotide level (GenBank accession nos. KP260925–7). The fox circovirus genomes had an ambisense organization characteristic of circovirus (online Technical Appendix Figure 3). Phylogenetic analysis revealed that the genomes were closely related to those of the recently described canine circoviruses (*3*,*13*), displaying ≈92% amino acid identity in the Rep protein and ≈89% nt sequence identity across the entire genome (online Technical Appendix Figure 4). On the basis of the suggested criteria demarcating species (*1*), the fox and canine circoviruses belong to the same species.

A diagnostic real-time fox circovirus PCR was performed targeting the Rep-coding sequence on 32 serum samples from foxes with and without neurologic signs (Table 1). Viral nucleic acid was extracted by using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Indianapolis, IN, USA) and amplified by real-time PCR by using primers VS756 (5′-TCCGAGATAGCC GGCGTGGTA-3′), VS757 (5′-CCCGGCCACAGATCAAGTACTTA-3′), and VS758 (5′-FAM-ATCCAACTCCGGAGGAGGAGGA-TAMRA-3′) and the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA). In addition to samples VS7100001–6, another 14 fox serum samples were positive for fox circovirus, indicating that the virus had infected foxes in multiple counties in the United Kingdom during past years (Table 1; online Technical Appendix Figure 5). Clinical data indicated that 77% of circovirus-positive foxes had signs of neurologic disease, compared with only 47% of circovirus-negative foxes (Table 2). Fox circovirus was present in male and female foxes and in adults and juveniles (Table 2). In addition, fox circovirus was detected by real-time PCR in brain samples of 2 of 4 foxes with neurologic disease (VS7100017 and 19; cycle threshold value >35) but not in the brain tissues of 2 foxes without disease. The detection of fox circovirus nucleic acid in the cerebrum of foxes with neurologic disease was confirmed by using the RNAscope 2.0 in situ hybridization kit (Advanced Cell Diagnostics, Hayward, CA, USA) and a Rep gene–specific probe according to the manufacturer's instructions. Negative controls consisted of circovirus-negative foxes without histopathologic disease. Multifocal fox circovirus RNA signal was detected and associated with the aforementioned histologic lesions in the cerebrum (Figure 2). Specifically, RNA signal was detected in mononuclear cells in perivascular cuffs, inflammatory infiltrates in the neuropil, and neuronal somata in cerebral gray matter of circovirus-positive foxes with neurologic disease. No circovirus signal was found in control foxes with lymphocytic cuffs due to other (known) viral infections or in control foxes without neurologic disease (Figure 2).

Conclusions

Our findings indicate that circoviruses commonly cause systemic infections in wild foxes in the United Kingdom and can be detected in the brains of foxes with neurologic disease. It has been suggested that circoviruses are involved in a plethora of diseases in pigs, dogs, and birds (*1*,*3–5*). The canine circovirus may be associated with development of vasculitis in dogs (*3*), and an overall virus prevalence in serum samples of ≈3% has been reported (*3*,*13*). However, we found that the prevalence of fox circovirus in serum samples from foxes with and without neurologic disease was much higher and more

Table 1. Overview of testing results for fox serum samples used in a study of the detection of circovirus in foxes with meningoencephalitis, United Kingdom, 2009–2013*

Animal	Year serum sample obtained	Age	Sex	County	Signs†	Outcome	454‡	PCR§	C_t	FFPE
VS7100001	2013	Adult	F	Nor	Yes	Euthanized	Yes	Pos	16.5	Yes
VS7100002	2013	Adult	F	Ess	Yes	Euthanized	Yes	Pos	39.3	No
VS7100003	2013	Adult	F	Nor	Yes	Euthanized	Yes	Pos	16.4	Yes
VS7100004	2013	Adult	M	Suf	Yes	Released	Yes	Pos	35.0	No
VS7100005	2013	Adult	F	Nor	Yes	Euthanized	Yes	Pos	16.4	Yes
VS7100006	2013	Adult	M	Bed	Yes	Euthanized	Yes	Pos	36.9	No
VS7100010	2009	Adult	F	Lei	Yes	Euthanized	No	Pos	23.4	No
VS7100014	2010	Adult	M	Suf	Yes	Euthanized	No	Pos	14.1	Yes
VS7100015	2010	Adult	M	Lin	Yes	Euthanized	No	Pos	14.3	No
VS7100017	2011	Adult	M	Nor	Yes	Euthanized	No	Neg	ND	No
VS7100018	2011	Adult	F	Nor	Yes	Euthanized	No	Neg	ND	No
VS7100019	2011	Adult	F	Cam	Yes	Euthanized	No	Pos	36.8	No
VS7100021	2011	Adult	M	Cam	Yes	Euthanized	No	Pos	25.3	No
VS7100025	2012	Adult	M	Suf	Yes	Euthanized	No	Neg	ND	No
VS7100030	2012	Adult	F	Nor	Yes	Euthanized	No	Neg	ND	No
VS7100032	2012	Adult	M	Ess	Yes	Euthanized	No	Pos	38.0	No
VS7100038	2013	Adult	F	Suf	Yes	Euthanized	No	Pos	17.9	Yes
VS7100008	2007	Adult	M	Cam	No	Euthanized	No	Neg	ND	No
VS7100011	2009	Adult	M	Lin	No	Euthanized	No	Pos	29.7	No
VS7100012	2010	Adult	M	Nor	No	Died	No	Neg	ND	Yes
VS7100020	2011	Adult	M	Cam	No	Euthanized	No	Neg	ND	No
VS7100022	2012	Adult	M	Cam	No	Euthanized	No	Pos	39.5	No
VS7100023	2012	Adult	M	Cam	No	Died	No	Pos	37.3	No
VS7100024	2012	Adult	F	Lin	No	Euthanized	No	Neg	ND	No
VS7100026	2012	Juvenile	F	Lei	No	Released	No	Pos	14.4	No
VS7100027	2012	Juvenile	F	Nor	No	Released	No	Pos	14.6	No
VS7100028	2012	Juvenile	F	Suf	No	Released	No	Neg	ND	No
VS7100029	2012	Juvenile	F	Lin	No	Released	No	Pos	14.2	No
VS7100031	2012	Juvenile	M	Suf	No	Released	No	Neg	ND	No
VS7100033	2012	Juvenile	M	Nor	No	Euthanized	No	Neg	ND	No
VS7100035	2013	Juvenile	F	Cam	No	Released	No	Neg	ND	No
VS7100036	2013	Adult	M	Cam	No	Released	No	Pos	39.2	No

*Bed, Bedfordshire; Cam, Cambridgeshire; C_t, cycle threshold values of real-time PCR; Ess, Essex; FFPE, formalin-fixed paraffin-embedded tissue; Lei, Leicester; Lin, Lincolnshire; ND, not determined; Neg, negative; Nor, Norfolk; Pos, positive; Suf, Suffolk.
†Neurologic signs were abnormal behavior, lack of fear, reduced alertness, aimless wandering, circling, facial muscle twitching, progressive weakness of hind legs, and visual abnormalities.
‡Samples were analyzed by using a viral metagenomics approach with the 454 sequence platform (GS Junior; Roche, Basel, Switzerland).
§TaqMan real-time PCR.

comparable to the prevalence of porcine circoviruses among pigs (*14*). No association of virus infection with vasculitis was apparent. Instead, fox circoviruses may be associated with development of neurologic disease directly or as a contributory complicating cofactor.

Cycloviruses, which belong to a proposed new genus in the family *Circoviridae*, were recently found in serum and cerebrospinal fluid of humans with paraplegia and acute infections of the central nervous system (*11*,*15*), suggesting that viruses from the family *Circoviridae* may have

Table 2. Univariate statistical analysis of age, sex, disease signs, and circovirus real-time PCR results for foxes in a study of the detection of circovirus in foxes with meningoencephalitis, United Kingdom, 2009–2013*

Parameter	PCR results for foxes, no. (%)†		p value by χ^2 test	OR (95% CI)
	Without neurologic signs	With neurologic signs		
All foxes	15 (46.9)	17 (53.1)		
Sex				
M	9 (60.0)	8 (47.1)	0.502	1.69 (0.41–6.88)
F	6 (40.0)	9 (52.9)		
Age, y				
Juvenile	7 (46.7)	0 (0)	0.002	3.13 (1.77–5.53)
Adult	8 (53.3)	17 (100)		
Circovirus positive				
No	8 (53.3)	4 (23.5)	0.144	3.71 (0.82–16.84)
Yes	7 (46.7)	13 (76.5)		

*OR, odds ratio.
†Neurologic signs were abnormal behavior, lack of fear, reduced alertness, aimless wandering, circling, facial muscle twitching, progressive weakness of hind legs, and visual abnormalities.

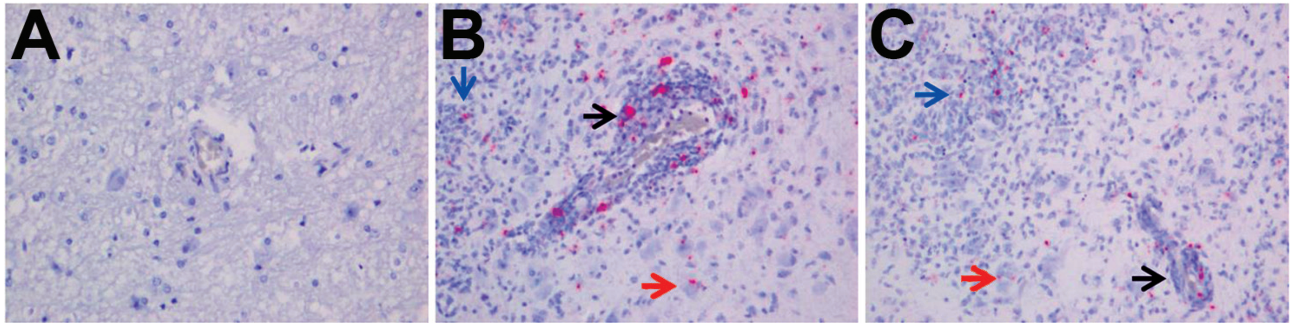

Figure 2. Detection of fox circovirus–specific transcripts in brain tissue of foxes with neurologic disease showing in situ hybridization of cerebrum with fox circovirus replication initiator protein gene–specific probe (original magnification ×200). A) Negative control fox VS7100012. The serum sample from this fox was negative for circovirus, and the animal did not exhibit signs of neurologic disease. B, C) Affected foxes VS7100005 and VS7100003, respectively. Both animals had neurologic disease, and their serum samples were positive for fox circovirus (see Table 1 for more information regarding these foxes). Black arrows indicate mononuclear cells in perivascular cuffs, blue arrows show inflammatory infiltrates in the neuropil, and red arrows point to staining in neuronal somata in cerebral gray matter of circovirus–positive animals with neurologic disease.

neurologic tropism more commonly than previously anticipated. However, a causal link between circovirus infection and disease in humans and animals remains to be proven. Because the prevalence of circoviruses in foxes was relatively high and closely related circovirus species seem pathogenic for both dogs and foxes, additional surveillance is warranted to clarify the epidemiology and pathogenicity of circoviruses in foxes.

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Determination of Predominance of Influenza Virus Strains in the Americas

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During 2001–2014, predominant influenza A(H1N1) and A(H3N2) strains in South America predominated in all or most subsequent influenza seasons in Central and North America. Predominant A(H1N1) and A(H3N2) strains in North America predominated in most subsequent seasons in Central and South America. Sharing data between these subregions may improve influenza season preparedness.

During 2002–2008, infection with influenza viruses caused 40,880–160,270 deaths each year throughout the Americas (*1*). To prevent illness and death, medical staff in 35 countries throughout the Americas administer influenza vaccines (*2*). However, producing the vaccine takes ≈6 months, and selecting virus strains necessitates assessing which strains are likely to predominate during upcoming epidemics (*3*).

Surveillance for influenza has improved dramatically, especially in the American tropics (*4*). Nevertheless, it remains unclear whether virus strains identified in North America subsequently become predominant in South America and vice versa (*3*). Such information could help public health officials in each hemisphere prepare for upcoming influenza seasons. We describe influenza epidemics in North, Central, and South America and explore whether the virus strains that caused them were similar.

The Study

We obtained the number of respiratory swabs tested throughout each year and the number that were positive for influenza virus from the Global Influenza Surveillance and Response System (*5*). Data from Canada, Mexico, and the United States (population 458 million) collected during 2002–2013 were aggregated to represent North America; data from Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panama (population 42 million) to represent Central America; and data from Argentina, Brazil, Chile, Paraguay, and Uruguay (population 262 million) to represent South America (*6*). We obtained antigenic characterization data from the Centers for Disease Control and Prevention (Atlanta, GA, USA).

We determined the proportion of respiratory specimens that tested positive for influenza virus each month in North, Central, and South America and then determined the annual median for each subregion; months in which the proportion exceeded the annual median were considered epidemic (*7*). The timing and length of epidemics in each subregion were also explored, and the proportion of samples testing positive for influenza virus was used as a proxy for epidemic severity. Antigenic virus strains were defined as predominant if they made up the largest proportion of positive samples by type or subtype during an influenza season.

We assessed whether predominant virus strains identified in South America were subsequently identified in Central and North America and whether strains identified in North America were subsequently identified in Central and South America. We also investigated whether predominant strains were represented by components of available Southern or Northern Hemisphere vaccine formulations.

During 2002–2013, South America reported 877,770 influenza-positive respiratory samples (2.8/10,000 persons/y) and North America 4,535,508 results (9.0/10,000 persons/y) to the Global Influenza Surveillance and Response System (*5*). During 2006–2013, Central America reported 82,163 results (2.4/10,000 persons/y). In each subregion, the number of reports increased during the study period ($p = 0.02$). During 2006–2013, the Centers for Disease Control and Prevention analyzed 2,971 samples from South America, 1,279 from Central America, and 25,127 from North America for antigenic characterization.

In South America, influenza epidemics started in April, in Central America in June, and in North America in December. With the exception of 2 (25%) of 8 years in Central America and 2 (17%) of 12 years in South America, when there was 1 southern temperate winter epidemic and a smaller northern temperate winter epidemic, all subregions had 1 annual influenza epidemic that lasted ≈5 months.

The predominant influenza A(H1N1) virus strains in South America predominated in 9 of 9 subsequent seasons

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in Central America and 12 (92%, 95% CI 78%–107%) of 13 subsequent seasons in North America (Table 1; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/7/14-0788-Techapp1.xlxs). Similarly, A(H3N2) virus strains in South America predominated in all 11 (92%, 95% CI 76%–107%) of 12 subsequent seasons in Central America and 10 (71%, 95% CI 48%–95%) of 14 subsequent seasons in North America. Predominant influenza B virus strains in South America only predominated in 8 (67%, 95% CI 40%–93%) of 12 subsequent seasons in Central America and 8 (57%, 95% CI 31%–83%) of 14 subsequent seasons in North America. Virus strains in South America during 1 season typically did not predominate in subsequent seasons in South America (54%, 95% CI 38%–70%).

The predominant A(H1N1) virus strains in North America predominated in 7 (78%, 95% CI 51%–105%) of 9 subsequent seasons in Central America and 10 (83%, 95% CI 62%–104%) of 12 subsequent seasons in South America. A(H3N2) virus strains in North America predominated in 8 (67%, 95% CI 40%–93%) of 12 subsequent seasons in Central America and 10 (77%, 95% CI 54%–100%) of 13 subsequent seasons in South America. Influenza B virus strains in North America predominated in 9 (75%, 95% CI 51%–100%) of 12 subsequent seasons in Central America and 7 (54%, 95% CI 27%–81%) of 13 subsequent seasons in South America. Virus strains that predominated in North America during 1 season were less likely to predominate in the subsequent season in North America (62%, 95% CI 46%–77%).

At least 1 component of the Southern Hemisphere vaccine composition recommendations matched a predominant antigenic characterization in South America in 13 (93%, 95% CI 79%–106% of 14 influenza seasons that occurred during 2001–2014, and at least 1 component of the Northern Hemisphere vaccine composition recommendations matched a predominant antigenic characterization in North America in all 14 influenza seasons that occurred during 2001–2014. Of 33 predominant antigenic virus strains identified in Central America during 2002–2014, 21 (64%, 95% CI 47%–80%) matched the Southern Hemisphere recommendations and 24 (73%, 95% CI 58%–88%) matched the Northern Hemisphere recommendations (Table 2).

Conclusions

Our findings suggest that virus strains identified during influenza epidemics in South America typically became predominant in subsequent epidemics in Central and North America. Almost as frequently, virus strains identified during epidemics in North America became predominant in the subsequent Central and South America epidemics. Although strain selection for 1 hemisphere's vaccine formulation typically occurs before influenza activity is widespread in the opposite hemisphere, health officials have an opportunity to anticipate which influenza virus strains may predominate by observing activity in other subregions. For example, influenza A(H1N1)pdm09 virus predominated in Brazil during 2013 (*8*) and became predominant in North America during 2013–2014. Health officials identifying influenza B virus strains in 1 hemisphere would have correctly predicted the predominant influenza B virus strains in the opposite hemisphere only half of the time unless they had also examined other co-circulating influenza B virus

Table 1. Most commonly identified antigenic characterizations of influenza strains in the Americas during 2001–2014*†

Year	Influenza A(H1N1) virus			Influenza A(H3N2) virus			Influenza B virus		
	South	Central	North	South	Central	North	South	Central	North
2001	A	NA	A	A	NA	A	A	NA	B‡
2002	A	NA	A	A	NA	A	B	B	B
2003	A	NA	A	A	B	B‡	B	NA	C‡
2004	NA	NA	A	B	B	C‡	C	C	C
2005	A	NA	A	C	C	C	D‡	E‡	D
2006	A	A	A	D‡	D	D	E	D/E	D
2007	A	A	B‡	E‡	E	E	F‡	F	F
2008	C‡	C	C	E	E	E	F	F	G‡
2009	D‡	D	D	E	E	F‡	F	G	G
2010	D	D	D	F	F	F	G	G	G
2011	D	D	D	F	F	F	G	G	G
2012	D	D	D	F	F	F	H‡	G	H
2013	D	D	D	G‡	G	H‡	G	I‡	I
2014	D	D	D	H	H	H	I	I	I

*Data from Canada, Mexico, and the United States were aggregated to represent North America; data from Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panama to represent Central America; and data from Argentina, Brazil, Chile, Paraguay, and Uruguay to represent South America.
†For influenza A(H1N1) virus, A, A/New Caledonia/20/99(H1N1); B, A/Solomon Islands/03/2006(H1N1); C, A/Brisbane/59/2007(H1N1); D, A/California/07/2009-(H1N1)pdm09. For influenza A(H3N2) virus, A, A/Panama/2007/99(H3N2); B, A/Fujian/411/2002(H3); C, A/California/07/2004(H3N2); D, A/Wisconsin/67/2005(H3N2); E, A/Brisbane/10/2007(H3N2); F, A/Perth/16/2009(H3N2); G, A/Victoria/361/2011(H3N2); H, A/Texas/50/2012(H3N2). For influenza B virus, A, B/Sichuan/379/99(YAM); B, B/Shandong/7/97(VIC); C, B/Shanghai/361/2002(YAM); D, B/Malaysia/2505/2005(VIC); E, B/Florida/07/2004(YAM); F, B/Florida/04/2006(YAM); G, B/Brisbane/60/2008(VIC); H, B/Wisconsin/01/2010(YAM) I, B/Massachusetts/02/2012(YAM). NA, not applicable.
‡Newly identified strain (8 in South America, 2 in Central America, and 7 in North America).

Table 2. Most commonly identified antigenic characterizations of influenza strains in Central America compared with composition of Southern and Northern hemisphere vaccines, 2002–2014*

Predominant strains in Central America	South America vaccine composition	Matched southern vaccine	North America vaccine composition	Matched northern vaccine
2002				
	A/New Caledonia/20/99(H1N1)-like	NA	A/New Caledonia/20/99(H1N1)-like	NA
	A/Moscow/10/99(H3N2)-like	NA	A/Moscow/10/99(H3N2)-like	NA
B/Shandong/7/97(VIC)	B/Sichuan/379/99-like	–	B/Hong Kong/330/2001-like	–
2003				
A/Fujian/411/2002(H3)-like	A/New Caledonia/20/99(H1N1)-like	NA	A/New Caledonia/20/99(H1N1)-like	NA
	A/Moscow/10/99(H3N2)-like	–	A/Moscow/10/99(H3N2)-like	–
	B/Hong Kong/330/2001-like	NA	B/Hong Kong/330/2001-like	NA
2004				
	A/New Caledonia/20/99(H1N1)-like	NA	A/New Caledonia/20/99(H1N1)-like	NA
A/Fujian/411/2002(H3)-like	A/Fujian/411/2002(H3N2)-like	+	A/Fujian/411/2002(H3N2)-like	+
B/Shanghai/361/2002(YAM)	B/Hong Kong/330/2001-like	–	B/Shanghai/361/2002-like	+
2005				
	A/New Caledonia/20/99(H1N1)-like	NA	A/New Caledonia/20/99(H1N1)-like	NA
A/California/07/2004(H3N2)-like	A/Wellington/1/2004(H3N2)-like	–	A/California/7/2004(H3N2)-like	+
B/Florida/07/2004(YAM)	B/Shanghai/361/2002-like	–	B/Shanghai/361/2002-like	–
2006				
A/New Caledonia/20/99(H1N1)-like	A/New Caledonia/20/99(H1N1)-like	+	A/New Caledonia/20/99(H1N1)-like	+
A/Wisconsin/67/2005 (H3N2)-like	A/California/7/2004(H3N2)-like	–	A/Wisconsin/67/2005 (H3N2)-like	+
B/Malaysia/2505/2005(VIC)	B/Malaysia/2506/2004-like	+	B/Malaysia/2506/2004-like	+
2007				
A/New Caledonia/20/99(H1N1)-like	A/New Caledonia/20/99(H1N1)-like	+	A/Solomon Is/3/2006 (H1N1)-like	–
A/Brisbane/10/2007 (H3N2)-like	A/Wisconsin/67/2005(H3N2)-like	–	A/Wisconsin/67/2005 (H3N2)-like	–
B/Florida/04/2006(YAM)	B/Malaysia/2506/2004-like	–	B/Malaysia/2506/2004-like	–
2008				
A/Brisbane/59/2007(H1N1)-like	A/Solomon Is/3/2006(H1N1)-like	–	A/Brisbane/59/2007 (H1N1)-like	+
A/Brisbane/10/2007(H3N2)-like	A/Brisbane/10/2007(H3N2)-like	+	A/Brisbane/10/2007 (H3N2)-like	+
B/Florida/4/2006(YAM)	B/Florida/4/2006-like	+	B/Florida/4/2006-like	+
2009				
A/California/7/2009(H1N1)-like	A/Brisbane/59/2007(H1N1)-like	–	A/Brisbane/59/2007 (H1N1)-like	–
A/Brisbane/10/2007(H3N2)-like	A/Brisbane/10/2007(H3N2)-like	+	A/Brisbane/10/2007 (H3N2)-like	+
B/Brisbane/60/2008(VIC)	B/Florida/4/2006-like	–	B/Brisbane/60/2008-like	+
2010				
A/California/7/2009(H1N1)-like	A/California/7/2009(H1N1)-like	+	A/California/7/2009 (H1N1)-like	+
A/Perth/16/2009(H3N2)-like	A/Perth/16/2009(H3N2)-like	+	A/Perth/16/2009 (H3N2)-like	+
B/Brisbane/60/2008(VIC)	B/Brisbane/60/2008-like	+	B/Brisbane/60/2008-like	+
2011				
A/California/7/2009(H1N1)-like	A/California/7/2009(H1N1)-like	+	A/California/7/2009 (H1N1)-like	+
A/Perth/16/2009(H3N2)-like	A/Perth/16/2009(H3N2)-like	+	A/Perth/16/2009 (H3N2)-like	+
B/Brisbane/60/2008(VIC)	B/Brisbane/60/2008-like	+	B/Brisbane/60/2008-like	+
2012				
A/California/7/2009(H1N1)-like	A/California/7/2009(H1N1)pdm09-like	+	A/California/7/2009(H1N1)pdm09-like	+
A/Perth/16/2009(H3N2)-like	A/Perth/16/2009(H3N2)-like	+	A/Victoria/361/2011(H3N2)-like	–
B/Brisbane/60/2008-like(VIC)	B/Brisbane/60/2008-like	+	B/Wisconsin/1/2010-like	–
2013				
A/California/7/2009(H1N1)-like	A/California/7/2009(H1N1)pdm09-like	+	A/California/7/2009(H1N1)pdm09-like	+
A/Victoria/361/2011(H3N2)-like	A/Victoria/361/2011(H3N2)-like	+	A/Victoria/361/2011	+
B/Massachusetts/02/2012(YAM)	B/Wisconsin/1/2010-like	–	B/Massachusetts/02/2012-like	+
2014				
A/California/07/2009(H1N1)pdm09-like	A/California/7/2009 (H1N1)pdm09-like	+	A/California/7/2009(H1N1)pdm09-like	+
A/Texas/50/2012(H3N2)-like GP	A/Texas/50/2012 (H3N2)-like	+	A/Texas/50/2012(H3N2)-like	+
B/Massachusetts/02/2012(YAM)	B/Massachusetts/2/2012-like	+	B/Massachusetts/2/2012-like	+
Total percentage matching strains		64%		73%

*Values are proportions of occurrences when predominant strains are represented in each vaccine formulation. +, match; –, no match; NA, influenza type not among predominant circulating strains; Solomon Is, Solomon Islands.

strains. Nevertheless, such findings underscore the importance of year-round surveillance, viral characterization, data sharing, and annual influenza vaccination.

Our analyses are based on a convenience sample of respiratory specimens obtained from heterogeneous surveillance systems using different diagnostic assays (e.g.,

PCR and immunofluorescence) and then aggregated by subregion. These samples may not be geographically representative. Additional data will be needed to determine whether the characteristics of 1 subregion reliably predict influenza epidemics in another. New viral strains that appear might be introduced from outside the Americas (*3*).

In summary, health officials in North and Central America may find clues about which influenza A virus strains are likely to predominate during an upcoming season by observing which were predominant in South America and vice versa. Our findings underscore the need to share timely and representative specimens with World Health Organization Collaborating Centres. In the future, shorter vaccine production times using novel technology might facilitate matching vaccine composition more closely to circulating virus strains.

This paper is dedicated to the memory of Alexander Klimov.

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Novel Arenavirus Isolates from Namaqua Rock Mice, Namibia, Southern Africa

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Arenaviruses are feared as agents that cause viral hemorrhagic fevers. We report the identification, isolation, and genetic characterization of 2 novel arenaviruses from Namaqua rock mice in Namibia. These findings extend knowledge of the distribution and diversity of arenaviruses in Africa.

Arenaviruses are known to cause severe hemorrhagic fevers across the globe with case fatality rates up to 30% (*1*). The viruses possess a bisegmented, single-stranded RNA genome with ambisense coding strategy consisting of a small segment coding for the nucleoprotein and glycoprotein and a large (L) segment coding for the RNA-dependent RNA polymerase and matrix protein.

In Africa, Lassa virus (LASV) and Lujo virus are the only known members of the family *Arenaviridae* that cause human disease (*2*,*3*); however, evidence for lymphocytic choriomeningitis virus, another *Arenaviridae* sp., was recently reported in Gabon (*4*). Several other arenaviruses of unknown pathogenic potential have also been found in Africa: Gbagroube, Kodoko, and Menekre viruses from western Africa (*5*,*6*); Ippy (IPPYV) and Mobala viruses from central Africa; Mopeia, Morogoro, Luna, and Lunk viruses from eastern Africa; and Merino Walk virus (MWV) from southern Africa (*7,8*). All of these viruses are carried by rodents of the family *Muridae*.

Until now, no molecular detection of arenaviruses has been reported from Namibia. A study in 1991 described a low seroprevalence (0.8%) for LASV antibodies in humans in northern Namibia (*9*). Because of lack of data about arenavirus occurrence and effects in southwestern Africa, we conducted a study of small mammals from Namibia to detect infection with arenaviruses.

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The Study

During 2010–2012, animal trapping was performed in 8 areas in central and northern Namibia (Figure 1), and samples from 812 rodents and shrews were obtained (Table 1). The animals were dissected in the field and stored individually in a field freezer at –20°C and later at −80°C. For primary arenavirus screening, lung sections of all animals were homogenized, and RNA was extracted and reversely transcribed by using random hexamer primers. Screening was performed by arenavirus genus-specific reverse transcription PCR (RT-PCR) (*10*) to detect the L genomic segment. From samples testing positive by

Figure 1. Screening for arenaviruses in Namibia. Trapping locations (named according to the nearest urban settlement) of small mammals. Sites where samples positive for new arenaviruses were found are marked by a crossed circle and underlined locality names. Geographic positioning system coordinates of the trapping sites: Ben Hur, 22°87.26′S, 19°21.10′E; Cheetah Conservation Fund (CCF), 16°39.0′E, 20°28.12′S; Mariental, 24°62.08′S, 17°95.93′E; Okahandja, (21°98.33′S, 16°91.32′E); Palmwag, 19°53.23′S, 13°56.35′E; Rundu, 17°56.645′S, 20°05.109′E; Talismanis, 21°84.30′S, 20°73.91′E; Windhoek, 22°49.93′S, 17°34.76′E.

Table 1. Small mammals captured in Namibia during 2010–2012 and tested for arenaviruses*

Mammal species	Common name	Localities of capture†	No. positive/no. tested
Aethomys chrysophilus	Red veld rat	Be, CCF, Ok, Pa, Ta	0/64
Micaelamys namaquensis	Namaqua rock mouse	CCF, Ma, Ok, Pa, Ru	4/266
Crocidura fuscomurina	Bicolored musk shrew	CCF, Pa, Ru	0/4
Crocidura hirta	Lesser red musk shrew	Ma	0/5
Dendromus melanotis	Gray climbing mouse	Ta	0/1
Elephantulus intufi	Bushveld sengi	CCF, Ma, Ok	0/14
Gerbilliscus spp.	Gerbil	Wi	0/6
Gerbilliscus leucogaster	Bushveld gerbil	Be, CCF, Ma, Ok, Pa, Ru, Ta	0/228
Gerbillurus paeba	Hairy-footed gerbil	Be	0/3
Gerbillurus setzeri	Namib brush-tailed gerbil	Be	0/1
Lemniscomys rosalia	Single-striped grass mouse	Be	0/2
Mastomys spp.	Multimammate mouse	Be, CCF, Ma, Ok, Pa, Ru, Ta	0/114
Mus indutus	Desert pygmy mouse	Ma, Pa	0/5
Petromyscus collinus	Pygmy rock mouse	Pa	0/3
Rhabdomys pumilio	Four-striped grass mouse	CCF, Ma, Pa, Ok, Wi	0/73
Saccostomus campestris	Pouched mouse	Be, CCF, Ok, Pa, Ru	0/17
Thallomys paedulcus	Acacia rat	Pa	0/4
u.u. Soricidae	Shrew	Wi	0/2
Total			4/812

*Morphologic species identification of the arenavirus positive rodent samples was confirmed by sequencing of partial mitochondrial cytochrome b gene (GenBank accession nos.: N27, KP752173; N73, KP752175; N80, KP752176; N85, KP752174).
†Abbreviations and sampling dates for trapping localities: Be, Ben Hur (11/2011); CCF, Cheetah Conservation Fund (02/2011); Ok, Okahandja (06/2012), Pa, Palmwag (09/2010), Ta, Talismanis (12/2011), Ma, Mariental (06/2012), Ru, Rundu (01/2011),Wi, Windhoek (09/2010 and 01/2012).

arenavirus PCR, frozen lung tissue aliquots were homogenized and added to confluent Vero-E6 cells (ATCC CRL-1586; American Type Culture Collection, Manassas, VA, USA) for virus isolation.

For genome sequencing, pellets from ultracentrifuged supernatant of infected cell cultures were lysed, and total RNA was purified. RNA was then subjected to random-primed RT-PCR as described (*11*). Next-generation sequencing was performed by using a 454 Genome Sequencer Junior (Roche, Indianapolis, IN, USA), and results were aligned against the virus database by using blastn and blastx algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequencing results matching arenavirus sequences were mapped to the LASV strain Josiah (GenBank accession no. AY628203). Because of low coverage for N27, an additional MiSeq (Illumina, San Diego, CA, USA) run was performed. De novo assembly of the data was performed with Geneious software (Biomatters, Auckland, New Zealand) (*12*). Sequence gaps or regions with low coverage were verified by Sanger sequencing (Applied Biosystems, Foster City, CA, USA). Genome segment outermost noncoding termini were sequenced after linkage by T4-RNA-Ligase (New England Biolabs, Ipswich, MA, USA) and RT-PCR amplification.

Of the 812 rodents and shrews tested (Table 1), arenavirus RNA was found in lung tissue samples of 4 Namaqua rock mice (*Micaelamys* [*Aethomys*] *namaquensis*), 3 from Okahandja (N73, N80, N85) and 1 from Mariental (N27). Sanger sequencing of PCR products from a 338-nt fragment of the viral polymerase gene confirmed arenavirus-like origin. Initial phylogenetic analysis showed that the Okahandja specimens were related to MWV, but the sample from Mariental was a highly divergent member of the genus *Arenavirus* (Figure 2, panel A). Cell culture isolation was performed with samples N27 and N73 and resulted in 2 novel arenavirus isolates: Mariental virus (MRTV) and Okahandja virus (OKAV), respectively.

The genomes of the 2 arenaviruses were investigated by using next-generation sequencing and RT-PCR Sanger sequencing. The genome data obtained for MRTV and OKAV showed a typical arenavirus nucleotide composition with the L segment (MRTV: 6,840 nt; OKAV: 7,170 nt) coding for RNA polymerase and matrix protein and the S segment (MRTV: 3,360 nt; OKAV: 3,379 nt) coding for glycoprotein and nucleocapsid protein. Table 2 shows the nucleotide and amino acid sequence identities of nucleocapsid and glycoprotein open reading frame with other Old World (i.e., Eastern Hemisphere locations such as Europe, Asia, Africa) representatives of genus *Arenavirus*. On the basis of the nucleocapsid amino acid identity, OKAV is most related to MWV (75.7% identity). Furthermore, MRTV has the highest amino acid identity with IPPYV (71.4% identity) and with Gbagroube, Lassa, Luna, and Mobala viruses (≈70% identity).

In the nucleocapsid-based phylogenetic tree, OKAV clusters with 100% bootstrap support with MWV detected in *Myotomys unisulcatus* rodents in South Africa (Figure 2, panel B), and MRTV forms a clade with IPPYV isolated from *Praomys* spp. in the Central African Republic. The bootstrap support of this monophyletic group of the tree lies at 56%. The analysis of the glycoprotein open reading frame (Figure 2, panel C) leads to a similar result; OKAV shares the most recent common ancestor with MWV, and MRTV clusters with IPPYV but with a weaker bootstrap support.

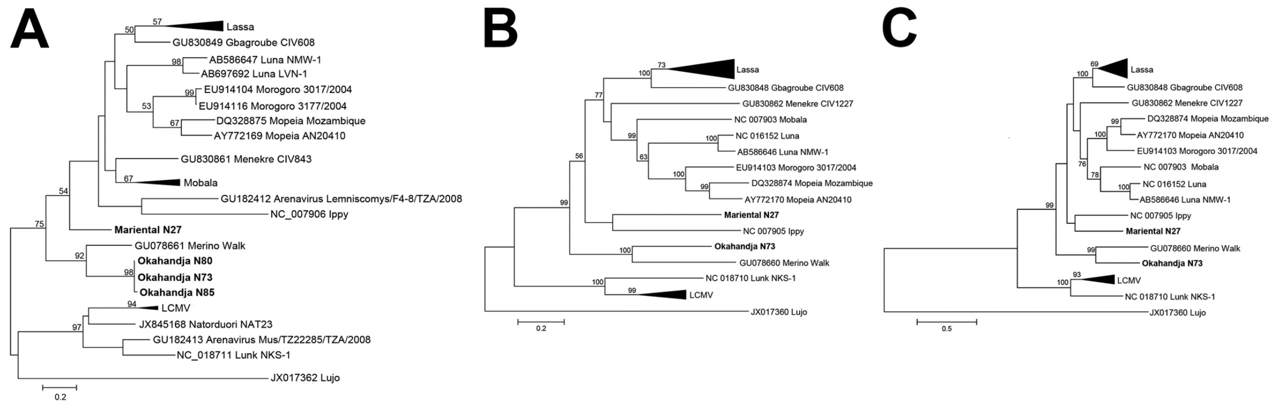

Figure 2. Phylogenetic analysis of Okahandja and Mariental viruses performed with maximum-likelihood method. A) Phylogenetic analysis of partial L segment sequence (338 nt) of Okahandja and Mariental viruses obtained from reverse transcription PCR screening and performed with MEGA 6.0 (*13*) with maximum-likelihood method (general time reversible plus gamma model with 7 discrete Gamma categories; 1,000 bootstrap replications). Values at the branches are bootstrap values of the corresponding neighbor-joining tree (maximum composite likelihood method); values <50% are not shown. Scale bar indicates an evolutionary distance of given substitutions per position in the sequence. B) Nucleocapsid open reading frame. C) Glycoprotein open reading frame. Scale bars indicate evolutionary distances of given substitutions per position in each sequence. LCMV, lymphocytic choriomeningitis virus.

Conclusions

We detected and isolated 2 novel arenaviruses in Namibia, OKAV and MRTV. OKAV clearly clustered in relationship with the MWV from southern Africa, but MRTV is a more divergent member of the Old World arenavirus clade. According to amino acid identity and phylogenetic analysis, MRTV was most closely related to IPPYV from the Central African Republic; however, the low bootstrap support precluded a stringent estimation of this closest relative.

These new strains comply with the arenavirus species definition (*14*) on the basis of amino acid differences in

Table 2. Nucleotide and amino acid identities of Mariental (MRTV) and Okahandja (OKAV) viruses compared with Old World representatives of the genus Arenavirus*

Virus species	S segment GenBank accession no.	GPC		NP	
		nt	aa	nt	aa
MRTV (N27)†	KM272987				
OKAV (N73)‡	KM272988	64.6	68.9	64.9	66.1
Gbagroube	GU830848	66.7	73.6	64.1	69.9
Ippy	NC_007905	66.4	**73.7**	64.1	**71.4**
Lassa	AY628203	67.4	73.2	65.5	69.8
LCMV	AB261991	57.4	57.2	61.1	63.6
Lujo	JX017360	47.7	38.2	60.1	56.7
Luna	AB586646	66.4	73.2	64.2	69.5
Lunk	NC_018710	57.4	54.1	61.2	62.5
Menekre	GU830862	66.5	72.3	65.1	68.3
Merino walk	GU078660	63.8	70.2	64.9	67.3
Mobala	NC_007903	63.8	72.1	64.6	70.5
OKAV (N73)‡	*KM272988*				
Gbagroube	GU830848	62.0	66.3	61.1	65.7
Ippy	NC_007905	62.9	69.4	62.3	66.2
Lassa	AY628203	64.6	68.2	60.8	65.9
LCMV	AB261991	58.9	57.1	62.0	63.6
Lujo	JX017360	47.6	38.4	60.0	57.8
Luna	AB586646	62.5	67.1	63.0	67.2
Lunk	NC_018710	56.1	55.1	60.6	62.6
Menekre	GU830862	63.7	70.4	62.9	65.9
Merino walk	GU078660	64.7	**76.1**	68.2	**75.7**
Mobala	NC_007903	62.0	66.5	63.6	64.5

*Identities are shown for glycoprotein and nucleocapsid open reading frames. Highest aa identity values are shown in **boldface**. S, small; GPC, glycoprotein; NP, nucleocapsid protein; nt, nucleotide; aa, amino acid; LCMV, lymphocytic choriomeningitis virus; L, large segment.
†Genome composition of MRTV (N27): (Z: 69–371; RdRP: 6,820–473; GPC: 49–1,527; NP: 3,297–1,710). Accession numbers for MRTV (N27) virus sequences: L, KP867641; S, KM272987.
‡Genome composition of OKAV (N73): Z, 58–336; RdRP, 7,121–435; GPC, 51–1,553; NP, 3,315–1,627. Accession numbers for virus sequences for OKAV N73: L, KP867642; S, KM272988; for OKAV N80: L, KM234277; for OKAV N85: L, KM234278.

nucleocapsid of ≥12% (>20% for both viruses), presence of specific host species, and existence of laboratory isolates. These properties indicate that MRTV and OKAV represent distinct arenavirus species.

These 2 viruses were found in the same host species located within a radius of 300 km. MRTV was found in only 1 sample (of 266); OKAV was detected in samples from 3 animals. Although more unlikely for OKAV than for MRTV, the possibility of a spillover infection to *M. namaquensis* from a still unknown reservoir host cannot be ruled out for either virus.

The Namaqua rock mouse's habitat includes the tree and shrub savannahs of Namibia and most parts of southern Africa, including Namibia, South Africa, Botswana, Zimbabwe, and parts of Mozambique (*15*). These locations imply the possible occurrence of MRTV or OKAV in larger regions of the continent. Cell culture isolates and genomic sequence data are the first prerequisites for evaluating the public health relevance of these new viruses. Our findings extend the knowledge of geographic distribution and genetic diversity of arenaviruses in Africa.

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Readability of Ebola Information on Websites of Public Health Agencies, United States, United Kingdom, Canada, Australia, and Europe

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Public involvement in efforts to control the current Ebola virus disease epidemic requires understandable information. We reviewed the readability of Ebola information from public health agencies in non–Ebola-affected areas. A substantial proportion of citizens would have difficulty understanding existing information, which would potentially hinder effective health-seeking behaviors.

The outbreak of Ebola virus disease (EVD) that originated in Guinea in April 2014 has become the largest known epidemic of this pathogen and was declared an international public health emergency (*1*). In addition, repatriation of health care workers and volunteers to Europe and the United States has resulted in human-to-human transmission in western health care organizations (*2*), thus bringing Ebola to the fore of public attention in settings far removed from local outbreak areas.

Currently, because there is no antiviral treatment or vaccine, surveillance and strict observation of recommended infection prevention and control measures, aided by public awareness regarding symptoms and prompt health care–seeking behavior, are essential efforts to control Ebola. In Africa, low awareness has led to community misunderstandings and unwillingness to cooperate with medical teams (*3*). In non–EVD-affected countries, nonrigorous information has resulted in unfounded fear among health care workers and citizens, disrupting the activity of hospitals caring for persons with EVD (*4*).

For health messages to be followed effectively, they must be tailored to the health literacy of the audience. Health literacy, which refers to "the cognitive and social skills which determine the motivation and ability of individuals to gain access to, understand and use information in ways which promote and maintain good health" (*5*), has been associated with better self-care (*6*). However, a substantial proportion of citizens worldwide have insufficient or inadequate health literacy (*7*).

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Several factors, including readability of information provided (*8*), can help reduce health literacy deficits. Readability refers to "the determination of the reading comprehension level a person must have to understand written materials" (*9*). It is recommended that health information materials should be written at a level typically understandable by an 11-year-old person (*10*). Such recommendations for clarity and understandability might be more effective if one considers that anxiety or panic attributed to a highly virulent infection, such as Ebola, might hinder comprehension of related information (*11*).

We examined readability of EVD public information available from selected public health agencies in non–EVD-affected countries. Countries that have EVD should explore how well this information would serve to reduce panic and anxiety and perform as an effective source of advice for the public.

The Study

Current information on Ebola aimed at the public was downloaded from various websites; a list is provided in online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/7/14-1829-Techapp1.pdf). Information was retrieved from the European Centre for Disease Control (Ebola factsheet for the general public); the US Centers for Disease Prevention and Control (CDC; Questions and answers on Ebola); Public Health England (PHE) in the United Kingdom (Ebola: public health questions and answers); and the government of Canada (Ebola virus disease) on September 1, 2014 and from the government of Australia (Ebolavirus disease outbreaks in West Africa: important information for travellers, patients and consumers) and the World Health Organization (WHO; Advice for individuals and families. Ebola guidance package) on November 11, 2014.

Any figures, such as maps or pictograms, were removed, and content was then formatted as plain text and uploaded to a free online tool (http://www.readability-formulas.com/free-readability-formula-tests.php/) from which different readability indicators were obtained (online Technical Appendix). The causes, symptoms, risks, treatment, prevention, and surveillance pages in the Canadian website were individually opened and

analyzed. We calculated measures of central tendency and dispersion for scores obtained in indicators reported by using Stata version 10.1 (StataCorp LP, College Station, TX, USA).

Results are shown in the Table. In terms of reading difficulty, mean Flesch Reading Ease score for all information was 48.85 (SD 7.76; 95% CI 40.69–57.00) and indicated difficult to read. WHO information was easiest to read (score 62.3); information from Australia was most difficult to read (score 42). Mean Gunning FOG Index was 12.6 (SD 1.68; 95% CI 10.83–14.36) and indicated difficult to read. Again, written content from WHO and Australia were at the easiest and most difficult reading levels, respectively.

Factsheets from PHE and Canada required a 12th US school grade reading level to be understood, and the CDC and WHO factsheets required a 9th US school grade reading level. Comparable results were obtained with Coleman-Liau Index and the SMOG (simple measure of gobbledygook) formula. The Automated Readability Index for all materials was 10.7 (SD 1.97; 95% CI 8.62–12.77) and required an age of 15–16 years to understand the text. Finally, information from PHE and Australia was written at the most demanding level according to the Linsear Write Formula (score 14.1, or college level), and the CDC content required an 8th US grade reading level for comprehension (score 8.4). The mean result for all content was 11.95 (SD 2.42, 95% CI 9.40–14.49).

Conclusions

Our analyses indicate that the information on EVD provided on websites of different public health agencies is written at a higher than recommended reading level. For such a reason, a substantial proportion of citizens with low literacy in the United States, United Kingdom, Canada, Australia, and Europe would have difficulty understanding key EVD messages. These results are of concern because poor readability might prevent or delay adoption of appropriate health-seeking behaviors, prolong ineffective self-care strategies, and perpetuate stigmatizing attitudes toward Ebola.

Providing adequate EVD information for the public might be arduous. Uncertainties remain regarding optimal clinical management for Ebola patients and disagreements in infection prevention and control protocols. The continued modification of procedures also demands constant public engagement efforts to avoid dissemination of conflicting messages and to ensure that information released is up to date and presented at a level that can be adequately understood. Because there have been limited national communication campaigns in non–EVD-affected countries, it is likely that other outlets, including traditional mass media and social media, might have been used by the public to meet their information needs (*13*), with probable trade-offs between immediacy and accuracy or reliability of information provided. The variation of readability identified in our study suggests that with contributions from health literacy specialists, public health agencies could further adapt the EVD information provided.

We recognize that persons accessing health information online are not representative of the average population because they are more educated and benefit from better information-seeking skills and health literacy (*14*). Thus,

Table. Readability of Ebola public information published by selected public health agencies*

Readability formula	Selected website: ECDC (20.0)†	PHE (16.40)†	CDC (17.49)†	Government of Canada (16.38)†	WHO (NA)	Government of Australia (12.55)†	Mean ± SD (95% CI)
Gunning Fog Index	13.7 (hard to read)	13.9 (hard to read)	10.7 (hard to read)	12.9 (hard to read)	10.3 (fairly easy to read)	14.1 (hard to read)	12.6 ± 1.68 (10.83–14.36)
Flesch Reading Ease Score	48.2 (difficult to read)	45.4 (difficult to read)	53 (fairly difficult to read)	42.2 (difficult to read)	62.3 (standard/avg)	42 (difficult to read)	48.85 ± 7.76 (40.69–57.00)
Automated Readability Index	11.6 (17–18 y old)	12.5 (18–19 y old)	7.8 (12–14 y old)	11.8 (17–18 y old)	8.6 (13–15 y old)	11.9 (17–18 y old)	10.7 ± 1.97 (8.62–12.77)
Coleman-Liau Index	12 (12th grade)	12 (12th grade)	10 (10th grade)	13 (college)	9 (9th grade)	11 (11th grade)	11.16 ± 1.47 (9.62–12.71)
SMOG Index	10.7 (11th grade)	11 (11th grade)	9.4 (9th grade)	11.1 (11th grade)	8.4 (8th grade)	11.5 (12th grade)	10.35 ± 1.19 (9.09–11.60)
Linsear Write Formula	13 (college)	14.1 (college)	8.4 (8th grade)	12.6 (college)	9.5 (10th grade)	14.1 (college)	11.95 ± 2.42 (9.40–14.49)
Flesch-Kincaid US Grade Level	11.3 (11th grade)	12.1 (12th grade)	9.2 (9th grade)	11.8 (12th grade)	8.8 (9th grade)	12.4 (12th grade)	10.93 ± 1.54 (9.31–12.55)

*ECDC, European Centre for Disease Control; PHE, Public Health England; CDC, US Centers for Disease Control and Prevention; WHO, World Health Organization; NA, not applicable; avg, average; SMOG, simple measure of gobbledygook. Items in parentheses are general assessments, age levels, or US-equivalent grade levels.
†Percentage of adults 16–65 years of age with literacy proficiency below reading level recommended for health information materials. ECDC percentage refers to a sample of 17 European Union Member States (*12*).

the online audience might be able to make more effective use of information on websites analyzed. However, such might not be the case for persons whose first language is not English, who might find information provided even more difficult to understand because of linguistic and cultural barriers.

It is accepted that readability measures alone may not reflect the level at which information is written (*15*). Because the Ebola epidemic has continued since our analysis, it might be possible for currently available information to have been modified and display greater readability. Our analysis was not exhaustive because we assessed selected public health agencies in non–EVD-affected countries and concentrated in English language materials. Therefore, our findings might not be representative of all health pages with EVD information. However, we evaluated key official websites.

Public health agencies in non–EVD-affected countries must improve the readability of EVD information currently provided so that the public could adopt effective self-care strategies, avoid fear, and reduce unnecessary panic and stigma toward persons affected by Ebola. In addition, agencies should consider multimodal Ebola awareness campaigns, including social marketing interventions, to encourage and strengthen public participation in Ebola control efforts.

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E.C.-S. was responsible for the design of the study and collected data. A.H.H. provided technical input during all stages of the project and analysis. All authors were responsible for data analysis, contributed substantially to writing the manuscript, approved its final version, had full access to all data in the study, and take responsibility for the integrity, accuracy, and presentation of data. E.C.-S. is the guarantor.

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Evaluation of Patients under Investigation for MERS-CoV Infection, United States, January 2013–October 2014

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Middle East respiratory syndrome (MERS) cases continue to be reported from the Middle East. Evaluation and testing of patients under investigation (PUIs) for MERS are recommended. In 2013–2014, two imported cases were detected among 490 US PUIs. Continued awareness is needed for early case detection and implementation of infection control measures.

Middle East respiratory syndrome coronavirus (MERS-CoV) infection was first reported in September 2012 in a patient with fatal pneumonia in Saudi Arabia (*1*). Subsequent investigation showed that earlier MERS-CoV infection had occurred in Jordan in April 2012 among a cluster of patients with pneumonia (*2*,*3*). As of February 5, 2015, the World Health Organization had reported 971 laboratory-confirmed cases worldwide and at least 356 related deaths (*4*). All known reported cases have been linked directly or indirectly to the Middle East region; most have been reported by Saudi Arabia and the United Arab Emirates. Typically, the initial symptoms for MERS patients seeking medical care are fever, chills, cough, shortness of breath, and myalgia. These symptoms often progress to severe lower respiratory tract infection, which may require mechanical ventilation and intensive care (*5*,*6*). Several asymptomatic or mild MERS cases have been reported (*7*), particularly in healthy young adults. Little is known about transmission routes, virus shedding, risk factors, and animal reservoirs, although bats and camels have been implicated in transmission and as reservoirs (*8*,*9*). Clusters of human-to-human transmission have been associated with household and health care settings (*2*,*3*,*5*).

Using World Health Organization guidelines and definitions (*4*), CDC developed guidance for evaluating a patient under investigation (PUI) for MERS-CoV infection, collecting specimens, conducting laboratory testing, and managing infection control (http://www.cdc.gov/coronavirus/mers/index.html). The PUI guidance was created to assist health care providers determine which patients should be considered for MERS-CoV evaluation and testing. To inform state and local health departments of the basic demographic and clinical characteristics of PUIs and on assay use, we summarized the descriptive analysis of PUIs in the United States.

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The Study

In October 2012, CDC developed real-time reverse transcription PCR (rRT-PCR) assays for detection of MERS-CoV (*10*). CDC initially performed the testing, but on June 5, 2013, a Food and Drug Administration–issued Emergency Use Authorization allowed for assay deployment in a kit to laboratories through the Laboratory Response Network. As of March 12, 2015, a total of 47 states and the District of Columbia had MERS-CoV testing capability. The assay kit is intended for detection of MERS-CoV RNA in respiratory, serum, and stool samples. CDC also developed serologic tests for detecting MERS-CoV antibodies; these tests have been used by CDC since the summer of 2013. Because MERS-CoV is an emerging pathogen, CDC guidelines and guidance regarding PUI characteristics are periodically updated as new MERS-CoV information and risk factors are identified. CDC recommends evaluating and testing PUIs for MERS-CoV and for other common respiratory pathogens.

On January 1, 2013, CDC began collecting data on PUIs for MERS-CoV infection. Health care providers for persons suspected of having MERS were to contact their state or local health department for consultation and to arrange for MERS-CoV testing, if indicated. PUIs were reported to CDC through state and local health departments by using the single-page PUI short form, which contains no personal identifiers (*11*). Since its implementation, the short form has been revised 3 times to reflect modifications to the PUI guidance. The short form collects information on basic demographic data, symptoms, disease severity, hospitalization, travel history, risk factors, and laboratory test results at the time of MERS-CoV testing. Follow-up data collection on missing information was not routinely performed. At least 370 (76%) PUIs met the guidance characteristics for PUI for MERS. The remaining 120 (24%) PUIs had incomplete clinical or travel data; the most common missing information was pneumonia data for persons with respiratory symptoms and a recent travel history. The short form was sent electronically to CDC by secure fax or email. Data collected on the short form was entered into a

CDC database by using Microsoft Access (Microsoft Corporation, Redmond, WA, USA). SAS version 9.3 (SAS Institute, Cary, NC, USA) was used for data analysis.

During January 1, 2013–October 31, 2014, a total of 490 PUIs were reported to CDC from 45 states and the District of Columbia (Figures 1, 2; Table 1). Of the 490 PUIs, 381 (78%) reported traveling from the Arabian Peninsula or neighboring countries to the United States within 14 days before illness onset (Table 2). A total of 113 (23%) PUIs also reported having close contact with a recently ill traveler from the Arabian Peninsula or neighboring countries within 14 days of symptom onset; the most common contacts were with persons from Saudi Arabia (55/113 [49%]), United Arab Emirates (10/113 [9%]), and Qatar (9/113 [8%]). Non-US residents accounted for 113 (23%) of the PUIs.

The most commonly reported symptoms were cough, fever, and shortness of breath (Table 1). A total of 292 (60%) PUIs were hospitalized, of whom 112 (38%) were admitted to the intensive care unit and 61 (21%) required mechanical ventilation. The most commonly reported underlying conditions among PUIs were immunosuppression and diabetes. Eleven (2%) PUIs died.

In total, 488 PUIs tested negative for MERS-CoV by rRT-PCR, serologic assay, or both. In May 2014, two PUIs tested positive for MERS-CoV by serologic assay and rRT-PCR in serum and respiratory samples, including lower respiratory tract specimens. These 2 patients were health care providers in whom respiratory symptoms had developed within 14 days of travel from Saudi Arabia; both cases were identified as imported MERS (*12*,*13*). Neither patient required mechanical ventilation.

The most commonly detected pathogens among the 490 PUIs were influenza A virus and rhinovirus/enterovirus; however, for 359 PUIs (73%), other pathogen testing was not performed or detected pathogens were not reported (Table 1). Timely reporting of PUIs to CDC may have influenced reporting of pending non–MERS-CoV etiologic pathogen test results.

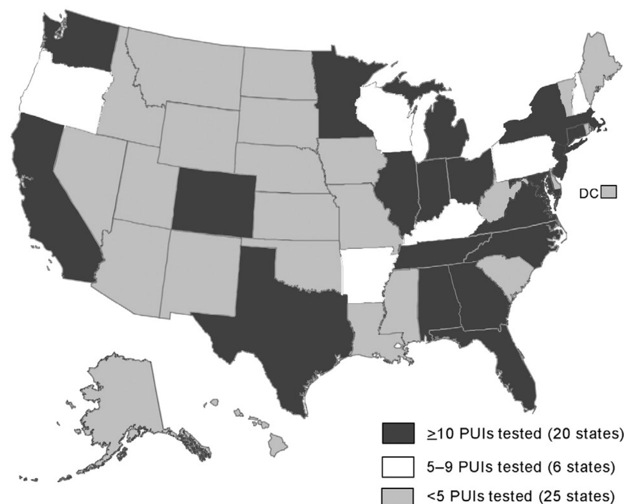

Figure 1. Number of PUIs tested for MERS-CoV (N = 490), by state, United States, January 1, 2013–October 31, 2014. DC, District of Columbia; PUIs, patients under investigation.

Conclusions

Currently in the United States, the preferred method for detecting MERS in PUIs with recent symptom onset is to test lower respiratory, naso-oropharyngeal, and serum specimens by using the CDC rRT-PCR assay. For PUIs in whom symptom onset occurred ≥2 weeks before specimen collection, testing using the CDC MERS-CoV

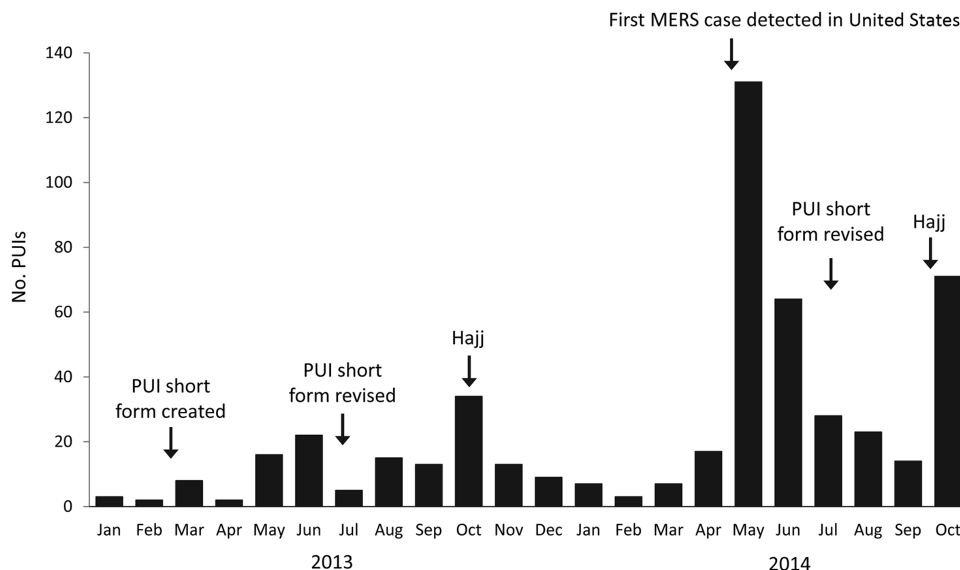

Figure 2. Number of PUIs tested for MERS-CoV (N = 490), by month reported, United States, January 1, 2013–October 31, 2014. PUIs, patients under investigation.

Table 1. Characteristics of 490 PUIs for MERS-CoV, United States, January 1, 2013–October 31, 2014*

Characteristic	Value
Sex, M/F	296 (60.4)/186 (38.0)
Median age, y (range)	48 (0.3–89)
Symptom	
Cough	393(80.2)
Fever	388 (79.2)
Shortness of breath	220 (44.9)
Chills†	153 (35.1)
Myalgia†	140 (32.1)
Sore throat	134 (27.4)
Headache†	106 (24.3)
Diarrhea†	58 (13.3)
Abdominal pain†	34 (7.8)
Hospitalized	292 (59.6)
Intensive care unit	112 (38.4)
Mechanical ventilation	61 (20.9)
Clinical finding	
Pneumonia‡	236 (48.2)
Acute respiratory distress syndrome‡	48 (9.8)
Renal failure	22 (4.5)
Died	11 (2.2)
Underlying condition†	
Immunosuppression	55 (12.6)
Diabetes	40 (9.2)
Hypertension	27 (6.2)
Cardiac disease	23 (5.3)
Asthma	20 (4.6)
Chronic pulmonary disease	11 (2.5)
Hyperlipidemia	9 (2.1)
Pregnant	8 (1.8)
Renal disease	7 (1.6)
Other	12 (2.8)
Specific job classification†	
Health care worker	35 (8.0)
US military	9 (2.1)
MERS-CoV rRT-PCR testing	
Specimen type	
Upper respiratory	414 (84.5)
Lower respiratory	242 (49.4)
Serum	235 (48)
Stool	40 (8.2)
MERS-CoV positive	2 (0.4)
Serologic testing for MERS-CoV	
Tested	67 (13.7)
MERS-CoV positive	2 (0.4)
Other pathogens detected§	
Influenza A virus	41 (8.4)
Rhinovirus/enterovirus	37 (7.6)
Influenza B virus	13 (2.7)
Streptococcus pneumoniae	11 (2.2)
Human metapneumovirus	6 (1.2)
Adenovirus	5 (1.0)
Coronavirus	4 (0.8)
Respiratory syncytial virus	4 (0.8)
Parainfluenzavirus	3 (0.6)
Chlamydophila pneumoniae	2 (0.4)
Legionella pneumoniae	2 (0.4)
Other	16 (3.3)

*Data are no. (%) unless otherwise specified. MERS-CoV, Middle East respiratory syndrome coronavirus; PUI, patient under investigation; rRT-PCR, real-time reverse transcription PCR.
†Included only in 2 most recent versions of PUI short form (N = 436).
‡Forty-one PUIs had pneumonia and acute respiratory distress syndrome.
§Etiologic pathogen not reported for 359 (73%) PUIs; $\geq$1 pathogen identified for some PUIs.

Table 2. Countries from which 381 PUIs for MERS-CoV infection had traveled within 14 days of symptom onset, United States January 1, 2013–October 31, 2014*

Country	No. (%)
Saudi Arabia	189 (49.6)
United Arab Emirates	60 (15.7)
Israel	45 (11.8)
Jordan	34 (8.9)
Qatar	27 (7.1)
Kuwait	22 (5.8)
Egypt	12 (3.1)
Bahrain	10 (2.6)
Oman	9 (2.4)
Iran	8 (2.1)
Iraq	8 (2.1)
Lebanon	8 (2.1)
Turkey	8 (2.1)
Pakistan	6 (1.6)
Palestinian Territories	6 (1.6)
Yemen	6 (1.6)
Other†	13 (3.4)

*Patients may have been in >1 country. PUI, patient under investigation.
†Azerbaijan (1); Afghanistan (2); Bangladesh (1); Greece (1); India (2); Indonesia (2); Kenya (1); Morocco (1); Somalia (1); Syria (1).

serologic assay on a single serum specimen is recommended. CDC also recommends testing for common respiratory pathogens (e.g., influenza virus), but identification of a respiratory pathogen should not preclude MERS-CoV testing (*14*). The PUI guidance serves to help health care providers and state health departments identify patients for evaluation and testing for MERS-CoV infection; however, because we are still learning about the natural history of MERS-CoV, it is reasonable to consider testing for MERS-CoV even when some PUI characteristics are not present, especially in the presence of strong clinical or epidemiologic judgment for MERS-CoV. During the evaluation process for MERS-CoV infection, appropriate infection control measures should be instituted as soon as possible for hospitalized and nonhospitalized PUIs (*15*).

The 2 US cases of imported MERS were detected in health care providers from Saudi Arabia. These cases prompted a CDC guidance update recommending evaluation and testing of persons with less severe respiratory illness who had strong epidemiologic risk factors, particularly heath care exposure, for MERS-CoV infection. Occupation, recent travel history, recent visit to a health care facility, and contact will ill persons should be determined when evaluating persons with respiratory illness. As testing increases, especially serologic testing, additional MERS cases, including mildly symptomatic cases and cases among younger persons are being identified. These cases highlight the range of severity of MERS-CoV infection and the need to consider testing persons with a compatible travel history who may not match the clinical profile of the initially described case-patients. CDC plans to revise MERS-CoV guidance as needed.

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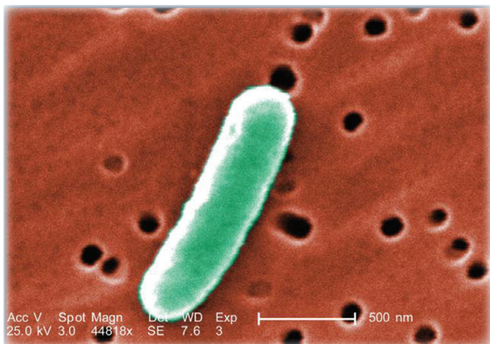
EID Podcast: Carbapenem-Resistant Enterobacteriaceae



Dr. Mike Miller reads an abridged version of the article, **Deaths Attributable to Carbapenem-Resistant Enterobacteriaceae Infections**

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Wildlife Reservoir for Hepatitis E Virus, Southwestern France

Sebastien Lhomme, Sokunthea Top, Stephane Bertagnoli, Martine Dubois, Jean-Luc Guerin, Jacques Izopet

Pigs are a reservoir for hepatitis E virus (HEV). To determine the relative contribution of game to the risk for human HEV infection in southwestern France, we tested wildlife samples. HEV RNA was in 3.3% of wildlife livers, indicating that in this region, eating game meat is as risky as eating pork.

Hepatitis E virus (HEV) is a causative agent of acute hepatitis worldwide. According to the Ninth Report of the International Committee on the Taxonomy of Viruses (http://ictvonline.org/), HEV is the sole member of the genus *Hepevirus* in the family *Hepeviridae*. HEV is a nonenveloped, single-stranded, positive-sense RNA virus containing ≈7.2 kb. Its genome contains 3 open reading frames (ORFs)—ORF1, ORF2, and ORF3—which encode nonstructural proteins, the capsid protein, and a small protein involved in virus egress, respectively (*1*).

Phylogenetic analysis of HEV sequences has led to the identification of 4 major genotypes (*1*). Genotypes 1 (HEV1) and 2 (HEV2) are pathogenic to humans only. HEV1 is present mainly in Asia and Africa, and HEV2 is in Africa and Mexico. In developing countries, HEV1 and HEV2 transmission is waterborne because of inadequate sanitary conditions. Genotypes 3 (HEV3) and 4 (HEV4) infect not only humans but also pigs, wild boars, deer, and other mammals. HEV3 is widespread, but HEV4 occurs mainly in Asia and was recently introduced into Europe (*1*). Pigs are a major reservoir of HEV3 and HEV4 (*2*); however, in recent years, the host range of HEV has expanded substantially (*3*).

HEV is hyperendemic to the Midi-Pyrénées area of southwestern France; annual incidence of cases among humans is 3.2% (*4*), and seroprevalence among blood donors has reached 52.5% (*5*). A multivariate analysis reported that the only factor associated with autochthonous HEV infection in this region was the consumption of game meat (*6*). However, the prevalence of HEV RNA in wildlife, especially wild boars and deer, has yet to be explored. Identifying the most commonly infected animals (sources of transmission) could help prevent zoonotic foodborne transmission. HEV strains have been recently identified in rabbits (*7*). Because HEV strains in rats have been recently described (*8*), we questioned the capacity of coypu to act as an HEV reservoir. Coypu are large, herbivorous, semiaquatic rodents that usually live in fresh or brackish water. In this study, we assessed the prevalence of HEV RNA among wild boars (Sus scrofa), deer (Cervus elaphus), rabbits (Oryctolagus cuniculus), and coypu (Myocastor coypus) and, thus, the potential for these animals to act as sources of HEV infection for persons living in the Midi-Pyrénées area.

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The Study

Samples of liver and bile were collected from 86 wild boars, 62 deer, 20 wild rabbits, and 78 coypu in the Midi-Pyrénées area. The wild boars and deer were hunted from February 2010 through January 2011, rabbits were hunted from October 2013 through February 2014, and coypu were hunted in April 2011.

RNA was extracted from 30 mg of liver by using RNeasy Mini Kits or from 140 μL of bile by using QIAamp Viral RNA Mini Kits as specified by the manufacturer (QIAGEN, Courtaboeuf, France). Real-time PCR based on ORF3 was used to detect and quantify HEV RNA in plasma samples as previously described (*9*). The limit of detection was 100 copies/mL.

HEV RNA was detected in 5 (5.8%) wild boar livers, 2 (3.2%) deer livers, 1 (5.0%) wild rabbit liver, and no coypu livers (Table). Thus, the overall prevalence of HEV RNA among wildlife, irrespective of species, was 3.3% (8/246) (95% CI 1.1%–5.5%). Because bile samples were available only from animals with negative HEV RNA liver samples, no bile sample was positive for HEV RNA.

Conclusions

The overall prevalence of HEV RNA in game (3.3%) is similar to that among pigs. A recent nationwide study in France reported that HEV RNA was present in the livers of about 4% of farmed pigs of slaughter age (*10*). These contaminated livers can then enter the food chain and be

Table. Hepatitis E virus RNA among wildlife, southwestern France*

Source, dates collected	No. tested	No. (%) HEV RNA positive	Virus concentration, median log copies/g (range)
Wild boars, 2010–2011			
Liver	86	5 (5.8)	2.80 (1.57–8.05)
Bile	29	0	NA
Deer liver, 2010–2011	62	2 (3.2)	2.78 (1.11–3.07)
Wild rabbits, 2013–2014			
Liver	20	1 (5.0)	8.70
Bile	13	0	NA
Coypu liver, 2011	78	0	NA

*NA, not applicable.

responsible for foodborne zoonotic HEV infection. Such pork products, especially raw or undercooked liver, are a major source of exposure to the virus (*11*). HEV has been found in pig liver sausages (*5*,*12*,*13*). Our findings suggest that game meat from wild boar, deer, and rabbit contribute to HEV epidemiology in the Midi-Pyrénées area. However, residents of this area eat less game meat than pork.

Prevalence rates of HEV RNA in the wild boar populations of other European countries like the Netherlands (2.0%) and Germany (5.0%) are similar (*2*), and rates of HEV RNA are higher among wild boars in Hungary (12.2%) and Italy (25.0%) (*2*) and among deer in Spain (13.6%), the Netherlands (15.0%), and Hungary (34.4%) (*2*). However, despite these HEV RNA prevalence figures, no HEV-hyperendemic region in these countries has been described. Thus, the prevalence of HEV RNA among wildlife alone is not enough to explain the high seroprevalence in a specific area. Contact with the animal reservoir and local dietary practices must be taken into account.

Prevalence of HEV RNA among wild rabbits in this study (5%) is lower than that found by a previous (2010) study of wild rabbits from the Haute-Garonne Department in southwestern France (6/12; 50%) (*7*). The low prevalence rate reported here could be the result of our shorter collection time (5 months in our study vs. 3 years in the previous study). It is also possible that HEV has become less prevalent since the previous study was done. The description of a strain from humans closely related to a strain from rabbits indicates that zoonotic transmission of HEV from rabbits is possible (*7*).

A recent study has shown that rats are competent hosts for HEV3, suggesting that rodents (e.g., coypu) may be an alternative reservoir for zoonotic strain HEV3 (*14*). Coypu meat is eaten as pâté and may be an unexpected source of HEV foodborne zoonotic HEV infection. However, none of the 78 coypu livers tested were positive for HEV RNA. Thus, coypu do not seem to carry zoonotic strains of HEV. However, significant nucleotide changes may be present in the HEV strains in coypu. Thus, the primers used may have not been able to amplify HEV RNA.

In conclusion, the prevalence of HEV RNA in wild boars, deer, and rabbits is similar to that previously reported for pigs. Consumption of the meat of these wild animals, and of pig liver sausage, all contribute to the HEV epidemiology in the Midi-Pyrénées area because of specific local eating habits. Game meat from this part of France should be cooked thoroughly to minimize the risk for HEV infection (*15*).

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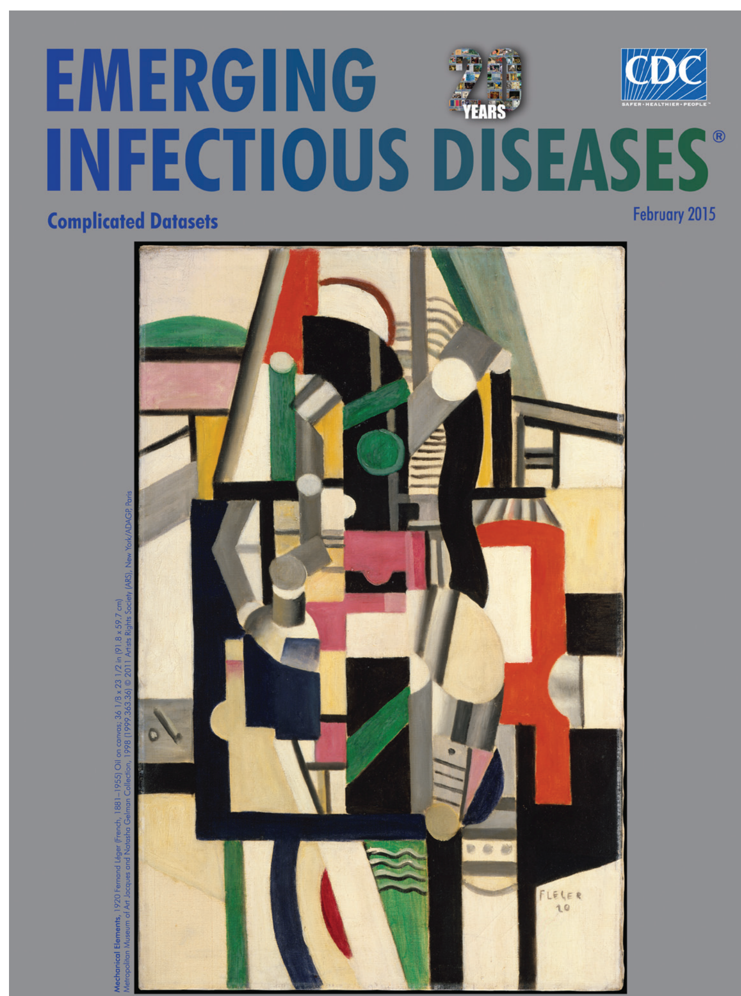

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Asymptomatic Malaria and Other Infections in Children Adopted from Ethiopia, United States, 2006–2011

Senait M. Adebo, Judith K. Eckerle, Mary E. Andrews, Cynthia R. Howard, Chandy C. John

We screened 52 children adopted from Ethiopia for malaria because they had previously lived in a disease-endemic region or had past or current hepatomegaly or splenomegaly. Seven (13.5%) children had asymptomatic malaria parasitemia by microscopy (n = 2) or PCR (n = 5). Our findings suggest that adoptees at risk for asymptomatic malaria should be screened, preferably by PCR.

International adoptees are at increased risk for infectious diseases (*1*). During 2007–2012, Ethiopia was 1 of the top 5 countries of origin for children who were adopted by persons in the United States (*2*), but few studies have been published on children from Ethiopia who were adopted by persons in the United States (*3*). Malaria caused by *Plasmodium falciparum*, *P. vivax*, and, less frequently, *P. ovale* is endemic to several regions in Ethiopia (*4*). Children adopted from Ethiopia are often living in orphanages in Addis Ababa, an area free of malaria, at the time of their adoption, but they may have lived in a malaria-endemic area before their transfer to the orphanage. The prevalence of asymptomatic malaria parasitemia among these children is not known.

The Study

We reviewed medical records of all children adopted from Ethiopia and seen at the University of Minnesota International Adoption Clinic (Minneapolis, MN, USA) during February 2006–June 2011 for results of standard infectious disease screening tests recommended by the American Academy of Pediatrics: tuberculosis (by tuberculin skin test or, in children ≥5 years old, by interferon-g release assay); intestinal parasites (fecal testing for ova, parasites, and *Giardia intestinalis* antigen); hepatitis B or C virus; HIV; and syphilis (*5*). Children were screened for hepatitis A virus at the discretion of the physician seeing the patient and for malaria by blood smear or PCR if they met screening criteria (i.e., history of living in a malaria-endemic region or a history of or current evidence of splenomegaly or hepatomegaly). The study was reviewed and approved by the University of Minnesota Institutional Review Board.

During the period studied, 255 international adoptees from Ethiopia were seen at the clinic. Adoptees' mean age at medical evaluation was 2.8 years (range 3.4 months–14.9 years); 148 (58%) were female and 107 (41.9%) were male. All 255 children were asymptomatic for malaria, but 52 met malaria screening criteria and were tested by peripheral blood smear (n = 24), PCR (n = 24), or both (n = 4). Of the 52 children, 7 (13.5%) had blood smear (2 children) or PCR (5 children) results positive for *Plasmodium* species. Table 1 outlines the sensitivity, specificity, and negative and positive predictive values of medical history questions and physical exam signs for asymptomatic malaria. The 2 children with a positive blood smears had low parasite densities (<0.1%), and the species could not be identified. These 2 children were treated before PCR testing was available. Subsequently, PCR became the preferred first-line diagnostic test, and 5 infections were diagnosed on the basis of PCR results: 3 *P. vivax*, 1 *P. falciparum*, and 1 mixed *P. vivax* and *P. falciparum*. Among the 7 children with parasitemia, 2 had a palpable spleen tip, 2 had a hemoglobin level of <11 g/dL (reference 11–15 g/dL), and none had thrombocytopenia. All children with a positive blood smear or PCR result were treated: atovaquone/proguanil for *P. falciparum* infections, chloroquine followed by primaquine for *P. vivax* infections, and atovaquone/proguanil followed by primaquine for the mixed infection and infections with no species identified.

In addition to the malaria results, of 217 children tested for intestinal parasites, 96 (44.2%) had positive results; *Giardia intestinalis* flagellates were most common (n = 75, 34.6%), followed by *Blastocystis hominis* protozoa (n = 34, 15.7%) (Table 2). Evidence of tuberculous infection was found in 49 (27.1%) children, hepatitis A virus in 14 (8.7%), hepatitis B virus in 6 (2.6%), and HIV in 1 (0.5%) (Table 2).

Conclusions

In this study, we show that 7 (13.5%) of 52 adoptees from Ethiopia who had lived in a malaria-endemic region or had hepatomegaly or splenomegaly by clinical history or on physical examination had asymptomatic malaria

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Table 1. Value of certain characteristics or findings for predicting asymptomatic malaria parasitemia in children adopted from Ethiopia who were seen at the University of Minnesota International Adoption Clinic, Minneapolis, Minnesota, USA, 2006–2011*

Characteristic or finding	Malaria, no. (%)		Sensitivity, %	Specificity, %	PPV, %	NPV, %
	Positive, n = 7†	Negative, n = 45				
History of hepatomegaly	0	1 (2.2)	0	97.8	0	86.3
History of splenomegaly	3 (42.8)	2 (4.4)	42.8	95.6	60.0	91.5
Presence of splenomegaly during examination	2 (28.6)	3 (6.7)	28.6	92.3	40.0	89.4
Presence of hepatomegaly during examination	1 (14.3)	7 (15.6)	14.3	84.4	12.5	86.4
History of splenomegaly or presence during examination	3 (42.8)	4 (8.9)	42.8	91.1	42.9	91.1
Hemoglobin level of <11 g/dL	2 (28.6)	6 (13.3)	28.6	87.7	25.0	88.7

*NPV, negative predictive value; PPV, positive predictive value.
†Children who were malaria-positive by blood smear or PCR testing.

parasitemia. We also confirm the findings of previous studies that showed high rates of infection with intestinal parasites (particularly *G. intestinalis* flagellates) (*3*), latent tuberculosis (*3*), and hepatitis A virus (*6*) in adoptees from Ethiopia.

The rate of asymptomatic malaria parasitemia in international adoptees is not known. As adoptions increase from countries in sub-Saharan Africa and other countries with areas of potential malaria transmission, such as India and Haiti, malaria screening will need to be considered for the adopted children. On the basis of the current data, we believe reasonable first-line criteria for malaria screening in international adoptees are residence in a malaria-endemic country plus either lack of documentation that the child lived for his or her whole life in a region of that country that was malaria free (e.g., Addis Ababa in Ethiopia) or past or current splenomegaly. However, a limitation of our study is that we used essentially these criteria to screen, and the prevalence of asymptomatic malaria might have differed if we used different criteria. For example, we did not screen all children with anemia (hemoglobin level of <11 g/dL); because malaria is a leading cause of anemia in malaria-endemic areas, anemia may be a useful additional screening criterion for malaria.

For over a century, microscopy has been the standard for documentation of malaria infection in persons with clinical malaria, but PCR has greater sensitivity for detection of low-level parasitemia (*7*) and is now a field standard for detection of asymptomatic parasitemia (*8*). Multiplex PCR also enables testing and identification of all 5 *Plasmodium* species that cause disease in humans and can provide species identification at low levels of parasitemia. In our study, microscopy testing on 2 children failed to determine the malaria species, a common difficulty in persons with low-level parasitemia. Without knowing the malaria species, we had to treat the children for both *P. falciparum* and *P. vivax* infection, which involved testing for glucose-6-phosphate dehydrogenase deficiency and treatment with multiple antimalarial medications. Knowledge of the prevalent species in immigrants from a specific area can also inform public health efforts and prophylaxis planning for travelers to that area. For these reasons, PCR is likely the test of choice for detection of asymptomatic parasitemia in children adopted from malaria-endemic areas.

For 3 reasons, we treated all adopted children with asymptomatic parasitemia, whether detected by PCR or microscopy. First, a diagnosis of malaria could be missed if these children became febrile. After their adoption, many lived in areas in which malaria is rarely if ever seen, so the diagnosis of malaria might not be considered. Second, malaria can cause severe disease, so a missed diagnosis could have major health consequences for the child. Third, most antimalarial medications have a low toxicity, so treatment is not a danger to the child.

Table 2. Prevalence of infectious diseases in children adopted from Ethiopia who were seen at the University of Minnesota International Adoption Clinic, Minneapolis, Minnesota, USA, 2006–2011

Infection	No. screening results available	No. (%) positive
Intestinal parasites	217	96 (44.2)*
Tuberculosis	181	49 (27.1)†
Malaria	52	7 (13.5)
Hepatitis A virus	161	14 (8.7)
Hepatitis B virus	233	6 (2.6)
Syphilis	215	0†‡
Hepatitis C virus	219	0†‡
HIV	218	1 (0.5)‡

*Evidence of infection with ≥1 of the following: *Giardia intestinalis* flagellates (n = 75, 34.6%), *Blastocystis hominis* protozoa (n = 34, 15.7%), *Hymenolepsis nana* tapeworms (n = 2, 0.9%), *Dientamoeba fragilis* protozoa (n = 2, 0.9%), *Ascaris lumbricoides* roundworms (n = 2, 0.5%), or *Trichuris trichiura roundworms* (0.5%).
†By tuberculin skin testing (induration ≥10 mm; n = 46), interferon-γ release assay (n = 1), or both (n = 2). Latent tuberculosis infection was diagnosed in 48 children. Tuberculosis disease was diagnosed initially in 1, but was later reassessed as latent tuberculosis infection; medications for disease were stopped after 4 months of treatment.
‡Initial screening tests results were positive in 2 additional children, but confirmatory tests were negative.

We now use PCR to screen asymptomatic children from malaria-endemic areas. We recommend this method for centers with rapid access to PCR for all 5 human *Plasmodium* species because increased sensitivity of detection is more important than rapid detection in asymptomatically infected children. However, any symptomatic child (e.g., a child with fever) must have microscopy or rapid diagnostic testing performed immediately, because these results are typically available quickly and can guide immediate decisions regarding treatment.

In summary, this study shows that children adopted from Ethiopia who lived in malaria-endemic regions of Ethiopia or had past or current splenomegaly are at risk for asymptomatic *P. falciparum* and *P. vivax* parasitemia. The study findings support the importance of obtaining a careful history to determine malaria risk and conducting PCR screening for asymptomatic infection in children with the noted risk factors. These findings may also be relevant to children adopted from other malaria-endemic countries.

Dr. Adebo works as a hospitalist pediatrician at the Children's Hospital of Philadelphia. Her primary research interests are global health and international adoption issues.

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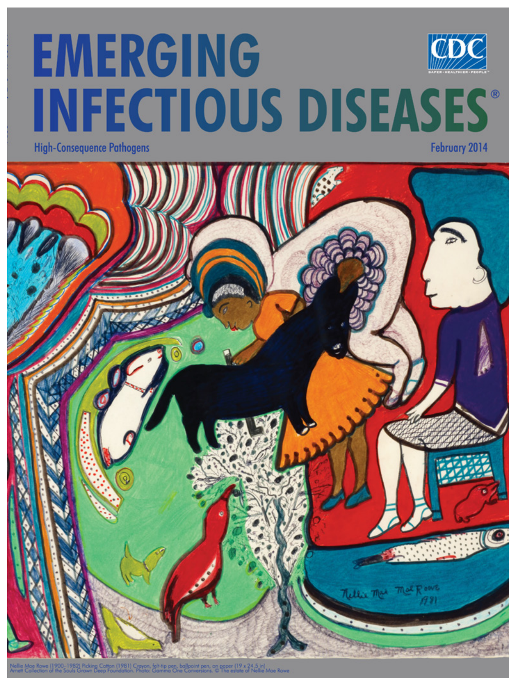

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Distinct Lineages of Bufavirus in Wild Shrews and Nonhuman Primates

Michihito Sasaki, Yasuko Orba, Paulina D. Anindita, Akihiro Ishii, Keisuke Ueno,[1] Bernard M. Hang'ombe, Aaron S. Mweene, Kimihito Ito, Hirofumi Sawa

Viral metagenomic analysis identified a new parvovirus genome in the intestinal contents of wild shrews in Zambia. Related viruses were detected in spleen tissues from wild shrews and nonhuman primates. Phylogenetic analyses showed that these viruses are related to human bufaviruses, highlighting the presence and genetic diversity of bufaviruses in wildlife.

Bufavirus (BuV), a recently described parvovirus, was initially discovered in the feces of a child with diarrhea in Burkina Faso in 2012 (*1*). Thereafter, BuV was identified in fecal samples from children and adults with gastroenteritis in Bhutan, Finland, and the Netherlands in 2014 (*2–4*), respectively. Genome sequences and phylogenetic analyses revealed that BuV comprised at least 3 genotypes and was distinct from all other known members of the *Parvoviridae* family (*1,2*). The International Committee on Taxonomy of Viruses assigned BuV as a new species of the genus *Protoparvovirus* in the subfamily *Parvovirinae* (*5*). Whether BuV is an etiologic agent of human gastroenteritis remains unclear, but knowledge about its distribution and genetic divergence in humans is accumulating. However, whether BuV infection exists in wildlife remains unanswered. Through use of metagenomics, we previously described the enteric virome of wild shrews of the *Crocidura* genus sampled at Mpulungu, Zambia, in 2012 (*6*). From this sequence dataset (GenBank/EMBL/DDBJ accession no. DRA002561), we identified sequence reads related to BuV. Here, we describe the genome of this new parvovirus.

The Study

We determined the nearly complete genome sequence of BuV, which we named Mpulungu BuV (MpBuV), by filling genome gaps with primer walking and rapid amplification of cDNA ends (GenBank/EMBL/DDBJ accession no. AB937988). The MpBuV genome comprises 4,613 nt and encodes open reading frames of the nonstructural protein (NS) 1 and the viral capsid protein (VP) 1 and VP2 (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/7/14-1969-Techapp1.pdf). blastp (http://blast.ncbi.nlm.nih.gov) searches showed that the MpBuV NS1, VP1, and VP2 proteins were closely related to those of human BuVs and the WUHARV parvovirus (E-value = 0.0). WUHARV parvovirus, identified in rhesus monkeys experimentally infected with simian immunodeficiency virus under laboratory conditions in the United States, was found to be a closely related to human BuVs (*7*).

In MpBuV, NS1 shares 52.5% aa identity with NS1 of human BuV (GenBank accession no. JX027296). We found that the amino acid sequence identity of VP1 between MpBuV and human BuV is 52.3%, whereas that of VP2 is 51.4%. Similar to human BuV, MpBuV showed potential splicing signals in the VP1 coding region. We also identified the parvovirus-conserved amino acid motifs in NS1, VP1, and VP2 of MpBuV (online Technical Appendix Figure) (*8–12*). Phylogenetic analysis was performed as described previously (*6*). A Bayesian phylogenetic tree was generated on the basis of the full-length NS1 proteins of MpBuV, human BuVs, and representative parvoviruses. MpBuV clustered with human BuVs and WUHARV parvovirus (online Technical Appendix Figure). According to the species demarcation criteria of the International Committee on Taxonomy of Viruses, each parvovirus species encodes an NS1 protein sharing <85% aa sequence identity with other known parvovirus species (*5*). The NS1 protein of MpBuV exhibited <58% sequence identity with that of any known parvovirus species; therefore, we propose that MpBuV is a new species within the *Protoparvovirus* genus.

Next, we performed PCR screening for MpBuV on shrews captured in Mpulungu. DNA was extracted from intestinal content suspensions and tissue specimens by using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and QIAamp DNA Mini kit (QIAGEN, Hilden, Germany), respectively. PCR was performed by using Tks Gflex DNA polymerase (TAKARA BIO, Otsu, Japan), forward primer MpBuV-F1 (position 2739–2763 in MpBuV genome, 5′-GAAGTGGTGTTGGTCATTCTACTGG-3′) and reverse primer MpBuV-R1 (position 3523 3546,

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Table. Sample information and nested PCR screening results for bufavirus, Zambia

Animal,* species (common name)	Location	Year	PCR positive/total
Primate			
Papio cynocephalus (yellow baboon)	Mfuwe	2009	2/50
P. ursinus (Chacma baboon)	Livingstone	2010–2011	1/50
Chlorocebus pygerythrus (vervet monkey)	Mfuwe	2009	0/50
	Livingstone	2010–2011	0/39
Shrew			
Crocidura hirta (lesser red musk shrew)	Livingstone	2011	0/2
	Mpulungu	2012	5/22
	Namwala	2012	0/2
	Mazabuka	2013	0/4
	Solwezi	2013	0/2
C. luna (moonshine shrew)	Mpulungu	2012	0/1
	Solwezi	2013	12/16
Rodent			
Mastomys natalensis (African soft-furred rat)	Livingstone	2011	0/35
	Mpulungu	2012	0/28
	Namwala	2012	0/29
	Mazabuka	2013	0/57
	Solwezi	2013	0/56
Other species†	Livingstone	2011	0/9
	Mpulungu	2012	0/20
	Namwala	2012	0/34
	Mazabuka	2013	0/16
	Solwezi	2013	0/14
Total			20/536

*For this analysis, the template DNA was prepared from the spleen tissues of the animals indicated.
†Other species from the genera.*Aethomys, Arvicanthis, Cricetomys, Gerbilliscus, Grammomys, Graphiurus, Lemniscomys, Mus, Paraxerus, Pelomys, Rattus, Saccostomus,* and *Steatomys*.

5′-GTTGGAGGTACACATGGATGAGGA-3′). We detected the MpBuV genome in 5 (22%) of the 23 samples from the intestinal contents of individual shrews captured in Mpulungu. We then tested for the presence of MpBuV in the lung, spleen, liver, and kidney tissues of the shrews that were PCR positive for MpBuV by screening of intestinal contents. The MpBuV genome was detected in 5 spleen and 4 liver samples from the 5 shrews.

This discovery of MpBuV urged us to further investigate BuVs and related parvoviruses in wildlife. We designed degenerate primers for nested PCR screening on the basis of a multiple sequence alignment of the NS1 gene from human BuV, WUHARV parvovirus and MpBuV as follows: BuV-F1 (position 190–215 in MpBuV genome, 5′-TCAAWRTMACCTGGAAAGACTACAGA-3′) and BuV-R1 (position 1503 1534, 5′-TCATTGGTTGTCATKAYWACTGGAGTTGGTTC-3′) for the first PCR round, and BuV-F2 (position 980–1006, containing an equimolar mixture of 5′-AGAAAAATGGATGCTCCAAGATCCAGA-3′ and 5′-AGAAAAATGGATGCTTGGTGAWCCWGA-3′) and BuV-R2 (position 1444 1465, 5′-ATTGCTTGGCCACTCATGATKG-3′) for the second PCR round. PCRs were performed by using Tks Gflex DNA polymerase and the annealing temperatures were set at 50°C and 55°C for the first and second PCR round, respectively.

We screened 536 spleen tissue specimens from wildlife. The specimens from 3 nonhuman primate species, 2 shrew species, and 14 rodent genera from 6 locations in Zambia were used for different research projects, as reported previously (*13,14*). All our sampling activities were conducted with the permission of the Zambia Wildlife Authority (Act No. 12 of 1998). We chose spleen tissue for nested PCR screening because specimens of intestinal contents were unavailable for almost all of the animals we sampled. As we described, MpBuV was detected in spleen samples in addition to samples of intestinal contents from the same animals.

Nested PCR screening for BuVs detected MpBuV from 5 shrews in Mpulungu; these were the same animals that were PCR-positive for MpBuV by using a specific primer set targeting MpBuV. Nested PCR was also positive in 3 primates and 12 shrews (Table). The PCR amplicons with expected size (≈480 bp) were subjected to direct sequence analysis. A Bayesian phylogenetic analysis was performed as described by using the partial nucleotide sequences obtained for the NS1 gene with the exception of the primer sequences (434–440 bp) and the corresponding genome regions of known protoparvoviruses and amdoviruses. The BuVs detected clustered with human BuVs and the WUHARV parvovirus and are distinguishable from other protoparvoviruses (Figure). The BuVs detected can be divided into MpBuV and 3 other strains; we have tentatively named these Solwezi BuV, Mfuwe BuV, and Livingstone BuV (Figure). Solwezi BuV, which shares 79% nt sequence identity with MpBuV, was identified in 12 shrews (*Crocidura luna*) in Solwezi. Mfuwe BuV and Livingstone BuV were identified in 2 yellow baboons (*Papio cynocephalus*) in Mfuwe and a chacma baboon (*P. ursinus*)

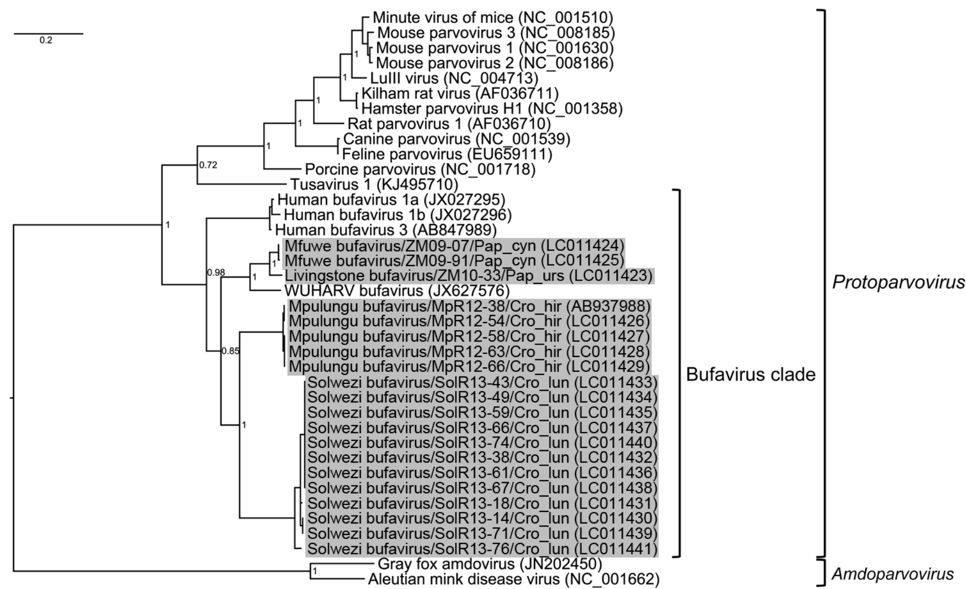

Figure. Partial nonstructural protein (NS) 1 gene phylogeny of newly identified bufaviruses, Zambia. The Bayesian phylogenetic tree was generated by using the partial NS1 gene fragments (434–440 bp) of bufaviruses and the corresponding region of known protoparvoviruses and amdoviruses. Gray shading indicates bufaviruses identified in this study. GenBank accession numbers of viral sequences are shown in parentheses. Bayesian posterior probabilities are indicated at each tree root. Scale bar indicates nucleotide substitutions per site.

in Livingstone, respectively. Both baboon-derived BuVs were closely related to the WUHARV parvovirus. These results indicate the presence of BuVs in wild nonhuman primates and in wild shrews.

Conclusions

The nearly complete genome sequence of a new parvovirus, MpBuV, was obtained from a wild shrew in this study. blastp searches indicated that each MpBuV open reading frame shared the highest amino acid identity with other known BuVs. Furthermore, our phylogenetic analysis showed that MpBuV clustered with BuVs but was distinct from any other known parvovirus. Accordingly, we propose that MpBuV should be considered a new species of BuV.

Our nested PCR screening identified 3 additional BuV strains: Solwezi BuV, Mfuwe BuV, and Livingstone BuV. These protoparvoviruses are also phylogenetically related to known BuVs and derived from wildlife (i.e., shrews and nonhuman primates). These results show the presence of human BuV-related genomes in wildlife expanding our knowledge of the distribution and genetic diversity of BuVs.

In summary, we investigated the situation regarding BuVs in Zambian wildlife. Thus far, no evidence exists of BuV transmission between humans and wildlife. Our nested PCR should be helpful for detecting BuVs in mammals and lead to better understanding of the distribution of BuVs.

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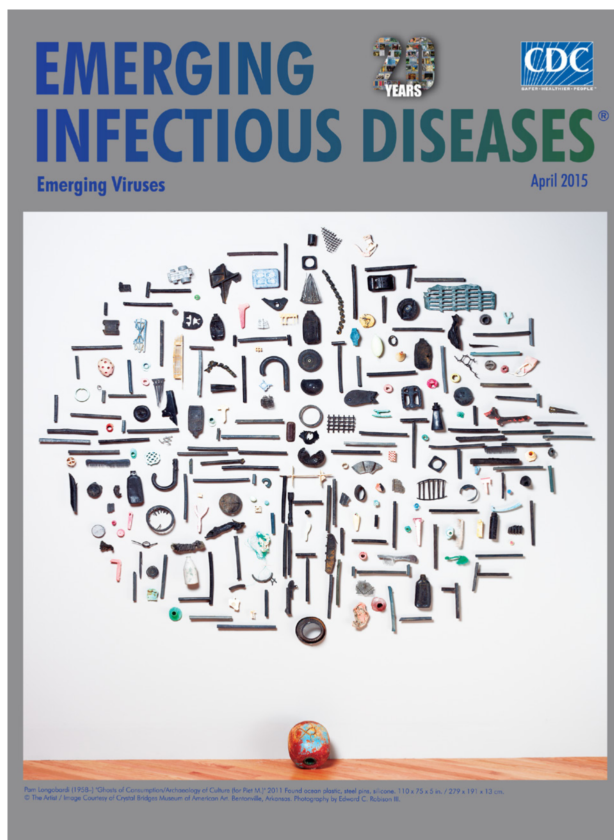

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Geographic Range Expansion for the Rat Lungworm in North America

Emily M. York, James P. Creecy, Wayne D. Lord, William Caire

Using quantitative PCR analysis and DNA sequencing, we provide evidence for the presence of the rat lungworm (*Angiostrongylus cantonensis*) in Oklahoma, USA, and identify a potentially novel rat host (*Sigmodon hispidus*). Our results indicate a geographic range expansion for this medically and ecologically relevant parasite in North America.

Emerging infectious diseases negatively impact humans and wildlife, causing disease outbreaks and deaths and local and global extinctions (*1*). Zoonotic disease emergence or re-emergence results from numerous factors (e.g., globalization of trade, increased interaction of humans and animals, anthropogenic climate change) that function independently or synergistically (*2,3*). Consequently, the means by which parasitic zoonoses are studied must be constantly advanced to promote identification, control, and prevention of outbreaks.

The rat lungworm, *Angiostrongylus* (*Parastrongylus*) *cantonensis*, causes eosinophilic meningitis in humans (*4*) and various disease manifestations (meningoencephalitis, neurologic disorders) in atypical host species, including wildlife and captive animals (*5*). Transmission of these worms occurs by ingestion of third-stage larvae in raw or undercooked intermediate or paratenic hosts (*6*). Although variable among geographic regions and within host species, the prevalence of rat lungworms might be high under favorable conditions (*7*).

The occurrence of *A. cantonensis* rat lungworms has been documented worldwide, and its distribution has been attributed largely to the spread of intermediate molluscan host species (e.g., *Achantina fulica*) and definitive rodent host species (e.g., *Rattus* spp.) (*8*). Moreover, host specificity of rat lungworms is highly plastic, which contributes to its continuous geographic expansion (*4*). These factors indicate that the rat lungworm is an emerging zoonotic pathogen of concern to humans and wildlife, and therefore provides an excellent opportunity to evaluate the sensitivity and effectiveness of epidemiologic surveying techniques.

The Study

We evaluated the current distribution and potential spread of the rat lungworm within areas of the Gulf Coast region and midwestern United States by sampling rodent populations in regions of Louisiana and Oklahoma that were predicted by an ecologic niche model to contain suitable and unsuitable habitat (*9*). We used a quantitative PCR (qPCR) TaqMan assay (Life Technologies, Foster City, CA, USA) (*10*) to test for the parasite in tissue samples and further evaluated these samples through DNA sequencing analysis.

We trapped animals during the spring, summer, and fall months during 2010–2012. A total of 42 rodents and 3 shrews were collected from McCurtain County in southeastern Oklahoma, and 47 rodents were collected in Louisiana (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/7/14-1980-Techapp.pdf). We also obtained 56 *Rattus norvegicus* rat brain and lung tissue samples from the City of New Orleans Mosquito, Termite, and Rodent Control Board. Blood, lung, and brain tissue samples were collected from the rodents. Flotation was performed on all 148 lung samples, and all samples were negative for adult *A. cantonensis* rat lungworms.

Known adult rat lungworms were used as controls for molecular analyses (online Technical Appendix). Cellular DNA was extracted from rodent blood and brain samples. We tested for rat lungworm internal transcribed spacer 1 (ITS1) DNA by using a TaqMan qPCR on an ABI 7500 system (*10*). A total of 134 blood samples and 137 brain samples contained DNA suitable for analysis. After qPCR, 34 of the 271 total tissue samples were classified as putatively positive for rat lungworm and sequenced, generating a 267-bp fragment of the ITS1 region (online Technical Appendix).

On the basis of DNA sequencing, 3 brain samples were identified as containing *A. cantonensis* DNA (GenBank accession nos. KP231729, KP231728, and KP231727). These brain tissue samples were obtained from 3 rodents (host catalog nos. 32, 70, and 76), which were identified as 1 Hispid cotton rat (*Sigmodon hispidus*) and 2 brown rats (*Rattus norvegicus*), respectively (Table 1). A comparison of the 3 brain samples with those in GenBank by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed a match with rat lungworm (GenBank accession nos. GU587762.1 and GU587759.1) (Table 2).

All sequences were aligned by using MUSCLE in MEGA 5.2 (http://www.megasoftware.net/), manually inspected for consensus, and compared with the 267-bp fragment generated from the known sample of rat lungworm. Maximum-likelihood phylogenetic analysis was performed by using sequence data for the ITS1 region of *A. cantonensis* (GenBank accession no. GU587759.1), 2 closely related species, *A. vasorum*

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Table 1. DNA sequences generated from 3 rat brain samples positive for *Angiostrongylus cantonensis* rat lungworms, United States*

Rat host species	Trapping location	Host catalog no.	Sequence, 5′→3′
Sigmodon hispidus	Red Slough WMA, OK	32	TTCATGGATGGCGAACTGATAGTATCATCGCATATCTACTATACGCAT GTGACACCTGATTGACAGGAAATCTTAATGACCCAAGTATAATGTTTC AATGGGCGCCAACGTAGCAACAGAACAGTTTTTCACACGTGAAAATG TGGAACGAGATACACAGGATGtatatataTATATATATATATATACACATA TATRTGTGTRTGGAAATAGATATACTAKCTTCAGMGAKGRWKCGSGY GATTCGCGTATCTAAGAAAAACACA
Rattus norvegicus	New Orleans, LA	70	TTCATGGATGGCGAACTGATAGTGTCATCGCATATCTACTATACGCA TGTGACACCTGATTGACAGGAAATCTTAATGACCCAAGTATAATGTTT CAATGGGCGCCAACGTAGCAACAGAACAGTTTTTCTACACGTGAAAA TGTGGAACGAGATACACAGGATGTATATATATATATATATATACACATAT ATATGTGTATGGAAATTGATATACTAGCTTCAGCGATGGATCGGTCG ATTCGCGTATCGATGAAAAACGCATCTA
Rattus norvegicus	New Orleans, LA	76	TTCATGGATGGCGAACTGATAGTATCATCGCATATCTACTATACGCAT GTGACACCTGATTGACAGGAAATCTTAATGACCCAAGTATAATGTTTC AATGGGCGCCAACGTAGCAACAGAACAGTTTTTCTACACGTGAAAAT GTGGAACGAGATACACAGGATGTATATATATATATATATATACACATATA TATGTGTATGGAAATTGATATACTAGCTTCAGCGATGGATCGGTCGA TTCGCGTATCGATGAAAAACGCAGCTA

*WMA, wildlife management area.

(the French heartworm, GU733324.1) and *A. costaricensis* (a parasitic nematode, (GU587745.1), and sequences obtained from the rodent hosts. Maximum-likelihood phylogenetic analysis grouped sequences from rat hosts 32, 70, and 76 with *A. cantonensis* with high bootstrap support (Figure).

Conclusions

Because the rat lungworm poses a major health risk to humans and wildlife worldwide, more work is needed to shed light on the location, dispersal, and influence of this parasite in new geographic regions. Although previous reports document rat lungworms in the Gulf Coast region of the United States (*5,11*), little is understood regarding their prevalence within definitive hosts and their dispersal throughout the southeastern United States. As expected, our analysis indicated that *R. norvegicus* rats from Louisiana harbored rat lungworms. Positive samples were collected from densely populated areas with high tourist activity, thereby increasing the risk for transmission to humans. Moreover, rat lungworms were identified outside their known habitat and in a new rat host species (*S. hispidus*) in Oklahoma, an area predicted to lack suitable habitat for the parasite (*9*). Our results provide a new perspective on the distribution of rat lungworms in the United States and indicate a northward range expansion that substantially increases the risk for disease spread within humans and wildlife.

Because endemic and novel pathogens require different and highly specialized disease management strategies, it is crucial to determine whether a pathogen is novel or endemic (*12*). Previous work has described the *A. cantonensis* rat lungworm as a novel pathogen in the southeastern United States. However, it is now characterized as endemic to this region, and our results strongly support this notion (*11*). Such changes in the epidemiologic classification of rat lungworms accentuate the need for techniques that monitor the extent to which parasites infiltrate new geographic areas and potentially pose threats to humans and native wildlife. One such threat includes an increasing prevalence of angiostrongyliasis, which should receive increased scrutiny in patients with eosinophilic meningitis from localities characterized by paratenic and intermediate hosts.

Rat lungworm was found in a previously undocumented mammalian host, *S. hispidus* rats, which strongly suggests that this parasite is an endemic pathogen. Although vegetation is their primary food source, *S. hispidus* rats will eat invertebrates (*13*). Whether these rats directly (by intentional consumption of host) or indirectly (by consumption of host or free third-stage larvae on vegetation) consume the parasite, we cannot rule out the possibility that acquisition of the parasite could occur in this species and enable further range expansion for rat lungworms. *S. hispidus* rats are a known host for another closely related *Angiostrongylus* species, *A. costaricensis*, which lends additional support for the notion that *S. hispidus* rats might act as a host for rat lungworms. Alternatively, *S. hispidus* rats might simply be an accidental/dead end host for this

Table 2. BLAST* results for sequences from 3 rat brain samples, United States†

Host catalog no.	Rat host species	Trapping location	Match, %	Coverage, %	e value
32	*Sigmodon hispidus*	Red Slough WMA, OK	92	98	5×10^{-105}
70	*Rattus norvegicus*	New Orleans, LA	99	100	3×10^{-130}
76	*R. norvegicus*	New Orleans, LA	99	100	1×10^{-133}

*http://blast.ncbi.nlm.nih.gov/Blast.cgi
†WMA, wildlife management area.

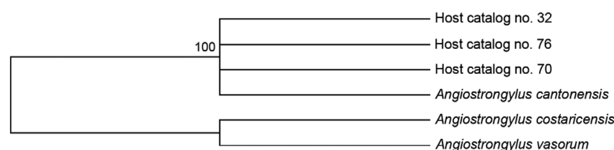

Figure. Maximum-likelihood bootstrap consensus phylogenetic tree showing the relationship between rat lungworm sequences generated in this study and other *Angiostrongylus* spp., United States. Tree was generated by using a Tamura 3-parameter model. Value along the branch is a bootstrap value.

parasite. Although wildlife might become infected with the parasite, not all wildlife are definitive hosts (*5,11*). Additional field and laboratory studies will clarify the role that *S. hispidus* rats play in the spread of the rat lungworm.

Because many terrestrial species remain taxonomically nondescribed, there is strong potential for continual emergence of unknown pathogens worldwide (*14*). Global travel, human encroachment into wildlife habitat, and climate change will influence distribution and emergence of disease (*2,15*). By incorporating field epidemiology with molecular genetic techniques to determine the geographic distribution of pathogens, major advances can be made in preventing the spread of wildlife diseases to human populations. Our results illustrate this point and highlight the need for future work to incorporate and refine these techniques and their application to epidemiology and wildlife disease surveillance.

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Slow Clearance of *Plasmodium falciparum* in Severe Pediatric Malaria, Uganda, 2011–2013

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Plasmodium falciparum resistance to artemisinin derivatives is emerging in Asia. We examined molecular markers of resistance in 78 children in Uganda who had severe malaria and were treated with intravenous artesunate. We observed in the K13-propeller domain, A578S, a low-frequency (3/78), nonsynonymous, single-nucleotide polymorphism associated with prolonged parasite clearance.

Resistance of *Plasmodium falciparum* parasites to artemisinin derivatives threatens the current first-line treatment for severe malaria. Artemisinin resistance was first reported in 2009 in Pailin, western Cambodia (*1*), and has since become prevalent in the greater Mekong Delta, Vietnam, where standard 3-day courses of artemisinin combination therapies for uncomplicated *P. falciparum* malaria are now failing (*2*–*4*).

Among several putative genetic determinants of parasite resistance to artesunate (*3*,*5*), polymorphisms in the propeller domain of a *kelch* gene on chromosome 13 (*PF3D7_1343700*; K13) are now recognized as the major determinant of artemisinin resistance observed in *P. falciparum* isolates from patients in Southeast Asia (*3*,*4*,*6*,*7*). Various single amino acid substitutions in the K13 protein are associated with a mean increase of 116% in the parasite clearance half-life ($t_{1/2}$) (*4*). The mechanism of resistance has been illuminated by a recent study of the *P. falciparum* transcriptomes from >1,000 acute malaria episodes (*6*). Slow-clearing parasites exhibited increased expression of unfolded protein response pathways (e.g., chaperone complexes); these pathways may mitigate protein damage caused by artemisinin. Slow-clearing parasites also exhibited decreased expression of proteins involved in DNA replication and decelerated development at the young ring stage. Haplotype analysis suggests that K13 mutations emerged independently in multiple geographic locations in Southeast Asia, causing concerns about the ability to contain resistant parasites (*7*).

With the widespread use of artemisinin treatment, resulting in continued pressure for natural selection of the most resistant parasites, resistance may emerge in regions beyond Asia, including Africa. The possible increase of parasite resistance to treatment highlights an urgent need to map K13 mutations throughout the malaria-endemic world (*7*). Consequently, recent molecular epidemiologic analyses of K13 in Senegal (*8*) and Uganda (*9*) and in a large collection of >1,100 infections from sub-Saharan Africa (*10*) have been undertaken, revealing the absence of nonsynonymous single-nucleotide polymorphisms (SNPs) associated with artemisinin resistance in Southeast Asia. Other distinct nonsynonymous SNPs have been discovered in parasites of African origin (*9*,*10*), but association of these mutations with a resistance phenotype has not been shown.

The Study

We examined parasite clearance kinetics and sequenced the parasite K13 gene in a cohort of 78 children with severe malaria (including 8 children who died) from the placebo group (being treated with artesunate alone) of a randomized, controlled trial conducted at the Jinja Regional Referral Hospital, Uganda, during July 12, 2011–June 14, 2013 (*11*). Inclusion and exclusion criteria have been described elsewhere (*11*). The median age of patients was 2.0 years (range 1.0–8.0 years), and 38 (49%) were female. All patients were treated intravenously with artesunate (Guilin Pharmaceutical, Shanghai, China) prequalified by the World Health Organization and according to the Organization's guidelines (*12*).

Giemsa-stained peripheral blood smears (thin and thick) were assessed for quantitative malaria parasite density by light microscopy at a quality-controlled central research laboratory, the Makerere University–Johns Hopkins University Research Collaboration Core Laboratory, which is certified by the College of American Pathologists. For each patient, 5 serial parasite densities were measured according to the following sampling schedule: 1) admission; 2) ≈12 hours later; 3) morning of the second day of

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Table. Characteristics of children infected with severe *Plasmodium falciparum* malaria parasites harboring the Kelch 13 A578S polymorphism compared with children infected with wild-type parasites, Uganda

Characteristic	A578S mutation, n = 3	Wild-type parasite, n = 75	p value
Parasite clearance time, median (interquartile range), h	80 (71–200)	45 (40–64)*	0.033
Clearance half-life, median, (interquartile range), h	5.9 (5.6–10.7)	4.5 (3.6–5.8)*	0.074
Prior artemisinin exposure, no. (%)	1 (33.3)	19 (25.3)	1.000
Deaths, no. (%)	0	8 (10.7)	1.000
Recrudescence or reinfection	0	0	1.000

*For 7 patients who died, parasite clearance time could not be calculated because of incomplete parasite clearance prior to death. For these same 7 children, the parasite clearance half-time ($t_{1/2}$) could not be calculated because of insufficient data points. For 1 child who died, clearance of parasitemia before death was documented; thus, the parasite clearance time and $t_{1/2}$ could be computed. Estimates of parasite clearance time and clearance $t_{1/2}$ are based on the remaining 68 patients. All fatal cases were associated with wild-type parasites.

admission; 4) morning of the third day of admission; and 5) morning of the fourth day of admission.

To measure parasite clearance kinetics, we used a standardized tool, the parasite clearance estimator, which expresses parasite clearance as the parasite's half-life ($t_{1/2}$), which was calculated by using the slope of the linear portion of the curve of log-transformed parasite densities over time (*13*). In addition, we computed the parasite clearance time, defined as the interval between the start of treatment and the first of 2 sequential negative peripheral blood films (*14*).

To determine molecular markers of resistance, we amplified and sequenced the *PF3D7_1343700* gene by using the nested PCR method, as described (*3*), with some modifications. DNA was extracted from cryopreserved erythrocyte fractions by using QIAGEN columns (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The K13-propeller domain was amplified by using K13-1 5′-CGGAGTGACCAAATCTGGGA-3′ and 5′-K13-4 GGGAATCTGGTGGTAACAGC-3′ for the primary PCR and K13-2 5′-GCCAAGCTGCCATTCATTTG-3′ and K13-3 5′-GCCTTGTTGAAAGAAGCAGA-3′ for the nested PCR. DNA sequencing of the 810-bp nested PCR product was performed to determine the amino acid haplotype of residues. Results were aligned to reference PF3D7 kelch protein, putative (PF13_0238) mRNA, complete coding sequence (National Center for Biotechnology Information reference sequence XM_001350122.1).

We identified limited diversity within the K13 gene. For 16 loci tested (amino acid positions 439, 441, 458, 465, 467, 476, 493, 522, 539, 543, 557, 558, 580, 617, 619, and 637), the wild-type sequence was found in all 78 parasite amplicons. We did not observe any of the most common amino acid substitutions in K13 associated with artemisinin resistance in *P. falciparum* isolates from Cambodia (C580Y, R539T or Y493H) (*3*), nor the I543T and N458Y mutations most strongly associated with increased clearance $t_{1/2}$ in another recent study (*4*), nor the M476I mutation selected in vitro under artemisinin pressure (*3*). Similarly, these point mutations were absent in isolates from Senegal and Uganda and in >1,100 *P. falciparum* parasites from 14 sites across sub-Saharan Africa (*8–10*). However, a previously reported point mutation, A578S (*9*,*15*), was found in 3 (3.8%) of 78 infections.

The Table shows infections with A578S parasites compared with infections caused by wild-type parasites. Parasite clearance time was prolonged in infections with A578S mutant parasites, and a similar trend was observed for clearance $t_{1/2}$. These 2 types of infections showed no differences in prior artemisinin exposure, number of deaths, or recrudescence or reinfection at day 14 from date of admission.

Conclusions

The role of the A578S amino acid substitution is unclear, but it occurs near the most common K13-propeller mutation (C580Y), which has been associated with delayed parasite clearance in Southeast Asia and with tolerance to artemisinin in vitro (*3*). Computational modeling suggests that A578S should considerably affect the tertiary structure of the K13 protein, thereby destabilizing the domain scaffold and altering its function (*15*). Isolates with A578S exhibited a phenotype of prolonged clearance under artesunate treatment in our study. Delayed clearance of A578S parasites was not observed in previous reports (*4*,*9*), although the number of isolates in our study and in others was small. Because multiple independent mutations in K13 have arisen in geographic regions engaged in intense treatment of malaria with artemisinin derivatives (*7*) and because only 2 point mutations were necessary to confer drug tolerance in vitro to a *P. falciparum* isolate from Tanzania (*3*), we are concerned that parasites with the A578S mutation are already causing severe malaria in children in Uganda, although with low frequency.

The nonsynonymous SNP A578S in the K13-propeller domain may represent another putative marker of delayed response to artesunate, although this indicator occurred infrequently in our cohort of children with severe malaria in Uganda. Our study documents an association between A578S and prolonged parasite clearance time, a finding relevant for future monitoring of *P. falciparum* response to parenteral artesunate in children in Africa.

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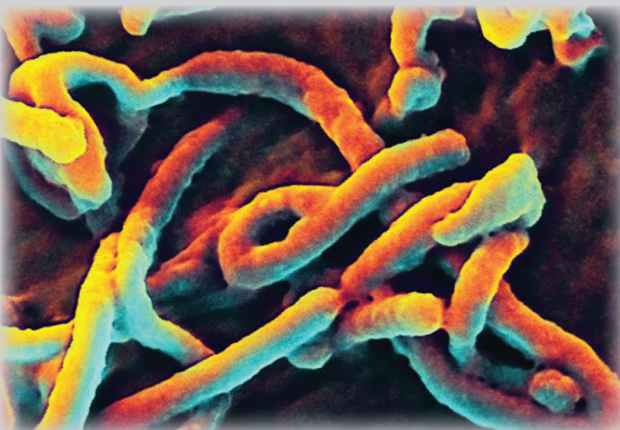

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Gastroenteritis Outbreaks Caused by Norovirus GII.17, Guangdong Province, China, 2014–2015

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In the past decade, the most prevalent norovirus genotype causing viral gastroenteritis outbreaks worldwide, including China, has been GII.4. In winter 2014–15, norovirus outbreaks in Guangdong, China, increased. Sequence analysis indicated that 82% of the outbreaks were caused by a norovirus GII.17 variant.

Norovirus infection is a leading cause of nonbacterial gastroenteritis outbreaks in industrialized and developing countries (*1*,*2*). On the basis of amino acid identity in viral protein 1, noroviruses can be divided into at least 6 genogroups (GI–GVI). GI and GII infect humans and can be further classified into genotypes; at least 9 genotypes belong to GI and 22 belong to GII (*3*). During the past decade, most reported norovirus outbreaks were caused by GII.4 norovirus (*4*,*5*). New variants of GII.4 have emerged approximately every 2–3 years and have caused norovirus gastroenteritis pandemics globally (*6*). Since 1999, the major circulating genotype in mainland China has been GII.4, accounting for 64% of all genotypes detected (*7*). In winter 2014–15, norovirus outbreaks in Guangdong Province, China, increased. Sequence analyses showed that the major cause of continuous gastroenteritis outbreaks in the region was a rarely reported norovirus genotype: GII.17.

The Study

In China, according to the National Public Health Emergency Contingency Plan, an outbreak with a cluster of at least 20 acute gastroenteritis cases (meeting the Kaplan criterion) within 3 days must be reported to Guangdong Provincial Center for Disease Control and Prevention. Samples from each outbreak are first tested for norovirus (Norovirus RT-PCR Kit; Shanghai ZJ Bio-Tech Co., Ltd., Shanghai, China) and for intestinal bacteria at the local Centers for Disease Control and Prevention. The norovirus-positive specimens are delivered to the Guangdong Provincial Center for Disease Control and Prevention for further genotyping.

From each outbreak, 5–10 samples are randomly selected for sequencing. Testing with One-Step RT-PCR (QIAGEN, Valencia, CA, USA) is performed with region C–specific primer, as previously described (*8*). The positive PCR products are sequenced, and norovirus genotypes are determined by using the Norovirus Automated Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool) or blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The major norovirus genotype causing each outbreak is defined as 1 genotype detected in >80% samples from the outbreak.

From January 2013 through January 2015, a total of 52 norovirus outbreaks were reported and were associated with 4,618 clinical cases; of these, 14 outbreaks were associated with ≈100 clinical cases. Of the 52 outbreaks, 44 (85%) occurred in schools and colleges, 5 (9.6%) in factories, and 3 (5.7%) in kindergartens. In Guangdong Province, norovirus outbreaks are highly seasonal; most (96%) outbreaks are reported from November through March (Figure 1). In late 2014, an increase in the number of norovirus outbreaks was noted. From November 2014 through January 2015, a total of 29 identified outbreaks were associated with 2,340 cases compared with 9 outbreaks and 949 cases the previous winter (2013–14).

Samples from 46 (88%) of the 52 outbreaks were successfully genotyped. GII norovirus was detected in samples from 96% of the outbreaks. From January 2013 through October 2014, the most common genotype found was GII.4/Sydney/2012, which was detected in samples from 48% of the outbreaks. Genotype GII.17 was first detected in the city of Guangzhou in November 2014 and thereafter spread rapidly (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/7/15-0226-Techapp1.pdf). From November 2014 through January 2015, GII.17 norovirus outbreaks were reported in 10 cities of Guangdong Province and represented 83% (24 of 29) of all outbreaks. In contrast, during 2013 and 2014, norovirus outbreaks caused by GII.4/Sydney/2012 were reported in only 5 cities in Guangdong.

The nucleotide sequences of the norovirus GII.17 strains from Guangdong have been deposited in GenBank

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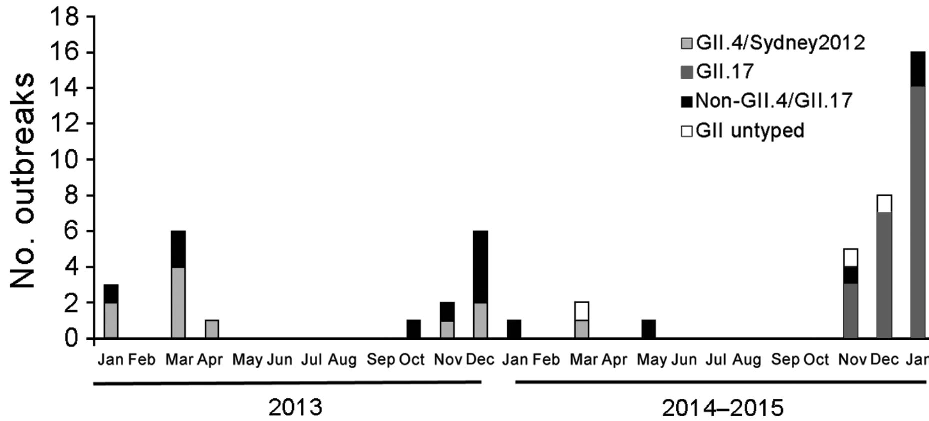

Figure 1. Norovirus outbreaks in Guangdong, Province, China, January 2013–January 2015.

(accession nos. KP718638–KP718738). For phylogenetic analysis, representative strains from Guangdong were compared with GII.17 reference strains from the GenBank database. On the basis of region C sequences, genotype GII.17 could be divided into 2 major clusters (a and b). GII.17 strains collected from Guangdong during 2014–2015 norovirus outbreaks all clustered together and belonged to cluster b, the cluster to which all strains identified after 2011 belonged. Sequence comparison suggested that the strains most closely related to Guangdong GII.17 were from neighboring regions (e.g., Taiwan, Korea, and Japan) and from groundwater in Kenya (Figure 2).

Conclusions

Outbreaks of nonbacterial gastroenteritis in Guangdong Province, China, during winter 2014–15 were caused by a rare norovirus, genotype GII.17. Previous epidemiologic data suggest that in the past 2 years, GII.4/Sydney/2012 has been the major circulating norovirus genotype worldwide (*4*,*5*,*9*). This GII.4 variant was first detected in Australia in March 2012 and was subsequently detected in France, New Zealand, Japan, the United Kingdom, the United States, and Hong Kong, and led to increased norovirus activity globally (*5*). In China, the GII.4/Sydney/2012 strain, first reported in October 2012, caused increased sporadic cases in the city of Shanghai (*10*). In early 2013 in Guangdong, GII.4/Sydney/2012 was the predominant norovirus genotype detected in norovirus outbreaks, while other genotypes including GII.3, GII.6, GI.2, GI.3, and GII.12 were occasionally detected. In winter 2013–14, detection of GII.4/Sydney/2012 decreased while detection of GII.3 and GII.6 increased. Norovirus genotype GII.17 was detected in the outbreak that occurred on November 18, 2014. Compared with GII.4/Sydney/2012, this variant of GII.17 displayed a high epidemic activity; in only 2 months, an increased number of related outbreaks were reported in 10 cities (Figure 1).

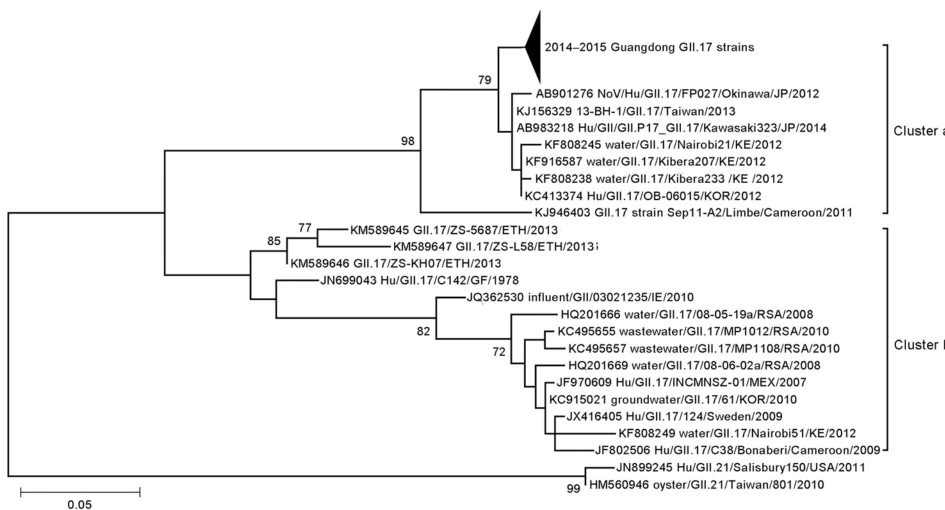

Figure 2. Phylogenetic tree of noroviruses based on the 282-bp region of the capsid N terminus/shell gene. Nucleotide sequences were analyzed by using the maximum-likelihood method. Supporting bootstrap values >70 are shown. The subtrees of GII.17 detected in Guangdong Province, China, during 2014–2015 were compressed. GII.21 genotype strains were used as outgroups. Scale bar indicates nucleotide substitutions per site. Sequences of 24 reference norovirus strains are included. Arrowhead represents number of strains from Guangdong, 2014–2015. ETH, Ethiopia; GF, French Guiana; IE, Ireland; JP, Japan; KE, Kenya; KOR, Korea; RSA, The Republic of South Africa; MEX, Mexico.

Sequence comparison with archived GII.17 strains from GenBank suggests that the GII.17 genotype identified in Guangdong is a newly emerged variant, differing from GII.17 strains detected before 2011. The recent detection of this new variant in samples from patients with sporadic cases in several regions of Asia (e.g., Korea, Japan, and Taiwan) and from groundwater in Kenya (*11*) suggests that this variant of GII.17 has circulated in a wide range of areas in recent years. For GII.17, most (66 [83%] of 80) sequences from the GenBank database are restricted to region C, the short conserved sequences of the N terminus of the capsid gene. This conserved region has been widely used for genotyping strains (*12*) and phylogenetic studies (*13*). To include more reference strains and to illustrate the relationship between GII.17 from Guangdong and other regions, we mainly used region C for phylogenetic analyses in this study. Similarly, phylogenetic analysis based on the nearly full length of capsid sequences also showed that the newly emerged GII.17 variant in Guangdong clustered with the strains from Japan and Taiwan in 2013 and 2014 and differed from GII.17 strains detected before 2011 (online Technical Appendix Figure 2).

In conclusion, a norovirus genotype GII.17 variant emerged in winter 2014–15 and caused outbreaks in multiple cities in Guangdong Province, China. The distribution of GII.17 genotype among patients with sporadic cases of gastroenteritis remains unknown. In future studies, epidemiologic and virologic surveillance should be broadened to better clarify virologic, clinical, and epidemiologic patterns of this newly emerged norovirus.

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Ebola Virus Stability on Surfaces and in Fluids in Simulated Outbreak Environments

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Kerri Miazgowicz, Trenton Bushmaker,
Joseph Prescott, Vincent J. Munster

We evaluated the stability of Ebola virus on surfaces and in fluids under simulated environmental conditions for the climate of West Africa and for climate-controlled hospitals. This virus remains viable for a longer duration on surfaces in hospital conditions than in African conditions and in liquid than in dried blood.

Since March 2014, >22,000 cases of Ebola virus disease (EVD) and ≈ 9,000 deaths have been reported in West Africa (*1*). Thousands of health care professionals have been mobilized to West Africa to assist with the ongoing outbreak of EVD (*2*). More than 800 Ebola virus (EBOV) infections have been reported in health care professionals (*1*).

Determining the persistence of EBOV on surfaces and under environmental conditions specific to outbreak settings and disease-endemic areas is critical to improving safety practices for these health care workers (*3*), as well as answering questions about EBOV transmission among the public (*4*). Researchers have experimentally assessed the stability of other EBOV strains on plastic, glass, and steel within dried media or guinea pig serum (*5*); in the dark on glass (*6*); and during exposure to UV light (*7*). However, the environmental conditions of these studies do not reflect the higher temperatures and relative humidities (RHs) in outbreak regions, or the current outbreak strain. No infectious EBOV could be found during environmental sampling in a ward with EVD patients; however, this result could be more indicative of cleaning measures than actual virus stability (*8*).

We report stability of EBOV with a current outbreak strain from Guinea (Makona-WPGC07) (*9*) on 3 clinically relevant surfaces: stainless steel, plastic, and Tyvek (Dupont, Wilmington, DE, USA). We also determined the stability of EBOV in water, spiked human blood, and blood from infected nonhuman primates (NHPs). These experiments were conducted in 2 environmental conditions, 21°C, 40% RH, and 27°C, 80% RH, to simulate a climate-controlled hospital and the environment in West Africa, respectively.

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The Study

We tested the stability of EBOV on 3 materials commonly found in an Ebola treatment unit (ETU) in West Africa: 1) utility-grade (308) stainless steel washers (McMaster-Carr, Atlanta, GA, USA); 2) plastic (Teflon [polytetrafluoroethylene]; McMaster-Carr); and 3) Tyvek (from the front of a coverall). For each time point, 3 disks (4-cm diameter) of each material were placed individually into wells of a 6-well plate. Five samples (10 μL/sample) containing a total dose of 10^6 50% tissue culture infectious doses ($TCID_{50}$s) of EBOV in cell-free medium were evenly distributed on the disks. The plates were divided into groups, and each group was placed into a plastic HEPA-filtered box and placed at 21°C, 40% RH, or 27°C, 80% RH. The samples were dried naturally, and virus titers were determined over a 14-day period.

In the surface and fluid stability experiments, all samples were stored at –80°C until titration (1 freeze–thaw cycle of EBOV samples that did not change virus titer). Titrations were performed on Vero E6 cells as described (*10*,*11*). The $TCID_{50}$ per milliliter for each sample at each time point was calculated by using the Spearman-Karber method (*11*).

Because viral decay rates often exhibit first-order kinetics (*12*), we $\log_{10}$ transformed our $TCID_{50}$ calculations to represent virus titer and used a linear regression analysis (Prism version 6.05; GraphPad, San Diego, CA, USA) to determine the $\log_{10}$ reduction rate of EBOV on each surface at both environmental conditions (Figure 1; Table 1). We also determined whether linear regression models were significantly different from each other at the $p<0.05$ level by using an analysis of covariance equivalent test in Prism. Overall, virus remained viable longest in hospital conditions and on Tyvek. Viable EBOV was detectable for 3 days on Tyvek at tropical conditions.

The stability of EBOV in water was assessed by diluting 150 μL virus stock in 2.85 mL of Ambion diethylpyrocarbonate–treated water (Thermo Fisher Scientific, Pittsburgh, PA, USA) and removing residual protein and medium with 1 initial and 2 rinse spins on Amicon Ultra Centrifugal Filters 100K MWCO (Merck, Darmstadt, Germany). EBOV was more stable in water at 21°C and had an ≈1 $\log_{10}$ reduction/day in water at 27°C (Table 1; Figure 2, panel A).

The stability of EBOV in human blood was assessed by spiking blood samples from a healthy human volunteer

[1]These authors contributed equally to this article.

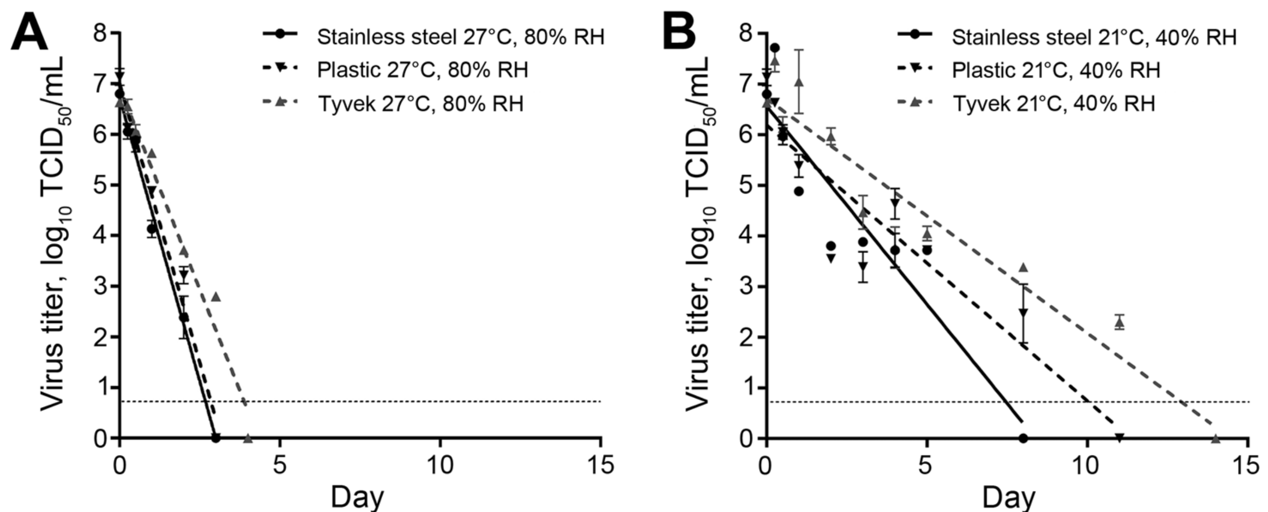

Figure 1. Linear regression model showing the effect of different environmental conditions and surfaces on survival of Ebola virus (EBOV). Virus was dried on 3 surfaces found in outbreak settings at A) 27°C, 80% relative humidity (RH) (West African tropical conditions) and B) 21°C, 40% RH (climate-controlled hospital conditions). Virus concentration was reduced at a significantly slower rate on all surfaces in hospital conditions than in tropical conditions ($p<0.0001$ for all surfaces). Triplicate samples were taken at each time point. Error bars indicate mean ± SEM virus titer. Dashed line indicates the limit of detection for the assay. An analysis of covariance equivalent test was used to compare linear regression models and determine differences in virus reduction rates. $TCID_{50}$, 50% tissue culture infectious dose.

to achieve a 10^5 $TCID_{50}$/mL virus titer. The spiked blood was distributed in 1-mL aliquots into closed screw-top vials to maintain a liquid state, spread in 50-µL aliquots onto the bottom of a 24-well plate, and dried. One group of samples was stored at 21°C, 40% RH, and the other group was stored at 27°C, 80% RH. EBOV stability in drying blood exhibited first-order kinetics and was viable for up to 6 days at tropical conditions (Table 1; Figure 2, panel B).

To approximate the stability of EBOV in naturally infected human blood, we used blood from cynomolgus macaques (*Macaca fascicularis*) as a proxy. Blood was collected during necropsy from 3 macaques that were previously enrolled in an Animal Care and Use Committee–approved EBOV pathogenesis study and were euthanized because they exhibited signs of EVD and viremia. Blood samples were divided into 2 groups with 2 sets of 150-µL aliquots for each time point; each group was stored at the conditions described above with each set in the liquid or drying state, and virus viability was assessed over a 14-day period. Because of variation in calculated virus titer from each of the individual NHPs, the $\log_{10}$ reduction rate could not be approximated and only the initial titer and the duration of viability are shown (Table 2). In general,

Table 1. Linear regression models for survival of Ebola virus on surfaces and in fluids at different environmental conditions*

Condition	Temperature, °C	Relative humidity, %	Model†	r^2	Virus log reduction time, d‡
Stainless steel	27	80	Y = –2.240X + 6.729	0.9798	0.45
Stainless steel	21	40	Y = –0.7829X + 6.564	0.8544	1.3
Plastic	27	80	Y = –2.205X + 7.008	0.9745	0.45
Plastic	21	40	Y = –0.5445X + 6.188	0.8303	1.8
Tyvek	27	80	Y = –1.599X + 6.939	0.9713	0.63
Tyvek	21	40	Y = –0.4631X + 6.709	0.8878	2.2
Drying human blood	27	80	Y = –0.6806X + 4.951	0.8724	1.5
Drying human blood	21	40	Y = –0.6917X + 4.828	0.9037	1.5
Liquid human blood	27	NA	Y = –0.1148X + 4.651	0.2892	8.7
Liquid human blood	21	NA	Y = –0.05000X + 4.231	0.05293	20
Water	27	NA	Y = –1.133X + 4.483	0.9607	0.88
Water	21	NA	Y = –0.5694X + 4.201	0.9139	1.8

*NA, not applicable.
†Y, $\log_{10}$ 50% tissue culture infectious dose/mL; X, days.
‡In hospital conditions, virus titer on steel was reduced significantly faster than on plastic (p = 0.004) and on Tyvek ($p<0.0001$), but there was no significant difference in reduction between Tyvek and plastic (p = 0.13). In tropical conditions, there was no significant difference in virus titer reduction on steel and on plastic (p = 0.78). However, virus decayed more slowly on Tyvek than on steel ($p<0.0001$) and on plastic ($p<0.0001$). There was no significant difference in reduction rate in virus titer in drying human blood in hospital or tropical conditions (p = 0.92). Stability of virus in liquid blood did not fit a linear regression model. Virus was reduced significantly faster at 27°C than in water at 21°C (p = 0.0001).

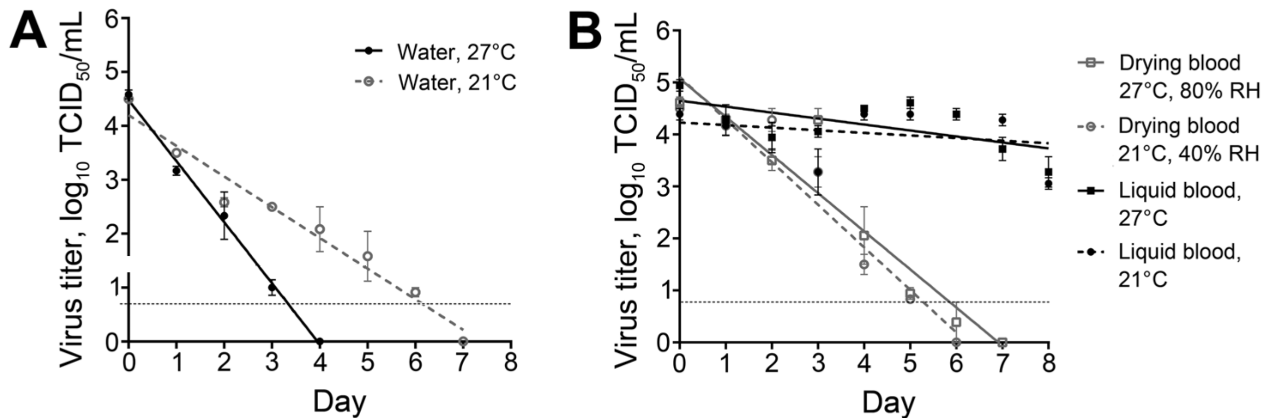

Figure 2. Linear regression model showing stability of Ebola virus (EBOV) in fluids under different environmental conditions. A) EBOV stability in water at 2 environmental temperatures. Virus concentration was reduced at a significantly faster rate in 27°C water than in 21°C water (p = 0.0001). B) Stability in drying or liquid EBOV-spiked human blood samples at 2 environmental conditions. Virus concentration was reduced at a significantly faster rate by drying than in liquid blood at both conditions (p<0.0001 for each condition). No significant difference between reduction rates in virus titer in drying human blood at both conditions was found (p = 0.92). Triplicate samples were taken at each time point. Error bars indicate mean ± SEM virus titer. Dashed line indicates the limit of detection for the assay. An analysis of covariance equivalent test was used to compare linear regression models and determine differences in virus reduction rates. $TCID_{50}$, 50% tissue culture infectious dose.

EBOV maintained viability for a longer duration in liquid than in drying blood regardless of initial titer or environmental condition.

Conclusions

We found that EBOV can persist on surfaces common in an ETU, highlighting the need for adherence to thorough disinfection and doffing protocols when exiting the ETUs and careful handling of medical waste. In addition, EBOV maintains viability for a longer duration in liquid than in dried blood. EBOV in blood of experimentally infected NHPs persists for a similar duration as EBOV in spiked human blood. A recent study showed that blood in the body cavity of an NHP contained viable EBOV for up to 7 days after death (*13*). We detected viable EBOV in drying blood for up to 5 days at both environmental conditions in human and NHP blood. Therefore, dried and liquid blood from an infected person in their home or ETU should be treated as potentially infectious. The finding that EBOV remains viable in water for as long as 3 (27°C) or 6 (21°C) days at the experimental concentration warrants further investigation into the persistence of the virus in aqueous environments, such as in wastewater or sewage canals. Viable EBOV has been isolated from urine (*14*) but not from human stool (*8*). Therefore, the potential for dissemination of EBOV through wastewater remains unknown.

This study is subject to several limitations. First, because standard volumes for samples were used, different volumes or matrices could influence the stability of EBOV under the tested conditions. Second, blood samples from the NHPs might have different immunologic or biochemical conditions, which can potentially influence virus stability. Third, the experimental conditions in the laboratory are sterile, but in disease-endemic areas and ETUs, bacteria or chemicals could influence EBOV viability.

Overall, we found that different environmental conditions, fluids, and surfaces influence the persistence of EBOV. These findings demonstrate that such factors are crucial in understanding transmission and improving safety practices.

Table 2. Stability of Ebola virus in infected nonhuman primate blood under different environmental conditions*

Blood sample, condition	Initial virus titer, $\log_{10}$ $TCID_{50}$/ mL	No. days viable
NHP 1		
Drying 27°C, RH 80%	6.5	5
Drying 21°C, RH 40%	6.5	1
Liquid 27°C	7.2	14
Liquid 21°C	7.2	14
NHP 2		
Drying 27°C, RH 80%	2.8	5
Drying 21°C, RH 40%	2.8	1
Liquid 27°C	4.2	11
Liquid 21°C	4.2	1
NHP 3		
Drying 27°C, RH 80%	7.2	4
Drying 21°C, RH 40%	7.2	4
Liquid 27°C	6.5	8
Liquid 21°C	6.5	14

*$TCID_{50}$, 50% tissue culture infectious dose; NHP, nonhuman primate; RH, relative humidity.

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Dr. Fischer and Dr. Judson are researchers in the Virus Ecology Unit at Rocky Mountain Laboratories in Hamilton, Montana. Their research interests are the ecology and evolution of emerging infectious diseases and relationships between human and environmental health.

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Outbreak of Ciprofloxacin-Resistant *Shigella sonnei* Associated with Travel to Vietnam, Republic of Korea

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We investigated an October 2014 outbreak of illness caused by *Shigella sonnei* in a daycare center in the Republic of Korea (South Korea). The outbreak strain was resistant to extended-spectrum cephalosporins and fluoroquinolones and was traced to a child who had traveled to Vietnam. Improved hygiene and infection control practices are needed for prevention of shigellosis.

Shigella spp. are etiologic agents of gastrointestinal disease worldwide and are frequently associated with outbreaks because of their low infectious doses and person-to-person transmission (*1*,*2*). For the treatment of persons who have severe infections, fluoroquinolones are among the first-line agents for adults; additionally, oral extended-spectrum cephalosporins are used to treat young children. However, the current emergence and spread of drug resistance in *Shigella* strains could hinder empirical antimicrobial therapy, leading to treatment failure. *S. sonnei* is the most frequently isolated species among all cases of *Shigella* infection in industrialized countries (*3*), and it has become increasingly prevalent across Southeast Asia in recent decades (*4*). Recently, international travel to areas where the disease is highly endemic has accelerated the global spread of drug-resistant *S. sonnei* to nonendemic regions. Here, we describe a travel-associated outbreak of illness caused by a *S. sonnei* strain that was resistant to extended-spectrum cephalosporins and fluoroquinolones.

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The Study

In the beginning of October 2014, six children who were vomiting and experiencing abdominal cramping and diarrhea were admitted to the local hospital in Gyeongsangnam-do, Repulic of Korea (South Korea). All patients attended the same daycare center that provided care and food to children from low-income families. Fecal specimens from 6 patients were submitted to the local public health laboratory and were processed according to a standard bacterial culture method. On October 6, Korea Centers for Disease Control and Prevention was notified that *S. sonnei* phase II were identified from all fecal samples. An epidemiologic investigation was conducted to determine the extent of the outbreak and to identify the mode of transmission. A confirmed case was identified by passive and active case-finding on the basis of laboratory-identified *S. sonnei* isolates in the fecal specimens of center attendees and staff members, families of the children, and persons in the community. A probable case was defined as a person with any shigellosis symptoms and an epidemiologic link to infected patients whose cultures were negative. The children's guardians were interviewed by using a standardized questionnaire that requested information on symptoms, food consumption, recent travel history, and contact persons. This investigation was part of a public health emergency response and was accordingly exempt from institutional review board approval.

The investigation revealed that an 8-year-old boy (the index case-patient in this outbreak) had recently returned after visiting family in Vietnam, where *S. sonnei* infection is highly endemic. He had experienced sustained diarrheal episodes since his return, and after returning to the daycare center, children in the daycare center began having similar symptoms. Cases of shigellosis were also identified among the grandparents of the index case-patient and a person the family visited in a geographically distant location on September 27. No isolates were obtained from the environmental samples collected, including foods, drinking water, and surface swab specimens of the daycare facility.

Eleven laboratory confirmed and 4 probable cases were identified during this outbreak. The median age of the patients in the daycare center was 7.8 (range 4–13) years. Overall, the reported symptoms were diarrhea (≥3 loose stools during 24 hours) and abdominal cramping; 4 patients were asymptomatic but their stool samples were

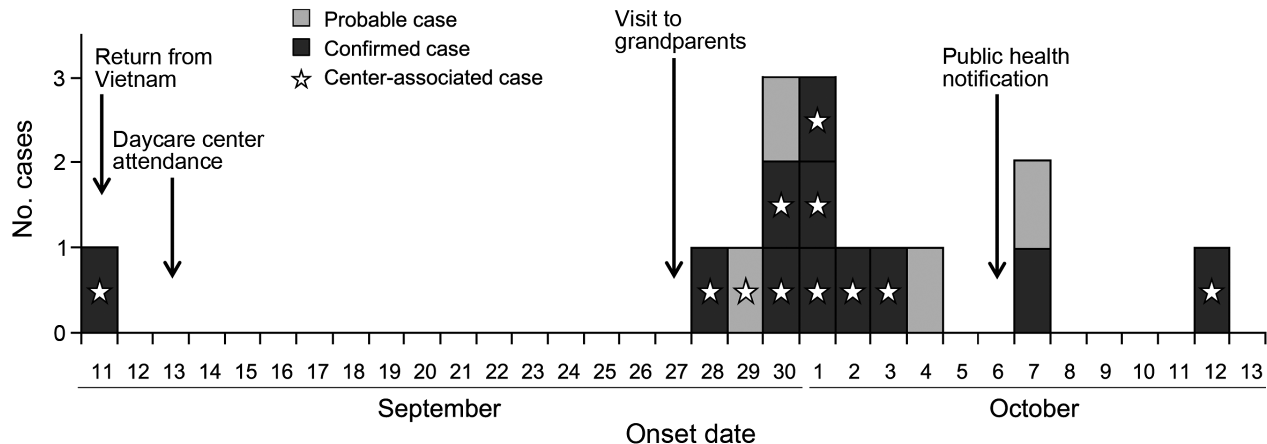
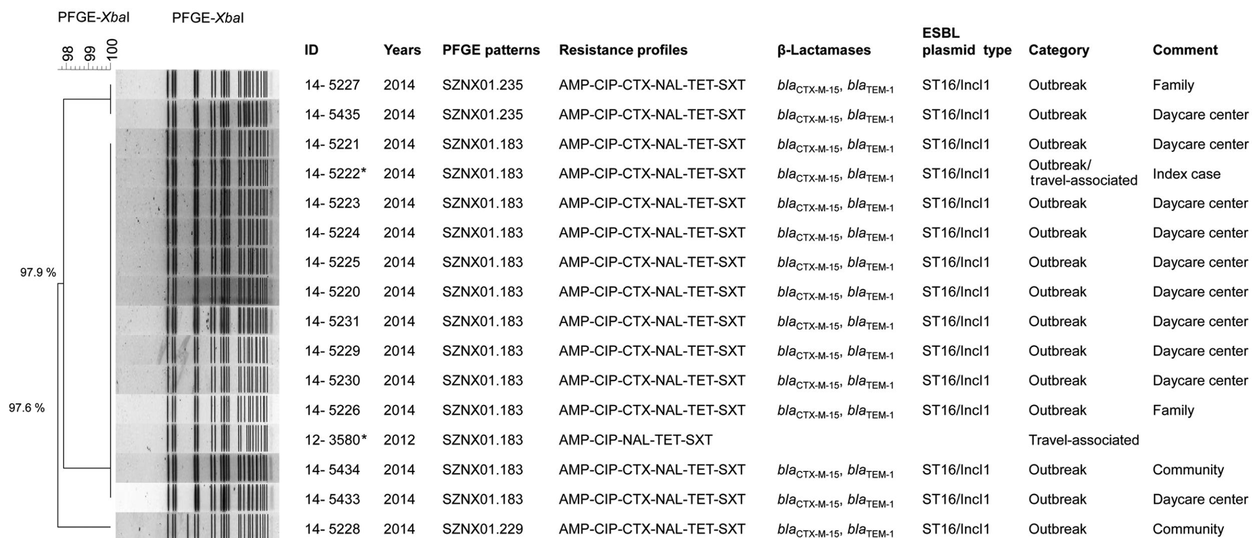


Figure 1. Epidemic curve of the outbreak of illness caused by *Shigella sonnei* infection, by symptom onset date, South Korea, 2014. Black bar sections indicate laboratory-confirmed cases; white bar sections indicate probable cases; stars indicate cases found in daycare center. Arrows indicate dates of the events for an index case-patient with travel history to Vietnam and of public health notification of the outbreak.

culture-positive. Of the 15 persons who became ill (Figure 1), 10 were treated with cefotaxime or ciprofloxacin, after which their stool samples were culture-negative. For 5 patients with continuing positive fecal culture, antibiotic drug treatment was later changed to cabapenems (meropenem or imipenem). According to local infection control guidelines, symptomatic patients were isolated in single-bed rooms until 2 consecutive fecal cultures tested negative for *S. sonnei*. To prevent the further spread of the disease, public health interventions were encouraged during the outbreak period: enforced handwashing at predetermined times at the daycare facility, strict hygiene measures in affected households, education about shigellosis, and environmental disinfection of the facility.

Laboratory-confirmed strains of *S. sonnei* were sent to Korea National Institute of Health for further characterization. All 15 isolates had identical or highly similar pulsed-field gel electrophoresis (PFGE) patterns after the *Xba*I digestion of chromosomal DNA. The main PFGE pattern of this outbreak (SZNX01.183; PFGE pattern number assigned by Korea National Institute of Health) had not been previously reported in domestic cases, and the isolate was genetically indistinguishable



ID	Years	PFGE patterns	Resistance profiles	β-Lactamases	ESBL plasmid type	Category	Comment
14- 5227	2014	SZNX01.235	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Family
14- 5435	2014	SZNX01.235	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5221	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5222*	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak/ travel-associated	Index case
14- 5223	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5224	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5225	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5220	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5231	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5229	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5230	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5226	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Family
12- 3580*	2012	SZNX01.183	AMP-CIP-NAL-TET-SXT			Travel-associated	
14- 5434	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Community
14- 5433	2014	SZNX01.183	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Daycare center
14- 5228	2014	SZNX01.229	AMP-CIP-CTX-NAL-TET-SXT	$bla_{CTX-M-15}$, bla_{TEM-1}	ST16/IncI1	Outbreak	Community

Figure 2. *Xba*I pulsed-field gel electrophoresis patterns of *Shigella sonnei* strains identified during a 2014 outbreak in South Korea and 2 isolated from samples from persons in Vietnam. The dendrogram was constructed by using Dice coefficient and UPGMA clustering, with 1.5% optimization and 1.5% position tolerance. Antibiotic resistance profiles and resistance determinants to extended-spectrum cephalosporins and fluoroquinolones are plotted next to the dendrogram. All strains had QRDR mutations GyrA(S83L,D87G) and ParC(S80I). *Strains 14-5222 and 12-3580 originated in Vietnam. AMP, ampicillin; CIP, ciprofloxacin; CTX, cefotaxime; NAL, nalidixic acid; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole; QRDR, quinolone resistance–determining region. Scale bar indicates percentage relatedness.

Table. Susceptibility profiles of outbreak *Shigella sonnei* isolate from index case-patient and *Escherichia coli* transconjugant strain used for testing, South Korea, 2014

Antimicrobial agent(s)	MIC, µg/mL		
	Shigella sonnei 14-5222	*E. coli* J53	*E. coli* J53, TC-14-5222
Nalidixic acid	>128	4	4
Ciprofloxacin	8	<0.12	<0.12
Ampicillin	>64	>64	>64
Ampicillin/sulbactam	16	4	16
Amoxicillin/clavulanate	16	8	16
Cefoxitin	4	4	4
Ceftazidime	8	<0.25	8
Cefotaxime	64	<0.25	64
Cefotaxime/clavulanate	<0.12	<0.12	<0.12
Cefepime	4	<1	4
Ceftriaxone	128	<0.12	128
Cefpodoxime	>32	0.5	>32
Cephalothin	>64	32	>64
Meropenem	<1	<1	<1
Imipenem	<2	<2	<2
Piperacillin/tazobactam	<4	<4	<4
Streptomycin	>128	4	4
Tetracycline	128	<2	<2
Chloramphenicol	8	8	8
Trimethoprim/sulfamethoxazole	>16	<1	<1
Gentamicin	2	<1	<1
Amikacin	<4	<4	<4
Azithromycin	1	1	1

from a ciprofloxacin-resistant *S. sonnei* strain isolated from a traveler returning from Vietnam during 2012 (Figure 2).

On the basis of MICs of antimicrobial agents determined by using a broth microdilution method (*5*), the outbreak strains were found to be resistant to both extended-spectrum cephalosporins (cefotaxime, MIC >32 µg/mL) and fluoroquinolones (ciprofloxacin, MIC >8 µg/mL). The strains were also resistant to tetracycline and trimethoprim/sulfamethoxazole but were susceptible to chloramphenicol, gentamicin, amikacin, and carbapenem. For azithromycin, an alternative oral agent for shigellosis, MICs were 1–2 µg/mL (Table). Extended-spectrum β-lactamase (ESBL) typing by using PCR and further sequencing (*6*) showed that all isolates carried the $bla_{CTX-M-15}$ and bla_{TEM-1} genes. ESBL plasmid of *S. sonnei* isolate from the index case-patient were successfully transferred to the recipient *E. coli* J53 Azi^R strain. PCR-based *inc/rep* typing and pMLST analysis of a transconjugant strain (*7*,*8*) showed that this ESBL plasmid was of the ST16/IncI1 type, which was previously identified in strain pKHSB1 from Vietnam (*9*). The genetic environment of the $bla_{CTX-M-15}$ gene was analyzed by PCR and sequencing with specific primers for the insertion sequences IS*Ecp1* and *orf477* (*6*). An intact IS*Ecp1* and truncated *orf477* were identified at 48 bp upstream and downstream of the *bla* gene, which has also been found in CTX-M-15-encoding plasmids from *Enterobacteriaceae* (*6*,*9*,*10*).

The outbreak strains had 2 mutations in the quinolone resistance-determining region of *gyrA* (Ser83Leu and Asp87Gly) and 1 mutation in *parC* (Ser80Ile [Figure 2]), which have been reported to be responsible for ciprofloxacin resistance in *S. sonnei* (*11*). However, *gyrB* and *parE* mutations and plasmid-mediated quinolone resistance genes were not detected (*12*).

Conclusions

We describe a shigellosis outbreak affecting children attending a daycare center, their family members, and residents of the surrounding community. To limit the extent of the outbreak, laboratory investigations of outbreak strains and infection-control measures including contact isolation and hand hygiene were immediately implemented, which may have contributed to preventing the further spread of this multidrug-resistant *S. sonnei* strain.

The outbreak strain was resistant to extended-spectrum cephalosporins and fluoroquinoloness and was introduced by a daycare center attendee who had returned from travel to Vietnam. The $bla_{CTX-M-15}$ gene in *S. sonnei* was first described in 2005 (*13*) and since then has been reported worldwide; we described an outbreak of CTX-M-15–producing *S. sonnei* in Korea in 2008 (*6*). The PFGE pattern of the 2008 outbreak strain (SZNX01.176) showed only 82.8% genetic similarity with that of the outbreak strains of the current study but was observed in several traveler-associated cases originating from China. These findings suggest that, despite the lack of direct evidence, various antimicrobial drug–resistant *S. sonnei* clones have been imported across geographic regions and may eventually spread globally and lead to increased illness and death rates.

In summary, we report a shigellosis outbreak in South Korea caused by a ciprofloxacin-resistant CTX-M-15–producing *S. sonnei* strain that originated from Vietnam.

Because international travel can contribute to the spread of multidrug-resistant pathogens, enhanced surveillance is necessary to control the dissemination of antimicrobial drug resistance. Improved hygiene, infection control plans, and better education for travelers are also required.

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Rapidly Expanding Range of Highly Pathogenic Avian Influenza Viruses

Jeffrey S. Hall, Robert J. Dusek, Erica Spackman

The movement of highly pathogenic avian influenza (H5N8) virus across Eurasia and into North America and the virus' propensity to reassort with co-circulating low pathogenicity viruses raise concerns among poultry producers, wildlife biologists, aviculturists, and public health personnel worldwide. Surveillance, modeling, and experimental research will provide the knowledge required for intelligent policy and management decisions.

The recent introduction of highly pathogenic avian influenza (HPAI) subtype H5N8 virus into Europe and North America poses major risks to poultry industries, zoologic collections, and wildlife populations; thus, this introduction warrants continued and heightened vigilance. First discovered in early 2014 in poultry and wild birds in South Korea, HPAI H5N8 virus apparently arose in China from reassortment events between HPAI subtype H5N1 virus (clade 2.3.4.4) and several low pathogenicity viruses (LPAIVs) (*1–3*). The H5N8 virus was subsequently detected in waterfowl in Russia in September 2014, and since then, H5N8 virus and reassortants have been detected in poultry and wild birds in Europe (Netherlands, Germany, Italy, the United Kingdom, Hungary, and Sweden), Taiwan, Japan, Canada (British Columbia), and the western and central United States (Washington, Oregon, California, Idaho, Utah, Minnesota, Missouri, Arkansas, Kansas, Iowa, Wyoming, and Montana).

Wild waterfowl are a primary natural host for LPAIVs, and infection rates in these populations peak at autumn migratory staging locations, where large numbers of immunologically naive juvenile birds congregate (*4*). The HPAI H5N8 virus has apparently adapted to wild waterfowl hosts: few or no clinical signs or adverse effects are apparent in these hosts when infected with the virus. Thus, it seems probable that the virus was disseminated out of Russia into Europe, East Asia, and North America by migrating waterfowl during autumn 2014 (*5*).

The HPAI H5N8 virus has encountered, interacted with, and reassorted with co-circulating LPAIVs in migratory and overwintering waterfowl populations, creating new HPAI viruses (HPAIVs). In Taiwan, new Eurasian lineage reassortant HPAIVs (i.e., H5N2 and H5N3 subtypes) and the parental H5N8 subtype virus have been detected in poultry and wild birds (*6*). In North America, HPAI H5N8 virus continues to circulate among waterfowl and commercial and backyard poultry flocks. In addition, new HPAIV reassortants (i.e., H5N2 and H5N1 subtypes) that are combinations of HPAI H5N8 virus and genetic elements from Eurasian and North American viruses are also circulating in these populations (*7*,*8*) (Figure).

Persistence of the original HPAI H5N8 virus for >1 year, the creation of multiple reassortant viruses that have maintained high pathogenicity in poultry, and adaptation of the virus to migrating waterfowl all indicate that these viruses could persist and spread in Northern Hemisphere waterfowl populations for an extended period. This dynamic of HPAIVs being transported by wild birds to new populations raises critical issues and poses a series of questions that researchers and modelers should examine in more detail. The risks are significant that these HPAIVs will continue to circulate and that new genetic combinations will arise in concentrations of overwintering waterfowl and then spill over into poultry operations and aviculture. The spillover risk is particularly high for operations with rudimentary biosafety practices (e.g., backyard flocks) and that trend toward outdoor access for organic poultry. Such events have already occurred in commercial poultry operations in Canada and some US states (California, Minnesota, Missouri, Iowa, and Arkansas), and subsequent culling operations and trade restrictions have caused substantial local economic losses. As wild birds begin their spring migrations and disperse into their breeding ranges, will they be transporting these viruses to new regions, including the rest of North America? Is this an inevitable outcome of HPAI H5N8 transmission in wild bird populations? Can these viruses be transported from Europe to eastern North America by migratory birds via North Atlantic routes (*9*)? Are there risks of these viruses reassorting with viruses from other species, such as swine, particularly feral swine whose populations are rapidly expanding, and will these reassortant viruses present greater risk of zoonotic disease?

These HPAIVs do not appear to pose substantial risks to waterfowl populations, but they may have detrimental effects on other, perhaps more sensitive, wildlife populations. Birds of prey seem to be particularly susceptible to HPAIV infection (*10*), including the HPAIV H5N8 virus that killed captive gyrfalcons (*Falco rusticolus*) that

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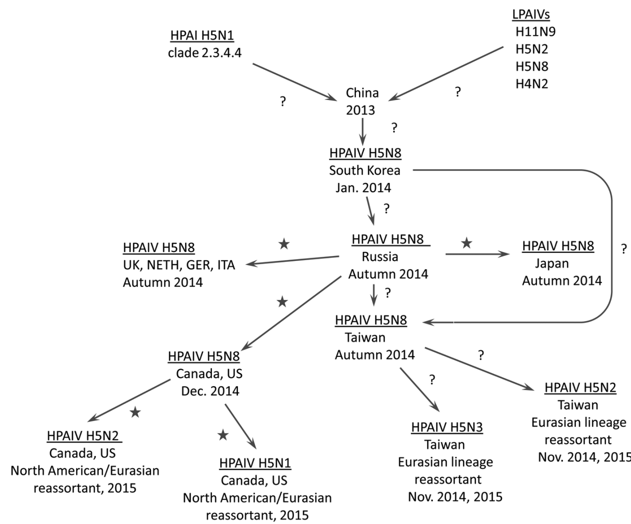

Figure. Genealogy of subtype H5N8 HPAIV, its spread from China to other countries, and its evolution in wild birds. Stars represent probable spread of virus and/or reassortment in wild birds; question marks indicate unknown mode. GER, Germany; HPAIV, highly pathogenic avian influenza virus; ITA, Italy; LPAIVs, low pathogenicity avian influenza viruses; Neth, the Netherlands; UK, United Kingdom; US, United States.

were fed infected duck carcasses (*7*). In North America, other raptor species have been found infected with H5N8 or H5N2 virus: Cooper's hawk (*Accipiter cooperii*), great horned owl (*Bubo virginianus*), red-tailed hawk (*Buteo jamaicensis*), peregrine falcon (*Falco peregrinus*), and bald eagle (*Haliaeetus leucocephalus*). It is not known what effect these viruses will have on small, at-risk wild bird populations, such as California condors (*Gymnogyps californianus*), that may prey on or scavenge infected birds, but the possible effects should be considered in conservation management decisions.

As HPAIVs continue spreading and evolving, the questions posed here, along with many more questions, will need to be answered to understand the risks to agriculture, zoologic collections, wildlife, and, potentially, human populations. As other researchers have recently pointed out, robust, targeted surveillance programs among wild birds (*11*) and poultry, modeling of the movements of HPAIV-infected wild birds, and experimental research studies will provide the knowledge required for intelligent policy and management decisions regarding agriculture, wildlife, and public health.

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Cluster of Ebola Virus Disease, Bong and Montserrado Counties, Liberia

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Lack of trust in government-supported services after the death of a health care worker with symptoms of Ebola resulted in ongoing Ebola transmission in 2 Liberia counties. Ebola transmission was facilitated by attempts to avoid cremation of the deceased patient and delays in identifying and monitoring contacts.

Reports of what has become the largest and longest epidemic of Ebola virus disease (EVD) began in March 2014 in West Africa (*1*). To interrupt Ebola transmission, health care authorities must promptly isolate and treat persons with EVD and identify and monitor exposed persons before symptoms develop (*2*). Effective contact tracing can limit the number of new cases; however, a single missed contact can result in many new cases (*3*). Gaps in contact tracing have been reported as challenges for infectious diseases such as sexually transmitted infections and tuberculosis (*4*–*6*). Because contact tracing requires patients to reveal names of persons with whom they have had contact and whom they may have exposed to illness, public health officials must quickly establish trust with sick persons and those at risk for disease (*3*,*7*).

We describe a cluster of EVD cases involving transmission across 2 jurisdictions in Liberia. Data for this report were derived from interviews, case reporting forms, treatment records, and laboratory results. This EVD cluster highlights the challenges associated with public health measures to interrupt transmission of Ebola.

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The Investigation

On December 8, 2014, a 78-year-old man (patient 1) from Gbarnga (Bong County), Liberia, was admitted to the Bong County Ebola Treatment Unit (ETU) where test results were positive for Ebola by reverse transcription PCR. He reported recent travel to Monrovia (Montserrado County), where he cared for his 32-year-old son, a health care worker who died from an acute illness.

On December 9, another son of patient 1 (patient 2, 39 years of age), who lived in Monrovia, had fever, headache, and malaise and sought care at hospital A in Bong County. He did not report contact with patient 1, nor did he report that he provided care for his sick brother in Monrovia. On December 10, hematemesis developed, and the patient was transferred to the Bong County ETU and treated for laboratory-confirmed EVD. Contact tracing identified 20 contacts living in Gbarnga. All contacts were initially symptom free and were quarantined at a local holding center for 21-day monitoring. No contacts in Monrovia were reported by patients 1 or 2.

On December 16, Bong County health officials were notified that a 15-year-old girl (patient 3) with fever, subconjunctival hemorrhage, and thrush was at hospital A. She had traveled 4 hours by taxi from Monrovia to be near her ill grandfather and father (patients 1 and 2) and did not report exposure to EVD patients or contacts in Monrovia. She was admitted to the ETU, and EVD was confirmed.

The next day, 4 additional family members who traveled by taxi from Monrovia were stopped at a roadside monitoring station in Gbarnga. All had fever and nonhemorrhagic symptoms and were transferred by ambulance to the ETU for evaluation; 2 family members (patients 4 and 5) had positive test results for EVD. The 2 family members whose results were negative for EVD, along with the taxi driver and a nonfamilial passenger, were transferred to a local holding center for 21-day monitoring. Contact investigations for patients 4 and 5 revealed no new contacts in Monrovia, but the patients reported that they resided in the same house in Monrovia with patients 2 and 3, who were receiving treatment in Bong County. Because family members with EVD had recently arrived from Monrovia and were being treated in Bong County, yet sources of infection and additional contacts were uncertain, Bong County requested that Montserrado County health officials conduct an investigation to identify patients and contacts at the Monrovia address so that potential EVD patients could be isolated and monitored.

Table. Characteristics of family members in Ebola cluster, Bong and Montserrado Counties, Liberia, November–December 2014

Patient no.	Relationship to patient 0	Age, y/Sex	Occupation	Date of symptom onset	Date admitted to ETU*	Outcome, date	City where Ebola exposure likely occurred†
0	–	32/M	Nurse's aide	Nov 14	–	Died, Nov 24	Monrovia
1	Father	78/M	Farmer	Dec 1	Dec 8	Recovered, Dec 24	Monrovia
2	Brother	39/M	Auto Mechanic	Dec 9	Dec 10	Recovered, Dec 23	Monrovia
3	Niece	15/F	Student	Dec 10	Dec 16	Recovered, Dec 30	Monrovia
4	Mother	55/F	Vendor in the market	Dec 15	Dec 17	Died, Dec 22	Monrovia
5	Son	3/M	–	Dec 16	Dec 17	Died, Dec 21	Monrovia
6	Cousin	29/F	Rubber plantation worker	Dec 16‡	Dec 18	Recovered, Dec 30	Gbarnga
7	Sister§	32/F	Vendor in the market	Dec 18	Dec 19	Died, Dec 21	Monrovia
8	Brother-in-law#	41/M	Construction worker	Dec 18	Dec 19	Died, Dec 27	Monrovia
9	Niece	10/F	Student	Dec 20‡	Dec 20	Recovered, Jan 9 (2015)	Gbarnga

*ETU, Ebola treatment unit.
†Gbarnga is located in Bong County; Monrovia is located in Montserrado County.
‡Became symptomatic while under observation at the Bong County Holding Center.
§Twin sibling of patient 0.
#Husband of patient 7.

The Monrovia investigation revealed that patients 1–3 had contact with patient 1's ill son, who was designated the putative source-patient (patient 0). Patient 0 was a nurse's aide at a community clinic. Fever, headache, joint pain, and abdominal pain developed in patient 0 on November 14, 2014, and he was cared for at home by his family for 7 days while his symptoms worsened. Although the patient and family members were aware of the EVD epidemic, they did not think patient 0 had EVD because he had no vomiting, diarrhea, and hemorrhagic symptoms; they believed he had a spiritual illness. On November 21, he was taken to a church with the hope that he would be healed through prayer. He died there on November 24, and his body was carried to his residence for mourning and burial preparation. Because all unexplained deaths were presumed to be Ebola related, an EVD burial crew retrieved his body for cremation the following day, despite resistance from the family and only after persuasion by local community leaders. No postmortem specimen was collected for EVD testing. After the body was removed, the home was sprayed with disinfectant, and the mattress, clothes, and other personal items used by patient 0 were burned. An attempt was made to identify additional contacts; however, the family was reluctant to cooperate with health officials and reported being angry about the cremation of patient 0 and destruction of property, although these practices were routine at that time for controlling EVD in Monrovia. The family in Monrovia began cooperating with Montserrado County health officials 3 weeks later, on December 18, after learning that 5 family members (patients 1–5) had EVD and after being provided with new mattresses and a small ration of food. At this time, they revealed 2 previously unreported symptomatic family members (patients 7 and 8). As of January 11, 2015, a total of 10 cases were included in this cluster. Eight (80%) patients in this cluster were not identified as contacts before their EVD diagnosis, and 4 (40%) sought care outside the county where they resided (Table; Figure).

Conclusions

Identifying sources of infection for index patients and tracing contacts are major components of EVD prevention and control efforts (*3*), yet carrying out these policies is challenging when those ill with EVD do not reveal the names of possible sources or contacts who could have been exposed to disease. Detection delays and ineffective contact tracing occurred in this cluster in part because the family believed that the mandatory cremation and property destruction taken as public health actions in Monrovia harmed more than helped. Consequently, some family members sought care in Bong County, riding 4 hours in a taxi from their home in Monrovia, a distance of ≈197 kilometers. Furthermore, family members were reluctant to reveal contact names in Monrovia and initially concealed knowledge of symptomatic persons.

This cluster may have been prevented if patient 0, presumably infected at the clinic where he worked, had been trained in infection control procedures and had access to personal protective equipment. Additional exposures and subsequent infections could have been prevented

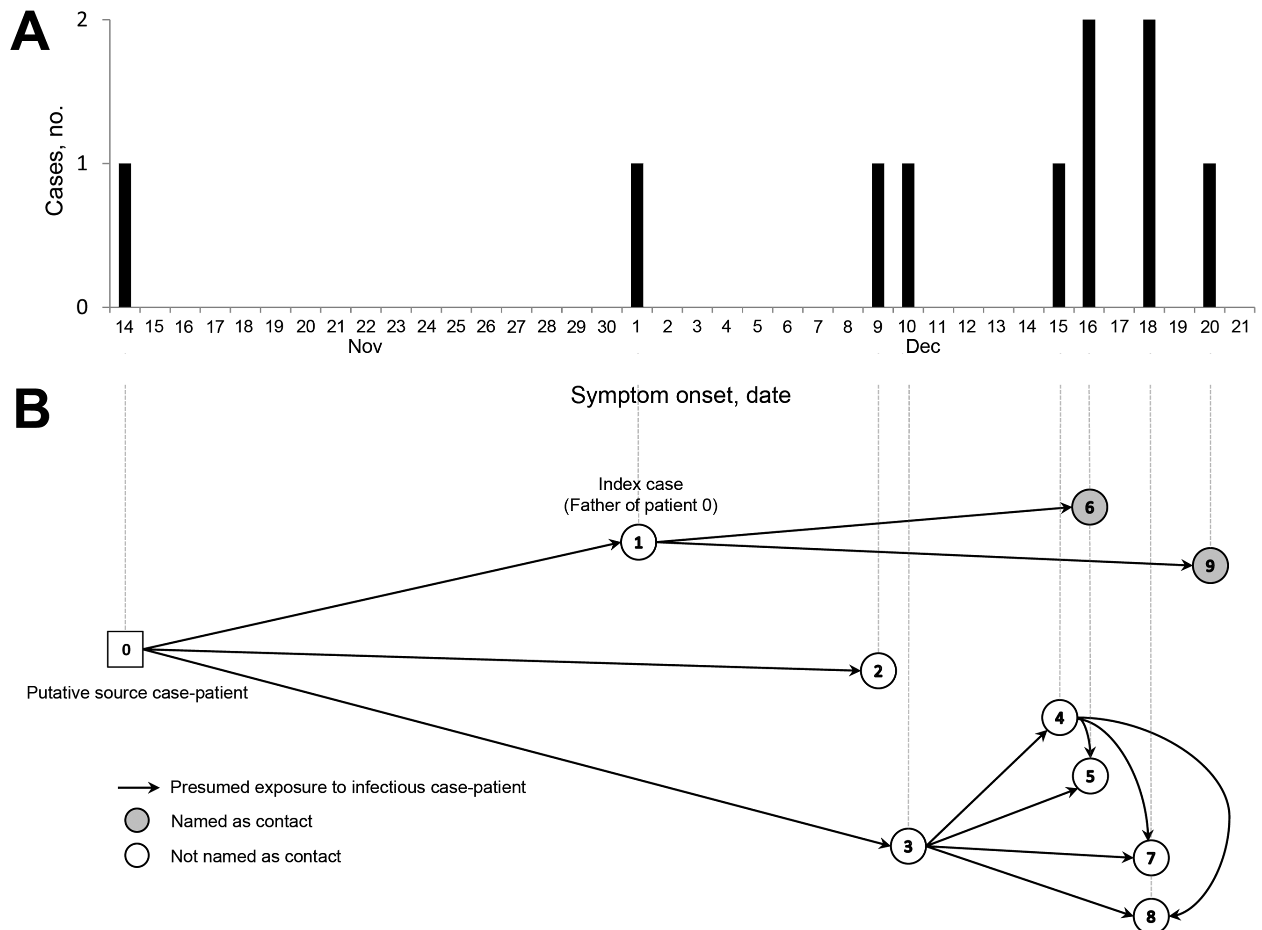

Figure. Timeline (A) and transmission diagram (B) of Ebola virus disease cluster, Bong and Montserrado Counties, Liberia, November–December 2014.

if he had been identified earlier as a suspected EVD patient, if testing had been performed on his body, if the results had been reported to the family, and if the Monrovia contacts had been followed daily to identify, isolate, and treat symptomatic persons. Had contact tracing identified patients 1–3 as patient 0's contacts and isolated them immediately after symptoms developed, 6 cases of EVD (in patients 4–9) and 4 deaths (patients 4, 5, 7, and 8) might have been prevented.

Rapid implementation of contact tracing to prevent disease transmission and increased coordination and communication between jurisdictions are critical to control of EVD. These efforts can identify case-patients who may have entered the community from another jurisdiction (to better understand importation and transmission patterns) and improve case finding and contact tracing to ensure that no cases are missed (*8*,*9*). The effectiveness of these efforts depends on trust between public health officials and the communities they serve.

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Mr. Nyenswah is the Head Minister of Health for the Ministry of Health and Social Welfare, Monrovia, Liberia. He has led the Ebola response in Liberia since 2014.

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EMERGING INFECTIOUS DISEASES

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Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

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ANOTHER DIMENSION

The Past Is Never Dead—Measles Epidemic, Boston, Massachusetts, 1713

David M. Morens

The recent measles epidemic in the United States has aroused public disbelief that a disease well-controlled for decades is reemerging to threaten children in the United States. Controversy surrounds measles vaccination in the United States; some parents have even avoided vaccinating their healthy children by exposing them to measles-infected children. However, measles has repeatedly reemerged in the United States during the past 3 centuries or longer (*1*,*2*), and its emergence patterns and means of preventing and controlling it are well understood. Until measles is globally eradicated—a goal within reach—it will continue to reappear, sicken, and kill almost anywhere, and we must energetically control each outbreak.

When we consider modern measles prevention, it is worth recalling what epidemics were like before vaccines and organized public health systems. One vivid account of measles describes the disease's deadly spread through a prominent Boston household >300 years ago. In 1713, America's first important medical figure (*3*), Puritan minister Cotton Mather (1663–1728), called by one authority "the Dr. Spock of the colonial New England" (*4*), wrote about a measles epidemic in the American colonies, describing not only its epidemiology and devastation but also the fear it elicited. Mather's account reminds us of the need for such modern medical and public health tools as vaccination, patient isolation, and prevention policies in saving families from the once-unpreventable diseases that compelled us to develop effective medical advances in the first place.

The following account, condensed from Cotton Mather's personal diary (*5*), focuses on illnesses in his own household, including those of his wife, 9 children, and a maidservant, over the course of 6 weeks during October–November, 1713.

Diary Excerpts

[18 October] …The Measles coming into the Town, it is likely to be a Time of Sickness…

[19 October] [I must]… lay hold on the Occasion to awaken Piety, and Preparation for Death, in the Souls of the children.

[24 October]… [on ≈18 October] my Son *Increase* fell sick…

[26 October] I must quicken the preparation of my Domesticks…

[27 October] My desirable Daughter *Nibby*, is now lying very sick of the *Measles*...

[28 October] … a very sensible Calamity is begun upon the Town… [with] some Degree of Mortality.

[30 October] The Spreading of the Measles… [is much worse in] Families, where they conflict with Poverty… This day, my Consort [wife], for whom I was in much Distress, lest she should be arrested with the Measles which have proved fatal to Women that were with child, after too diligent an Attendance on her sick Family, was… surprized with her Travail [went into labor]… [and] graciously delivered her, of both a Son and a Daughter… wherein I receive numberless Favors of God. My dear *Katy*, is now also down with the *Measles*…

[1 November] *Lord's Day*. This Day, I baptized my new-born twins… So I called them, *ELEAZAR* and *MARTHA*….

[4 November] In my poor Family, now, first, my Wife has the *Measles* appearing on her…

My Daughter *Nancy* is also full of them…

My Daughter *Lizzy*, is likewise full of them…

My Daughter *Jerusha*, droops and seems to have them appearing.

My Servant-maid, lies very full and ill of them.

Help Lord; and look mercifully on my poor, sad, sinful Family…

[5 November] My little son *Samuel* is now full of the Measles….

[7 November]… my Consort is in a dangerous Condition, and can gett no rest... Death… is much feared for her… So, I humbled myself before the Lord, for my own Sins... that His wrath may be turned away…

[8 November] …For these many Months… I have often, often express'd my Fear unto my Friends concerning [the measles]. And now, *the Thing that I greatly feared is coming upon me!*

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...this Day we are astonished, at the surprising Symptomes of death upon [my wife]… Oh! The sad Cup, which my Father has appointed me!... God made her willing to Dy. God extinguished in her the Fear of Death… God enabled her to Committ herself into the Hands of a great and good Savior; yea, and to cast her Orphans there too…

I pray'd with her many Times, and left nothing undone…

[9 November] On Munday… [9 November], between three and four in the Afternoon, my dear, dear, dear Friend expired…. [I] cried to Heaven…

[10 November] …I am grievously tried, with the threatening Sickness of my discreet, pious, lovely Daughter *Katharin.*

And a Feavour which gives a violent Shock to the very Life of my dear pretty *Jerusha*.

[11 November] This day, I interr'd the earthly part of my dear consort…

[12 November] …The epidemical Malady began upon this Town, is like to pass thro' the Countrey…. it [might] be a service unto the public, to insert in the News-paper, a brief Direction for the managing of the sick. I will advise with a Physician or two.

[13 November] …I hear of some aged and bedrid people, which I design speedily to visit…

[14 November] This Morning… the death of my Maid-servant, whose Measles passed into a malignant Feaver…

Oh! The trial, which I am this Day called unto in… the dying Circumstances of my dear little *Jerusha*!

The two Newborns, are languishing in the Arms of Death…

[15 November] … my little *Jerusha*. The dear little Creature lies in dying Circumstances. Tho' I pray and cry to the Lord… Lord she is thine! Thy will be done!...

[18 November] …About Midnight, little *Eleazar* died.

[20 November] …The distressed Families of the Poor to which I dispense… are now so many…

Little *Martha* died, about ten a clock, A.M.

I begg'd, I begg'd, that such a bitter Cup, as the Death of that lovely [Jerusha], might pass from me…

[21 November] …Betwixt 9 h. and 10 h. at night, my lovely *Jerusha* Expired. She was 2 years, and about 7 months old. Just before she died, she asked me to pray with her; which I did… and I gave her up unto the Lord. [Just as she died] she said, *That she would go to Jesus Christ*…

Lord I am oppressed; undertake for me!

[23 November] …My poor Family is now left without any Infant in it, or any under seven Years of Age…

This day, I followed my dear *Jerusha* to the Grave... with Resolutions… especially what I may do for my own and other Children.

[25 November] … several Things may I do for the Service of the Town in its Adversity… [including] Charitable Distributions among the Poor… I will procure, and I will dispense, as many of these, as I can…

[28 November] Breathing in the midst of so many Deaths, what can there be so needful and so proper for me, as for me to *Die Daily*, and become a man dead unto this World…

[17 December] This day was kept as a Day of Prayer in the several Churches of Boston, because of the heavy Calamity on the Town. And a liberal Collection was made, for the Relief of the Poor, under the Calamity of Sickness, and growing Scarcity. It was a most bitter season…

[23 December] …I have given to the Printer, a Letter about the *Right Management of the Sick under the Distemper of the Measles* [note: the actual publication bears a different title; see Mather, *6*]; which is now spreading and raging in the Countrey. I propose to scatter it into all parts… to save many lives….

Discussion

Mather's chronicle of explosive measles in his own family documents a shocking case-fatality rate (5 of 11 infected household members, or 45%). It also reminds us that emerging and reemerging diseases such as measles once appeared suddenly to kill almost anyone (*7*), a reality with which most persons lived until modern times (and with which many in the developing world who lack access to good nutrition and modern medical care still live today).

The fear that measles engendered in the citizens of Boston, expressed in Mather's many diary entries of tenderness, hope, and despair, should bring to mind another disease of similar case-fatality that arouses comparable fear and despair: Ebola virus disease. The ravages of this disease in West Africa have recently been broadcast on television screens in the United States. Like the stunned looks in the eyes of grieving West Africans, the account of a father watching his family suffer and succumb to death elicits sympathy for those struggling against fatal diseases that even modern parents may not fully understand, and that even aggressive public health measures may prevent only with great difficulty. The writer of these passages was not just America's preeminent theologian and medical authority, but a husband and father

whose grief can be universally understood. One of Mather's most noteworthy sermons, preached 24 years earlier, observed that: "Yet few outward Earthly Anguishes are equal unto these. The Dying of a Child is like the Tearing off [of] a limb…" (*8*).

Several medical aspects of Mather's entries are noteworthy. From Mather's brief notations, we cannot be certain of the exact dates of onset of most of the illnesses he described. However, he clearly chronicles 2 serial generations of measles within his family (one of no more than 17 days, the other of no more than 15). These intervals correspond to what textbooks began to describe, more than a century later, about the patterns of measles spread. Measles seems to have been brought into the household by Mather's son Increase around October 18, 1713. Increase apparently infected his mother, Elizabeth Clark-Hubbard Mather, and his 4 youngest siblings; these 5 became ill on or shortly before November 4. A second generation of measles involved twins, born on October 30 and said to be ill on November 14, consistent with infection at or shortly after birth (or, much less likely, given the apparent date of onset of disease in their mother, infected in utero). It is curious that Mather correctly understood measles to be especially dangerous for parturient women. He may have concluded this on the basis of reports circulating among physicians or because he knew that the same phenomenon had long been documented for influenza; an influenza pandemic complicated by pleuropneumonia had begun, and swept through the Americas, during 1697–1698.

Also noteworthy, and mentioned in his referenced letter (*6*) but not specifically in the diaries, is Mather's realization that pleuritick fever, probably corresponding to pneumonia, was a serious complication of measles. Viral pneumonia and secondary postmeasles bacterial pneumonia are now considered to be among the most fatal complications of this disease (*9*,*10*).

Today, high case-fatality rates for measles are seen only in ill and immunosuppressed persons or in those who are malnourished. However, in the premodern world measles was, confusingly, sometimes benign, sometimes deadly. The reasons for this documented pattern remain obscure: differences in virulence among the various extant measles clades have not been found. The deadliest historical measles outbreaks seemed to occur disproportionately in the poor and disadvantaged, especially including young children in orphanages or environments of desperate poverty, or in indigenous persons living in areas of potential relative nutritional deficiency (*9*). These findings suggest a key role for host and environmental factors in measles severity. Even a modest deficiency in vitamin A is now known to exacerbate measles severity, and postmeasles bacterial pneumonias appear to be much more common in situations of poverty, crowding, and high bacterial circulation.

The *Letter* Mather promised was "published for the benefit of the poor" (*6*) in December 1713. It informed those unfortunate citizens without access to a physician's care about the typical clinical appearance and course of measles, and about simple treatments for it. In recommending generic remedies for unbalanced "humours," it broke no new ground, suggesting (italics and capitalization preserved): *Syrup of Saffron and Treacle Water*, Syrup(s) of Maiden-hair or *Hyssop*, Tea of *Sage* or *Rosemary, Sugar-Candied*, or *Buttered Pills*, *Hot Beer and Rum*, *Hot Cyder*, *Hot Honey*, *Water with Roasted Apples* in it, Shavings of *Castile Soap* in a Glass of Wine or Beer, or *Tea* made of *Rhubarb*, and sweetened with a Syrup of *Marshmallow* (*Althaea officinalis*). These were all ingredients that the poor could afford, and that might at least be comforting, if not life-saving.

The 1713 Boston measles epidemic occurred 21 years after the Salem Witch Trials, in which historians still debate Mather's role as instigator or mitigator; 7 years after Mather discovered that inoculation might be able to prevent smallpox; and 8 years before Mather passionately advocated inoculations in response to a deadly smallpox epidemic. Because Mather died 30 years before preventive measles inoculation is known to have been attempted (*11*) and 225 years before the first effective measles vaccine was developed, we have no way of knowing what he would have thought about measles immunogens, their use in public health programs, or policies to ensure universal vaccination of children.

In this writer's opinion, however, there is little doubt that Mather—were he alive today—would strongly support all reasonable measles control efforts, including universal and publicly enforced vaccination. After all, he was a proponent of smallpox inoculation, and he fought energetically in public forums against all who tried to prevent inoculations on the grounds that it was inherently risky and might theoretically prolong or even start epidemics. He also had lived through the most devastating tragedy of his life: the loss of his own wife and children from epidemic measles. Moreover, as the first person in the New World to espouse an "animalcular" theory (germ theory) of disease (*3*), Mather would surely have been predisposed to accept the scientific basis of immunization, and he surely would have been impressed that aggressive global measles vaccination has, in little more than a decade, reduced the death rate by a factor of at least 5-fold and saved ≈1 million lives each year. It seems highly likely that Mather would not only advocate measles prevention and control, on the basis of the most up-to-date medical tools and public health information, but also measles elimination and eradication.

Mather's grief and despair, expressed in line after line of his diary, should remind us not only that the risks of infectious diseases like measles are real and ongoing but

also why previous generations of physicians and scientists, supported by a public desperate for medical advances to save their children, worked so long and so well to develop and deploy the very vaccines that some people now avoid and decry. As we debate today how best to deal with yet another measles epidemic in the United States, we should look closely at the lessons Cotton Mather and his contemporaries learned 3 centuries ago. Emerging infectious diseases like measles keep reminding us that "the past is never dead. It isn't even past" (*12*).

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Influenza A(H5N6) Virus Reassortant, Southern China, 2014

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To the Editor: Avian influenza A viruses generally do not cause disease in aquatic birds, the natural reservoir of these viruses (*1*). Influenza A(H5N6) was first isolated from mallards by García et al. in 1975 (*2*). Influenza viruses continue to evolve and reassort to generate novel, highly pathogenic viruses. Novel H5 highly pathogenic avian influenza virus subtypes, such as H5N2, H5N5, and H5N8, have been reported (*3*,*4*). Highly pathogenic influenza A viruses are endemic to many countries (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/), cause tremendous economic losses to the poultry industry, and represent a serious threat to public health.

In March 2014, an influenza A(H5N6) outbreak caused the death of 457 birds in Laos (http://www.oie.int/wahis_2/public%5C..%5Ctemp%5Creports/en_imm_0000015052_20140507_182757.pdf). During the same month, a flock of ducks in Guangdong Province in southern China exhibited typical respiratory signs of influenza A virus infection. This flock also had 70% decreased egg production and a slightly increased mortality rate. Throat swab specimens were taken from the symptomatic and dead ducks, and the samples were used to inoculate chicken embryos for virus isolation. Hemagglutination (HA) and neuraminidase (NA) inhibition assays were performed to identify the subtype of the isolated virus, which was designated A/duck/Guangdong/GD01/2014 (H5N6) (GD01/2014). The complete RNA genome was amplified by reverse transcription PCR and cloned into the pMD-19T vector for sequencing (*5*). The complete genome sequence of the GD01/2014 virus was submitted to GenBank (accession nos. KJ754142–KJ754149).

Multiple-sequence alignments showed that the HA gene of GD01/2014 shared 97.5% nt identity with A/wild duck/Shandong/628/2011 (H5N1) and NA genes shared 96.6% and 98.3% nt identity with A/swine/Guangdong/K6/2010 (H6N6) and A/duck/Shantou/1984/2007 (H6N6), respectively. All internal genes shared high levels of nucleotide identity (97.6%–98.5%) with A/wild duck/Fujian/2/2011(H5N1). The whole genes of GD01/2014 and the H5N6 viruses in Laos (LAO/2014) shared 98.2%–99.7% nt identity, indicating the same genotype. Phylogenetic analysis of the HA gene revealed that the isolated virus belonged to clade 2.3.4.6 (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/21/7/14-0838-Techapp.pdf) (*6*). The NA gene of GD01/2014 was clustered with some H6N6 viruses circulating in China (online Technical Appendix Figure, panel B). The 6 internal genes of GD01/2014 were closely related with A/wild duck/Fujian/2/2011(H5N1) and A/wild duck/Fujian/1/2011(H5N1) (online Technical Appendix Figure, panels C–H). Phylogenetic analysis showed that all 8 genes of GD01/2014 and LAO/2014 were closely related although genetically distant from the earlier isolated H5N6 viruses (online Technical Appendix Figure). These findings suggest that GD01/2014 and LAO/2014 are reassortants of wild duck H5N1 and H6N6 viruses, both of which have pathogenic and potential pandemic capacity in southern China. A previous report that H5N1 and H6N6 co-infected a duck suggests that GD01/2014 might be generated from the co-infection of H5N1 and H6N6 in the same host (*7*).

The intravenous pathogenicity index of GD01/2014 was 3.0, which indicates that the isolate is highly pathogenic for chickens. GD01/2014 had multiple basic amino acids (LRERRRKR/GLF) at the cleavage site between HA1 and HA2; this characteristic is typical of highly virulent influenza viruses (*8*). The HA protein contained E190, R220, G225, Q226, and G228 (H3 numbering) residues at the receptor-binding pockets, indicating that the virus preferentially binds to the sialic acid-2,3-NeuAcGal of the avian-like receptor (*9*). The HA protein has 7 potential N-glycosylation sites (PGSs); the HA1 protein has 5 PGSs; the HA2 protein has 2 PGSs. The NA protein of GD01/2014 and LAO/2014 had a deletion of 11 aa residues at positions 59–69 (N6 numbering) in the NA stalk region. Moreover, a deletion of 5 aa residues from positions 80–84 in the nonstructural 1 protein was found in GD01/2014 and LAO/2014. The position 627 and 701 of the polymerase basic 2 protein were E and D, respectively, characteristics of the avian influenza virus (*10*)

In summary, in 2014, outbreaks of H5N6 virus occurred in China, Laos, and Vietnam and caused the deaths of infected humans in Sichuan province, China (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2014/; http://www.wpro.who.int/china/mediacentre/releases/2014/20140507/en/). We characterized the novel reassortant H5N6 virus in China and found that it

[1]These authors contributed equally to this article.

was the same genome type as and was highly homologous with the H5N6 virus in Laos. The findings in this study are also supported by the previous genetic characterization of these viruses by Wong et al. (*11*). However, the adaptation, host range, and virulence of this reassortant H5N6 virus are still unclear and should be further investigated. Furthermore, the potential for infection, outbreaks, and pandemic in other poultry and mammals should be carefully monitored.

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Characterization of 3 Megabase-Sized Circular Replicons from *Vibrio cholerae*

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To the Editor: Prokaryotes typically have a single circular chromosome. However, some bacteria have >1 chromosome. *Vibrio* bacteria, for example, have 2 circular chromosomes: 1 (Ch1) and 2 (Ch2) (*1–3*). Most recognizable genes responsible for essential cell functions and pathogenicity are located on Ch1. Ch2 is also thought to encode some genes essential for normal cell function and those associated with virulence. Both chromosomes are controlled coordinately in their replication and segregation (*4*). Evidence suggests that Ch2 was originally a mega-plasmid captured by an ancestral *Vibrio* species (*2,5*). We report the characterization of recent isolates of *V. cholerae* O1 from Thailand that carry a novel gigantic replicon (Rep.3) in addition to Ch1 and Ch2.

Cholera outbreaks occurred in Tak Province, Thailand, during March–December 2010. We obtained 118 isolates of *V. cholerae* O1 and subjected their *Not*I digests to pulsed-field gel electrophoresis (PFGE), which differentiated the isolates into 8 different patterns (*6*). The profile of PFGE type A6 was identical to that of PFGE type A4, except that a large DNA band existed in type A6. The PFGE profile of the intact (undigested) DNA of the type A6 isolates exhibited a unique genome structure consisting of 3 large replicons (Figure, http://wwwnc.cdc.gov/EID/article/21/7/14-1055-F1.htm).

Three isolates of PFGE type A6 (TSY216, TSY241, and TSY421) were obtained during June 3–July 5, 2010, from 3 unrelated residents of a village near the Thailand–Myanmar border. The isolates were classified as multilocus variable-number tandem-repeat analysis type 16, suggesting that they are of clonal origin (*6*). Next, we performed whole-genome sequencing of TSY216, as a representative of PFGE type A6 isolates, by using the GS FLX Titanium

system (8 kb–span paired-end library; Roche, Indianapolis, IN, USA). Using Newbler version 2.6, the Roche 454 GS De Novo Assembler software (454 Life Sciences, Branford, CT, USA), we assembled 424,273 reads into 3 large scaffolds comprising 119 contigs at 18.3-fold coverage. The gaps between contigs were closed by PCR, and the PCR products were then sequenced. Illumina sequence data (Illumina, Inc., San Diego, CA, USA) were used to improve low-quality regions. The whole-genome sequence of TSY216 was completed and deposited in GenBank (accession nos. CP007653–55).

Full-genome sequencing revealed that *V. cholerae* O1 El Tor TSY216 consists of 3 circular replicons, Ch1 (3,053,204 bp), Ch2 (1,051,284 bp), and Rep.3 (896,006 bp), with an average G+C content of 47.7%, 47.0%, and 37.3%, respectively. In total, 4,579 coding sequences were detected and annotated by using the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The whole-genome comparison between 2010EL-1786 (an outbreak isolate from Haiti) (*7*) and TSY216 revealed that Ch1 and Ch2 shared nearly identical gene content and showed conserved synteny, but integrative and conjugative elements were distinguishable. Strain TSY216 carries CTX-3, whereas strain 2010EL-1786 possesses CTX-3b. These CTXs represent wave 3 of the seventh cholera pandemic (*8*). Rep.3 of TSY216 did not share a conserved region with Ch1 and Ch2. Thus, this replicon may have been gained fairly recently through horizontal gene transfer from unknown organisms.

Rep.3 encodes 999 coding sequences and 66 transfer RNAs, among which 39 have been assigned putative functions and 960 encode hypothetical proteins and proteins of unknown function. The origin of the replicon could not be traced from the coding sequences in the public databases. Of note, Rep.3 encodes a specific transfer RNA for each amino acid, for a total of 20 amino acids. In addition, Rep.3 carries 2 genes encoding the histone-like nucleoid-structuring protein. In this regard, a 165-kb plasmid, pSf-R27, in *Shigella flexneri* encodes a histone-like nucleoid-structuring protein that was claimed to be a transcriptional repressor of the plasmid (*9*). Rep.3 may have a stealth strategy similar to that of pSf-R27.

We assessed the stability of the Rep.3 of the 3 A6 isolates. In total, 96 colonies for the 3 isolates were subcultured each day for 30 consecutive days. Then, using PCR and PFGE, we determined whether Rep.3 remained in the 96 subcultures. The Rep.3-specific primer set (Rep3hns-F: 5′-TTCAATGCGTCCAGCGTTGC-3′ and Rep3hns-R: 5′-TCGCACCTCTATCAATAGCC-3′) for PCR was designed for detection of the histone-like nucleoid-structuring protein gene encoded on the third replicon. All subcultures maintained Rep.3 in an unchanged state. However, when the organisms were cultured at 42°C, ≈70% of the subcultures lost Rep.3. The growth rates of the organisms with and without Rep.3 showed no substantial difference when the organisms were cultured in Luria-Bertani medium at 37°C.

The appearance of *V. cholerae* O1 variants with additional circular replicons may contribute to evolution of the bacteria in unexpected manners. Clones from the seventh cholera pandemic, which began in 1961, share nearly identical gene content (*8,10*). However, some clones, such as TSY216, can gain a replicon of megabase class and maintain it stably. Eventually, epidemic *V. cholerae* O1 may gain the ability to incorporate genes that change properties such as antigenicity or pathogenicity. The function of Rep.3 remains under investigation.

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Severe Malaria Not Responsive to Artemisinin Derivatives in Man Returning from Angola to Vietnam

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To the Editor: Partial artemisinin-resistant *Plasmodium falciparum* malaria, characterized by delayed parasite clearance after treatment with artesunate or artemisinin-based combination therapy, was first detected in western Cambodia and has now spread to or emerged de novo in 5 countries of the Greater Mekong Subregion (GMS) (*1*). However, most reported cases of malaria have been in Africa, and detecting artemisinin and multidrug resistance in Africa will have consequences for policy and containment plans (*2*).

Thus, vigilant monitoring is pivotal, and it is therefore with great interest that we read the case report on a patient in Vietnam with severe *P. falciparum* malaria, acquired in Angola in 2013, that was not responsive to artesunate or several other antimalarial combinations (*3*). We believe that there are several issues that challenge the conclusion that artemisinin resistance has reached Angola: 1) the phenotypic and genotypic characteristics of the infecting strain in this patient were very different from artemisinin-resistant strains in the GMS; 2) pharmacokinetic issues cannot be ruled out; and 3) perhaps of most relevance, the study documents severely delayed clearance of multiple strains in this polyclonal *P. falciparum* infection, suggesting splenic hypofunction as an important contributor.

The parasite clearance half-life calculated with the World Health Organization (WHO) online slope analyzer from the log linear segment of the clearance curve after start of artesunate therapy was 102.5 hours, which is ≈10 times longer than observed in the most artemisinin-resistant parasites in Cambodia. Postpublication genotyping of the infecting strain provided by the authors to WHO showed a wild-type Kelch (K13) gene, which is a recently discovered molecular marker for artemisinin resistance strongly correlated to the resistant phenotype in the GMS (*1*).

No pharmacokinetic assessment was made, and subtherapeutic artesunate and dihydroartemisinin (as well as clindamycin, piperaquine, quinine, and doxycycline) blood concentrations cannot be excluded. The intravenous artesunate regimen used differed from the WHO guideline of 2.4 mg/kg on admission, after 12 h, then daily. Pharmacokinetic modeling of the split doses used in the described case indicate that this dosing schedule results in ≤20% artesunate and dihydroartemisinin blood concentrations. In addition, quality issues in the artesunate batch might have played a major role. Batch no. 511002 used for this patient (not 511004 as mentioned in the article) was manufactured by Pharbaco (Hanoi, Vietnam) in April 2011 and had a shelf-life of 3 years; it was quality controlled and passed the quantitative testing by high pressure liquid chromatography in January 2014 (National Institute of Drug Quality Control, Vietnam). However, according to information shared with WHO, a test for clarity after reconstitution was not performed, whereas other samples from the same batch had failed this specific test, which led the Drug Administration of Vietnam to withdraw this batch from the market. The patient was subsequently treated with nasogastric-administered dihydroartemisinin/piperaquine and quinine plus doxycycline. Reduced intestinal absorption in this severely ill patient, related to reduced splanchnic blood flow, could have resulted in reduced bioavailability (*4*).

Host factors can affect parasite clearance. In this case, the parasitological response to artesunate and clindamycin, dihydroartemisinin/piperaquine, quinine, and doxycycline were all unusually slow. Functional asplenia results in very slow parasite clearance after artesunate treatment, resembling the clearance characteristics in the described case (*5*). This interpretation is supported by finding genotypes representing ≥2 clones of parasites persisting >1 week after treatment with multiple antimalarial drugs. It seems very unlikely that this patient harbored multiple highly artemisinin-resistant parasite strains. Dead circulating intraerythrocytic parasites in patients who have hyposplenia can be recognized morphologically, but the article does not provide details on this.

Circulation of multidrug resistant malarial strains in sub-Saharan Africa can have disastrous consequences, and it is critical to detect its arrival at an early stage. The case report by Van Hong et al. implies the unlikely event of independent emergence of multidrug resistant strains in a traveler from Vietnam in Angola, without evidence of local declining artemisinin-based combination therapy efficacy. WHO and partners are investigating the phenotype and genotype of parasite strains from the same geographic area in Angola to address the concerns raised above. We believe that this single case report is insufficient to raise the alarm.

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In Response: We agree with Ringwald and Dondorp (*1*) that our report of a Vietnamese worker returning from Angola with severe Plasmodium falciparum malaria not responsive to artemisinins (*2*) is unlikely to indicate that artemisinin resistance has reached Angola. Nevertheless, this case, for its unusual clinical manifestation and response to treatment, had raised alarm in Vietnam, where the number of imported malaria cases and deaths among Vietnamese workers returning from Africa has recently increased (*3,4*). After our report was published in July 2014, we collected additional information that may be useful in putting such a case in perspective.

The results of an external quality control study by Sigma-Tau Pharmaceuticals on 10 vials of the same batch (no. 511002) of intravenous artesunate as administered to our case-patient (report available on request) confirmed acceptable drug concentration and showed that the opalescence observed after reconstitution was caused by precipitation of an impurity (representing 0.12% of the preparation) identified as an active metabolite of artesunate. Therefore, the treatment administered to the patient was of acceptable quality. The blood concentrations of artesunate and dihydroartemisinin may have been 20% lower than ideal (as predicted by a pharmacokinetic model), but this finding cannot explain why the parasite density remained >200,000/µL for several days.

Ringwald and Dondorp also mention functional asplenia as a possible cause of delayed parasite clearance. We argue that this would have resulted in a much longer (weeks/months) parasite clearance (*5*) than the observed sharp decrease after quinine and tetracycline administration. Moreover, we did not observe any accumulation of circulating dead parasites (Howell-Jolly bodies), which is against the hypothesis of functional asplenia. Furthermore, sharp decline of parasite density immediately after quinine and doxycycline administration by nasogastric tube is not consistent with the proposed hypothesis of reduced intestinal absorption.

In hindsight and after consideration of additional information, we agree that it is unlikely this patient harbored several resistant parasite clones. However, the reasons for the lack of response to arteminins in this patient remain unknown and are under continued investigation.

The discussion triggered by the publication of our case report raises the question of what should be reported to the attention of the scientific community and public health authorities. Besides being an obligation for clinical physicians, reporting unusual treatment failures such as our case is also an essential component of antimalarial resistance surveillance. As mentioned by Ringwald and Dondorp, “vigilant monitoring is pivotal” for the detection of possible foci of resistance. For early detection of artemisinin resistance, we would rather have a more sensitive than specific system, because the latter would probably miss the first emerging cases of resistance. Reporting cases similar to the one we published should be encouraged.

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Diversity of *Bartonella* spp. in Bats, Southern Vietnam

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To the Editor: To investigate bats as potential reservoirs for *Bartonella* spp. in Vietnam, we screened a range of bat species to determine the prevalence and genetic diversity of *Bartonella* spp. in bat populations in southern Vietnam. In a study of bat biodiversity in southern Vietnam, 60 bats were trapped at 6 sites in Dong Nai Culture and Nature Reserve and Cat Tien National Park, Vietnam, in May 2013. Bats were trapped by using mist nets and harp traps set at ground level, and were euthanized by using isoflurane (http://www.avma.org/KB/Policies/Documents/euthanasia.pdf) for cataloguing at the Vietnam Academy of Sciences and Technology, Hanoi. Blood specimens were collected by cardiac puncture, and external measurements were recorded. Bats were speciated according to morphology (*1*,*2*); trapped bats represented 10 species belonging to 5 genera. All species have been given a conservation status of least concern (http://www.iucnredlist.org/).

Total nucleic acid was extracted from blood samples by using the MagNApure automated nucleic acid extraction system (Roche, Basel, Switzerland). Extracted nucleic acid was subjected to conventional PCR to detect *Bartonella* spp. DNA by using primers specific for the citrate synthase A gene (*3*). A positive PCR result was determined by amplification of a 729-bp fragment. Twenty-one (35.0%) of 60 bat blood specimens had a result consistent with presence of *Bartonella* spp. (Table). Among insectivorous or carnivorous bats, *Bartonella* prevalence was 20 (45.5%) of 44 compared with 1 (6.2%) of 16 fruit-eating bats ($\chi^2 = 6.3$, $p = 0.01$). The prevalence of *Bartonella* spp. did not differ between sampling locations (Table) or by estimated age of the bat (determined by deviation above or below the median tibial length of each species); prevalence was 33.3% (9/27) in younger bats and 36.4% (12/33) in older bats.

DNA sequences from the 21 PCR citrate synthase A gene amplicons (GenBank accession nos. KP100340–KP100360) were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to assess sequence similarity. Potentially novel *Bartonella* phylogroups were identified as having <96% sequence similarity with all publicly available sequences in GenBank (*4*). The sequences were then manually aligned with those of a representative sample of *Bartonella* spp. and trimmed to the 327-nt region (positions 801–1127) commonly used for taxonomic classification (*4*). A neighbor-joining tree was constructed by using the Hasegawa–Kishino–Yano plus gamma model of nucleotide substitution in Geneious version 7.1.7 with 1,000 bootstrap replications (*5*).

Sequence analysis identified 10 distinct *Bartonella* phylogroups (I–X) among 21 *Bartonella*-positive blood samples from bats in Vietnam (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/7/14-1760-Techapp1.pdf). Nine of these phylogroups showed <96% sequence similarity to all previously identified *Bartonella* sequences, suggesting they might belong to new *Bartonella* species. *Bartonella* spp. in *Rhinolophus* spp. bats were classified into phylogroups I, III, VIII, IX, and X. Phylogroups II and VII were detected in samples from *Hipposideros* spp. bats, and phylogroups IV and V were detected in *Megaderma* spp. bats. Phylogroup VI was detected only in a *Megaerops* spp. bat.

Although 9 lineages (I, III–X) were novel, phylogroup II was identified in 4 *Hipposideros* spp. bats and showed 96.3%–97.2% similarity to *Bartonella* spp. isolated from a bat fly found on a *Hipposideros* spp. host in Malaysia (GenBank accession no. JX416238). This similarity might suggest widespread distribution of this *Bartonella* spp. lineage in *Hipposideros* spp. bats or their ectoparasites in Southeast Asia. Additional genetic characterization of strains is needed to determine whether any of these novel phylogroups represent new species and to investigate their evolutionary and ecological relationships with other *Bartonella* spp. identified in Vietnam and elsewhere.

The primary observation in this study was detection of *Bartonella* spp. (by DNA amplification) in bats in southern Vietnam at a prevalence of 35.0%, which is comparable with that reported in Kenya (30.2%) and Guatemala (33.0%) (Table) (*6*,*7*). However, the use of conventional PCR in this study might underestimate the true prevalence.

Although high prevalences have been proposed to be caused by persistent infection of bats with *Bartonella* spp., our findings indicate no increase in prevalence by age of bat, which would be expected if persistent infection were common. This finding, and detection of multiple lineages infecting individual bat species, may instead reflect high levels of transmission within and between bat species

Table. Prevalence of *Bartonella* spp. in bats from 2 sites in Dong Nai, Vietnam, 2013

Bat species	No. *Bartonella* spp.–positive bats/no. bats trapped (%)		
	Cat Tien National Park	Dong Nai Nature Reserve	Total
*Cynopterus sphinx**	0/0	0/14	0/14 (0)
Hipposideros armiger†	2/6	0/0	2/6 (33.3)
Hipposideros larvatui†	3/5	0/0	3/5 (60)
*Megaerops niphanae**	0/0	½	1/2 (50)
Megaderma spasma†	0/0	1/2	1/2 (50)
Megaderma lyra‡	1/1	0/0	1/1 (100)
Rhinolophus acuminatus†	0/0	9/17	9/17 (52.9)
Rhinolophus chaseli†	2/5	0/0	2/5 (40)
Rhinolophus sinicus†	0/3	2/4	2/7 (28.6)
Rhinolophus luctus†	0/1	0/0	0/1 (0)
Total	8/21 (38.1)	13/39 (33.3)	21/60 (35)

*Fruit-eating.
†Insectivorous.
‡Carnivorous.

caused by crowded roosting areas and sharing of roosts by multiple species. This behavior provides opportunities for transmission of *Bartonella* bacteria or exchange of infected ectoparasites, such as *Cyclopodia* spp. (*8*), although the precise roles of these 2 processes are unknown.

Although no human cases of *Bartonella* spp. infection have been reported in Vietnam, *Bartonella* spp. have been identified in febrile humans elsewhere in Southeast Asia (*9*) and are also common in rats in southern Vietnam (*10*). Because close contact with bats (i.e., through manure farming and consumption of bat meat) and potential arthropod vectors (i.e., through handling and consumption of fruit) is common in parts of Vietnam, targeted screening of bats and their human contacts might improve our understanding of the zoonotic potential of these bacteria and their potential effect on public health.

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Seropositivity for Avian Influenza H6 Virus among Humans, China

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To the Editor: Influenza virus subtype H6 was first isolated from a turkey in 1965 in the United States (*1*) and was subsequently found in other parts of the world (*2*). Over the past several decades, the prevalence of H6 virus has dramatically increased in wild and domestic birds (*2–4*). In China, highly pathogenic influenza A(H5N1), low pathogenicity influenza (H9N2), and H6 are the most prevalent avian influenza viruses among poultry (*5*). Although only 1 case of H6 virus infection in a human has been reported worldwide (*6*), several biological characteristics of H6 viruses indicate that they are highly infectious to mammals. Approximately 34% of H6 viruses circulating in China have enhanced affinity to human-like receptors (α-2,6 NeuAcGal) (*2*). H6 viruses can also infect mice without prior adaptation (*2*,*7*), and some H6 viruses can be transmitted efficiently among guinea pigs (*2*). To evaluate the potential threat of H6 viruses to human health, we conducted a systematic serologic study in populations occupationally exposed to H6 viruses.

During 2009–2011, a total of 15,689 serum samples were collected from live poultry market workers, backyard poultry farmers, large-scale poultry farmers, poultry-slaughter factory workers, and wild bird habitat workers in 22 provinces in mainland China. A/chicken/Y94/Guangdong/2011 (H6N2), a representative isolate of predominant H6 viruses in mainland China, was used for the serologic testing (online Technical Appendix Table 1, Figures 1, 2, http://wwwnc.cdc.gov/EID/article/21/7/15-0135-Techapp1.pdf). Hemagglutination inhibition (HI) assay was performed for all serum samples, and samples with an HI titer ≥20 were verified by a microneutralization (MN) assay, as indicated by World Health Organization guidelines (*8*). An MN result of ≥20 was considered positive.

The HI result was ≥20 for H6N2 virus in 298 of the 15,689 specimens, and the MN result was positive in 63 of the 298 specimens (overall seropositivity range 20–320, mean 32.7, 0.4%) (online Technical Appendix Table 2). The proportion of group members who were seropositive differed significantly according to occupational exposure (p = 0.0125). Seropositivity was highest among workers in live poultry markets, backyard poultry farmers, and workers in wild bird habitats (0.66%, 0.42%, and 0.51%, respectively) (Table). According to χ^2 test results, seropositivity among workers in live poultry markets was significantly higher than that among large-scale poultry farmers (p = 0.0015, adjusted α = 0.005. Analysis by unconditional logistic regression model showed that exposure to live poultry markets was a risk factor for human infection with avian influenza H6 virus (odds ratio 2.1, 95% CI 1.27–3.47).

Seropositivity did not differ significantly among male and female persons tested (p = 0.08) (Table). No children were positive for the H6N2 virus. For other age groups, seropositivity ranged from 0.25% to 0.45%, but differences were not significant (p>0.05) (Table).

Of the 22 provinces from which serum specimens were collected, 11 were northern provinces and 11 were southern provinces. Positive specimens were detected in all southern provinces. In northern China, no seropositive results were detected in Henan, Liaoning, or Jilin Provinces. According to χ^2 test results, seropositivity in southern China was significantly higher than seropositivity in northern China (p = 0.0375) (Table).

Human infection with influenza H6 virus in mainland China has not been reported, but 63 serum specimens tested in our study were positive for the H6 virus. This level of seropositivity is much higher than that for highly pathogenic

Table. Seropositivity of occupationally exposed populations for the influenza (H6N2) virus, China, 2009–2011*

Population	Total no. serum samples	Mean titer for MN ≥20	No. serum samples with MN ≥20	Seropositivity (95% CI)	Odds ratio† (95% CI)
Total	15,689	32.70	63	0.40 (0.40–0.41)	
Occupation					
Live poultry market	3,950	43.08	26	0.66 (0.64–0.68)	2.10 (1.27–3.47)
Poultry farm	3,762	25.71	7	0.19 (0.18–0.19)	0.40 (0.18–0.87)
Backyard poultry farm	4,324	26.67	18	0.42 (0.40–0.43)	1.05 (0.61–1.82)
Poultry slaughter factory	1,235	30.00	2	0.16 (0.15–0.17)	0.38 (0.09–1.57)
Wild bird habitat	788	20.00	4	0.51 (0.47–0.54)	1.28 (0.47–3.54)
Other	1,630	23.33	6	0.37 (0.35–0.39)	0.91 (0.39–2.11)
Sex					
F	7,620	24.29	28	0.37 (0.36–0.38)	Reference
M	8,069	39.39	35	0.43 (0.42–0.44)	1.18 (0.72–1.94)
Age group, y					
Children, ≤14	74	–	0	0	0 (0)
Youth, 15–24	1,168	20.00	3	0.26 (0.24–0.27)	0.75 (0.19–3.00)
Adult, 25–59	1,2450	34.07	54	0.43 (0.43–0.44)	1.27 (0.54–2.94)
Elderly, ≥60	1,748	13.33	6	0.34 (0.33–0.36)	Reference
No age record	249	–	0	0	–
Geographic distribution					
South	10,522	32.00	50	0.48 (0.47–0.48)	Reference
North	5,167	35.38	13	0.25 (0.24–0.26)	0.59 (0.30–1.15)

*MN, microneutralization; –, not applicable.
†Odds ratios were calculated by using unconditional logistic regression model (SPSS 17.0, Armonk, NY, USA).

avian influenza A(H5N1) virus, for which only 2 of the serum specimens we tested were positive (data not shown), but much lower than the seropositivity level for low pathogenicity avian influenza A(H9N2) virus; 3.4% of the samples tested were positive for A/Chicken/Hong Kong/G9/1997(H9N2)–like virus (data not shown). A previous US study has reported H6N2-positive antibodies in veterinarians (*9*). Our results and the veterinarian study indicate that the H6N2 virus could infect humans.

In our study, positive samples were detected in 19 of 22 provinces and in all tested worker populations, suggesting that the H6 virus has been broadly circulating in birds in China. Live poultry market exposure is the major risk factor for human infection with avian influenza H6 virus. The limitation of this study is that antigen selection may not accurately detect neutralization antibodies for different subtypes of H6 viruses. Surveillance of the H6 virus in birds and occupationally exposed populations should be strengthened for pandemic preparedness.

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Absence of MERS-Coronavirus in Bactrian Camels, Southern Mongolia, November 2014

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To the Editor: Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified among humans in 2012 in Saudi Arabia (*1*). As of February 5, 2015, a total of 971 MERS cases and 356 associated deaths had been confirmed (*2*). Because MERS is a zoonotic disease, it is essential that the animal reservoirs and hosts that sustain virus circulation in nature be identified.

Seroepidemiologic and virologic studies have demonstrated evidence of MERS-CoV infection in dromedary camels (*Camelus dromedarius*) in the Arabian Peninsula (*3*), and viruses isolated from dromedaries appear capable of infecting the human respiratory tract (*4*). In some instances, MERS-CoV infection in dromedaries has preceded infection in humans (*5*), indicating that dromedaries are a natural host for MERS-CoV and a possible source of human infection. Thus, it is important to define the geographic range of MERS-CoV infection in camels and the species of camelids that are infected by MERS-CoV in nature.

Two species of camels exist: 1-hump dromedaries (*C. dromedarius*) and 2-hump Bactrian camels (*C. bactrianus*).

[1]These authors contributed equally to the article.

Dromedaries are common in hot desert terrains of the Arabian Peninsula, the Middle East, Afghanistan, central Asia, India, and parts of Africa. Bactrian camels are found in colder steppes of Mongolia, Central Asia, Pakistan, and Iran. Studies have demonstrated a high seroprevalence (>90%) of MERS-CoV in adult (≥5 years of age) dromedaries from the Middle East and from northern, eastern, and parts of central Africa (*6*), but whether MERS-CoV circulates among Bactrian camels is unknown.

To determine whether MERS-CoV is circulating among both species of camels, we studied apparently healthy Bactrian camel herds in southern Mongolia during November 24–30, 2014. We investigated 11 herds in Umnugovi Province (170 sampled animals) and 1 herd in the adjacent Dundgovi Province (30 sampled animals) (Table). A convenience sample was collected from each herd; younger animals were oversampled. Serum and nasal swab samples were collected from each animal. The nasal swab samples were placed in virus transport medium and later tested by real-time PCR targeting open-reading frame 1a and upstream of envelope protein gene, as previously described (*7*); all samples were negative for MERS-CoV RNA. The serum samples were tested for the presence of MERS-CoV antibody by using a validated MERS-CoV (strain EMC) spike pseudoparticle neutralization test (*8*); no samples were positive, indicating a lack of recent or past MERS-CoV infection. A random sample of 5 serum samples each from camels in Umnugovi and Dundgovi Provinces was tested by using a microneutralization test against bovine coronavirus (BCoV) as previously described (*8*); all 10 samples were positive (titer range 1:20–1:640).

The sampled animals included 127 camels ≥5 years of age from 12 herds across 2 provinces in southern Mongolia. Thus, the negative test results indicate that MERS-CoV is not circulating among Bactrian camels in southern Mongolia. The seroprevalence of MERS-CoV among adult dromedaries in the Middle East and Africa is typically >90%, so the lack of any serologic reactivity in camels from Mongolia implies that MERS-CoV infection does not infect Bactrian camels or that the geographic range of the virus does not extend to northeastern Asia. In contrast, infection with a BCoV-like coronavirus seems ubiquitous in Bactrian camels, as it is in dromedaries (*7*).

Dipeptidyl peptidase-4 (DPP4; cluster of differentiation 26) is the receptor for MERS-CoV. As deduced from the human DPP4–MERS-CoV spike protein structural model, the differences in the amino acids in DPP4 molecules of dromedary and Bactrian camel were found in 2 small regions far from the binding interface of DDP4 and MERS spike protein (*9*). The 15 aa of DPP4 critical for binding with MERS-CoV spike protein are conserved between dromedaries and Bactrian camels. Definitive evidence of susceptibility, or lack thereof, of Bactrian camels to MERS-CoV can be established only by experimental infection of these animals.

Even if Bactrian camels are susceptible to MERS-CoV infection, geographic separation may be an alternative explanation for the absence of MERS-CoV among camels in Mongolia. So far, Australia is the only country where dromedaries appear to be free of MERS-CoV; however, as with dromedaries elsewhere, dromedaries in Australia are infected by a BCoV-like virus (*8*). Dromedaries in Australia originated from Afghanistan; these camels were shipped to Australia in the early part of the twentieth century to work on railroad construction projects. There are 2 plausible explanations for the lack of MERS-CoV in Australia: the small numbers of adult animals that were transported from Afghanistan to Australia might not have been sufficient to introduce the virus into Australia or the virus might have been absent from dromedaries in Afghanistan.

Our study was limited by sample size and by the breadth of the study area. Mongolia has 21 provinces and ≈349,300 Bactrian camels, but we studied just 2 southern

Table. Collection sites of nasal swab and serum specimens from Bactrian camels tested for Middle East respiratory syndrome coronavirus, southern Mongolia, November 2014

Herd no.	Province, district	Age, y			No. sampled/no. total in herd
		≤1	2–4	≥5	
1	Umnugovi, Khankhongor	9	5	9	23/56
2	Umnugovi, Khankhongor	7	2	9	18/31
3	Umnugovi, Khankhongor	8	0	5	13/28
4	Umnugovi, Khankhongor	0	9	17	26/65
5	Umnugov, Bayan-Ovoo	0	7	8	15/27
6	Umnugovi, Bayan-Ovoo	0	1	16	17/70
7	Umnugovi, Bayan-Ovoo	0	0	4	4/9
8	Umnugovi, Bayan-Ovoo	0	2	9	11/33
9	Umnugovi, Bayan-Ovoo	0	0	10	10/54
10	Umnugovi, Bayan-Ovoo	1	5	7	13/36
11	Umnugovi, Bayan-Ovoo	0	8	12	20/24
12	Dundgovi, Khuld	0	9	21	30/58
Total		25	48	127	200/491

provinces and a total of 200 camels. Umnogovi Province has the largest, and Dundgovi Province the fifth largest, camel population in the country (≈113,000 and ≈28,000 animals, respectively). Further studies on the epidemiology of MERS-CoV infection in dromedaries and Bactrian camels from central Asia, China, and Mongolia are warranted.

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Oligella ureolytica Bacteremia in Elderly Woman, United States

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To the Editor: *Oligella ureolytica* is an aerobic gram-negative coccobacillus found as a commensal organism in human urinary tracts (*1*). Previously referred to as CDC Group IVe, this bacterium is not commonly encountered as a source of infection and is difficult to isolate by using conventional laboratory procedures (*2*). The few cases of pathogenic infection with *O. ureolytica* described in the literature have occurred in patients ranging in age from newborn to 89 years and from the varied locations of India, Turkey, Canada, and the United States (*3*–*7*). We report a case of *O. ureolytica* bacteremia in a patient in whom sepsis was diagnosed and review the current literature on this emerging pathogen.

A 66-year-old woman sought treatment in our emergency department for a fever of 100.7°F, femur fracture, and a right buttock stage III decubitus ulcer. She reported having fallen 4 days earlier, after which she was unable to walk and spent 4 days laying in her own urine and feces. Blood tests revealed an elevated leukocyte count of 24.4 × 10^9 cells/L (76% neutrophils, 2% bands), and urinalysis showed trace leukocyte esterase, +3 bacteria, and 5–10 leukocytes. Chest radiograph and head computed tomography images were unremarkable. Her electrocardiogram showed nonspecific ST wave changes. Samples from the patient's blood, urine, and wounds were collected while the patient was in the emergency department and were sent for culture.

Wound cultures showed growth of *Proteus mirabilis* and *Enterococcus* spp. The urine culture grew >100,000 CFU *Escherichia coli*. The first set of blood cultures grew *O. ureolytica* in aerobic and anaerobic bottles, but another set drawn 30 min later showed no growth. The blood cultures were processed by using the Bact/Alert 3D (bioMérieux, Marcy l'Etoile, France) and Gram stained. Identification was from the Vitek 2 compact system (bioMérieux). The *O. ureolytica* sample was sensitive to amikacin, ampicillin/sulbactam, ceftazidime, ceftriaxone, gentamicin, imipenem, levofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole, and chloramphenicol. No resistance was found.

Because of the unique bacteremia, further diagnostics were conducted. The results of chest, abdomen, and pelvic computed tomography scans were unremarkable. HIV

Table. Documented cases of pathogenic *Oligella ureolytica* infection*

Year	Patient age, y	Patient sex	Location	Culture source	Concurrent conditions	Urinary disorder	Reference†
2014	30	M	India	Blood	Metastatic lung adenocarcinoma	Urinary incontinence	(*3*)
2013	Newborn	F	Turkey	Blood	None	Maternal urine exposure during delivery?	(*4*)
2013	89	M	United States	Urine	Adenocarcinoma of prostate	High post void residual	(*5*)
1996	49	F	Canada	Neck lymph node	Non-Hodgkin lymphoma	None	(*6*)
1993	40	M	United States	Blood	AIDS, sacral ulcer, diarrhea	None	(*7*)

*Some published cases that were believed to be contamination or for which the organisms did not fit the laboratory profile of *O. ureolytica* were excluded.
†Antimicrobial drug sensitivity has varied among reports; some resistant organisms have been encountered (*3–8*).

test results were negative. The nonspecific electrocardiogram changes prompted us to request a transesophageal echocardiogram, but the patient refused. For 10 days, the patient was given vancomycin (1 g/d), aztreonam (2 g/8 h), and metronidazole (500 mg/8 h). Cultures of blood that had been collected 5 and 8 days after the original culture were sterile. After 16 days, leukocytosis and fever had resolved, and the patient was discharged to a skilled nursing facility. Although we found no reports in the literature of endocarditis caused by *O. ureolytica*, the patient's refusal of a transesophageal echocardiogram and the presence of the uncommon bacterium led us to empirically continue aztreonam for endocarditis after her discharge.

The literature reports 5 cases of pathogenic *O. ureolytica* infection (Table). This bacterium has also been isolated from the respiratory tract of patients with cystic fibrosis (*9*). A 2-year study conducted in 1983 at a high-volume hospital in the United States demonstrated *O. ureolytica* growth in the urine of 72 patients (*8*). Of these patients, 71 had long-term urinary drainage systems and 14 had symptomatic urinary tract infections. Many of these patients were permanently disabled from spinal cord injuries (*8*). This study was the only one we found focused on *O. ureolytica* infection in the clinical setting. We found no cases in which a patient's death was attributed to *O. ureolytica* infection, and all reported cases resolved with antimicrobial drug treatment (*3–8*). The low virulence of this organism may contribute to the paucity of recognized cases.

Of the reported cases, all occurred as opportunistic infections in patients with a source of immunosuppression such as malignancy, HIV, or newborn status. The patient we reported in this article showed no evidence of malignancy and had no major source of immunosuppression besides malnutrition, tobacco use, and advanced age. The patient's wound had been contaminated by urine and feces, which was postulated to be the cause of bacteremia in the 1993 case.

Limitations in commonly available laboratory procedures make the identification of this bacterium difficult. The incubation period is long (4 days), and not all laboratories incubate cultures for that long, as occurred in the 2013 urinary tract infection case (*1,3,5*). Also, the identification of less commonly encountered bacteria is not always pursued to the genus and species level (*2*). Furthermore, it is believed that *Oligella* spp. can be misidentified as phenotypically similar organisms, such as *Bordetella bronchiseptica* and *Achromobacter* spp. (*4*,*10*).

We believe that many cases of *O. ureolytica* infection have gone unrecognized or were incorrectly identified. Some cases may also have been dismissed as contamination because of laboratorians' and clinicians' lack of familiarity with this bacterium. Our review suggests that advancing laboratory techniques will lead to more recognized cases and that further studies are necessary to understand this bacterium's clinical significance.

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Estimating Ebola Treatment Needs, United States

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To the Editor: By December 31, 2014, the Ebola epidemic in West Africa had resulted in treatment of 10 Ebola case-patients in the United States; a maximum of 4 patients received treatment at any one time (*1*). Four of these 10 persons became clinically ill in the United States (2 infected outside the United States and 2 infected in the United States), and 6 were clinically ill persons medically evacuated from West Africa (online Technical Appendix 1 Table 6, http://wwwnc.cdc.gov/EID/article/21/7/15-0286-Techapp1.pdf).

To plan for possible future cases in the United States, policy makers requested we produce a tool to estimate future numbers of Ebola case-patients needing treatment at any one time in the United States. Gomes et al. previously estimated the potential size of outbreaks in the United States and other countries for 2 different dates in September 2014 (*2*). Another study considered the overall risk for exportation of Ebola from West Africa but did not estimate the number of potential cases in the United States at any one time (*3*).

We provide for practicing public health officials a spreadsheet-based tool, Beds for Ebola Disease (BED) (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/21/7/15-0286-Techapp2.xlsx) that can be used to estimate the number of Ebola patients expected to be treated simultaneously in the United States at any point in time. Users of BED can update estimates for changing conditions and improved quality of input data, such as incidence of disease. The BED tool extends the work of prior studies by dividing persons arriving from Liberia, Sierra Leone, and Guinea into the following 3 categories: 1) travelers who are not health care workers (HCWs), 2) HCWs, and 3) medical evacuees. This categorization helps public health officials assess the potential risk for Ebola virus infection in individual travelers and the subsequent need for post-arrival monitoring (*4*).

We used the BED tool to calculate the estimated number of Ebola cases at any one time in the United States by multiplying the rate of new infections in the United States by length of stay (LOS) in hospital (Table). The rate of new infections is the sum of the rate of infected persons in the 3 listed categories who enter the United States from Liberia, Sierra Leone, or Guinea. For the first 2 categories of travelers, low and high estimates of Ebola-infected persons arriving in the United States are calculated by using low and high estimates of both the incidence of disease in the 3 countries and the number of arrivals per month (Table). Calculating the incidence among arriving HCWs required estimating the number of HCWs treating Ebola patients in West Africa (online Technical Appendix 1, Tables 2–4). For medical evacuations of persons already ill from Ebola, we calculated low and high estimates using unpublished data of such evacuations through the end of December 2014.

Although only 1 Ebola case has caused additional cases in the United States (*7*), we included the possibility that each Ebola case-patient who traveled into the United States would cause either 0 secondary cases (low estimate) or 2 secondary cases (high estimate) (Table). Such transmission might occur before a clinically ill traveler is hospitalized or between a patient and HCWs treating the patient (*7*). To account for the possibility that infected travelers may arrive in clusters, we assumed that persons requiring treatment would be distributed according to a Poisson probability distribution. Using this distribution enables us to calculate, using the BED tool, 95% CIs

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Table. Calculated monthly rates of Ebola disease among persons arriving in the United States and additional secondary cases, 2014

Arriving persons		Input 1: infections/mo*	Input 2: at-risk population	Input 3: US arrival rate/mo†	Output 1: importations/mo‡	Output 4: additional secondary cases§	Output 2: total cases/mo‡
Non-HCW	Low	1	10,000	2,000	0.2	0	0.2
	High	3	10,000	3,000	0.9	2	2.7
HCW	Low	1	100	30	0.3	0	0.3
	High	5	100	60	3.0	2	9.0
Medical evacuations¶	Low	NA	NA	1	1.0	0	1
	High	NA	NA	3	3.0	0	3

*Infections in travelers who are not HCWs were based on the monthly incidence identified in World Health Organization situation reports during June–October 2014 (online Technical Appendix 1 Table 1) (*5*). The high value was the highest monthly incidence [September] rounded to the nearest whole number; the low value was set at 30% of the high value. Infections in HCWs were based on estimates of the number of HCWs in West Africa with and without Ebola virus infection at different times in the epidemic [online Technical Appendix 1 and Appendix 1 Tables 2–4]. HCW, health care worker; NA, not applicable.
†The low estimate of US arrival rates for travelers who are not HCWs and both the low and high rates for HCWs were based on the count of screened airline passengers originating in Liberia, Sierra Leone, and Guinea in the month from mid-October through mid-November 2014 (Centers for Disease Control and Prevention [CDC], unpub. data). For the high US arrival rate for travelers who are not HCWs, we assumed a 50% increase over the low value [3,000 = 2,000 × 1.5] to approximate the arrival rate in 2013, before the epidemic (*3*). Rates of HCW arrivals were based on travelers who identified themselves as having worked in a health care facility during the previous 21 d during screenings at their airport of entry to the United States during November 5–December 1, 2014, and the exposure risk category assigned to them according to CDC's Interim US Guidance for Monitoring and Movement of Persons with Potential Ebola Virus Exposure (*4,6*). The low estimate value of arrivals of HCWs (30 arriving HCWs) was approximately the lowest rate of high-risk and some-risk HCWs entering the United States. The high estimate value (60 arriving HCWs) was approximately the highest rate of high-, some-, and low-risk HCWs entering the United States (CDC, unpub. data).
‡Output 1 = (Input 1 / Input 2) × Input 3; Output 2 = Output 1 + (Output 1 × Input 4). See online Technical Appendix 1 for further details.
§Assumed number of additional secondary transmissions occurring in the United States per primary case based on the range of experience from the outbreak: 1 imported case to the United States resulted in 2 secondary infections, and several case-patients have been treated without any secondary infections (*7*).
¶Number of medical evacuations was obtained from unpublished Medical Evacuation Missions Reports (US Department of Health and Human Services, unpub. data).

around the average estimate of arriving case-patients. The treatment length used in both the low and high estimate calculations was 14.8 days, calculated as a weighted average of the LOS of hospitalized case-patients treated in West Africa through September 2014 (online Technical Appendix 1 Table 5) (*8*). We conducted a sensitivity analysis using LOS and reduced case-fatality rate of patients treated in the United States (online Technical Appendix 1 Table 6).

For late 2014, the low estimate of the average number of beds needed to treat patients with Ebola at any point in time was 1 (95% CI 0–3). The high estimate was 7 (95% CI 2–13).

In late 2014, the United States had to plan and prepare to treat additional Ebola case-patients. By mid-January 2015, the capacity of Ebola treatment centers in the United States (49 hospitals with 71 total beds [*9*]) was sufficient to care for our highest estimated number of Ebola patients. Policymakers already have used the BED model to evaluate responses to the risk for arrival of Ebola virus–infected travelers, and it can be used in future infectious disease outbreaks of international origin to plan for persons requiring treatment within the United States.

Acknowledgments

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Highly Pathogenic Avian Influenza A(H5N1) Virus in Poultry, Nigeria, 2015

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To the Editor: In Nigeria, from February 2006 through July 2008, outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 virus infection in poultry negatively affected animal and public health as well as the agricultural sector and trade. These outbreaks were caused by viruses belonging to genetic clades 2.2 and 2.2.1 (*1*). In January 2015, seven years after disappearance of the virus, clinical signs of HPAI (swollen head and wattles, hemorrhagic shank and feet) and increased mortality rates were observed among backyard poultry in Kano and in a live bird market in Lagos State, Nigeria. The virus was isolated from 2 samples independently collected from the poultry farm (parenchymatous tissues) and the market (tracheal swab), and H5 subtype virus was identified by reverse transcription PCR. The samples were adsorbed onto 2 Flinders Technology Associates cards (GE Healthcare Life Sciences, Little Chalfont, UK), which were sent to the World Organisation for Animal Health/Food and Agriculture Organization of the United Nations Reference Laboratory for Avian Influenza in Italy for subtype confirmation and genetic characterization. Influenza A(H5N1) virus was detected in both samples, and sequencing of the hemagglutinin (HA) gene showed that the viruses possessed the molecular markers for HPAI viruses with a multibasic amino acid cleavage site motif (PQRERRRKR*G).

The complete genome of the virus from backyard poultry was successfully sequenced from the genetic material extracted from the Flinders Technology Associates cards by using an Illumina MiSeq platform (*2*) and was submitted to the Global Initiative on Sharing All Influenza Data database (http://platform.gisaid.org/) under accession nos. EPI556504 and EPI567299–EPI567305. Maximum-likelihood trees were estimated for all 8 gene segments by using the best-fit general time reversible plus invariant sites plus gamma 4 model of nucleotide substitution with PhyML (*3*). The topology of the phylogenetic tree of the HA gene demonstrated that the H5N1 virus from Nigeria (A/chicken/Nigeria/15VIR339-2/2015) falls within genetic clade 2.3.2.1c (Figure, panel A). In particular, the HA gene sequence clustered with H5 viruses collected in China in 2013 and with an H5N1 virus (A/Alberta/01/2014) isolated from a Canada resident who had returned from China (similarity 99.3%–99.5%) (*4*).

The remaining 7 genes were closely related to genes of A/Alberta/01/2014(H5N1), although the 2 viruses differed by 32 aa (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/7/15-0421-Techapp1.pdf). Just as for the virus from Canada (*4*), 7 of 8 gene segments of the virus from Nigeria clustered with HPAI A(H5N1) virus circulating in Vietnam and China, while the polymerase basic 2 gene segment (Figure, panel B) resulted from reassortment with viruses circulating in the same Asian countries but belonged to the H9N2 subtype. Differing from the strain from Canada (only 2 aa mutations compared with the 2.3.2.1c candidate vaccine strain; *5*), the strain from Nigeria possesses 6 aa differences: 3 in HA1 and 3 in HA2 (online Technical Appendix). The effect of these mutations on the antigenic relatedness of these strains should be further explored.

Molecular characterization demonstrated that the polymerase basic 2 sequence contains glutamic acid at position 627, establishing the lack of a well-known mammalian adaptation motif (*6*). Mutations associated with increased virulence in mice have been observed in the nonstructural protein 1 (P42S, D87E, L98F, I101M, and the 80–84 deletion) and in the matrix 1 proteins (N30D, T215A). In addition, the substitutions D94N, S133A, S155N (H5 numbering) associated with increased binding to α-2,6 sialic acid have been identified in the HA protein. However, most of these substitutions are present in the H5N1 virus sequences from Asia included in our phylogenetic analyses, suggesting that they may be common among the HPAI H5 virus subtype. Mutations associated with resistance to antiviral drugs have not been detected (*7*).

The results obtained from whole-genome analysis provide evidence that a novel clade of the A(H5N1) virus, specifically clade 2.3.2.1c, has reached Nigeria. Although ascertaining how and exactly when this has happened is difficult, it seems most likely that the virus entered the country in December 2014, as evidenced by unverified

[1]These authors contributed equally to this article.

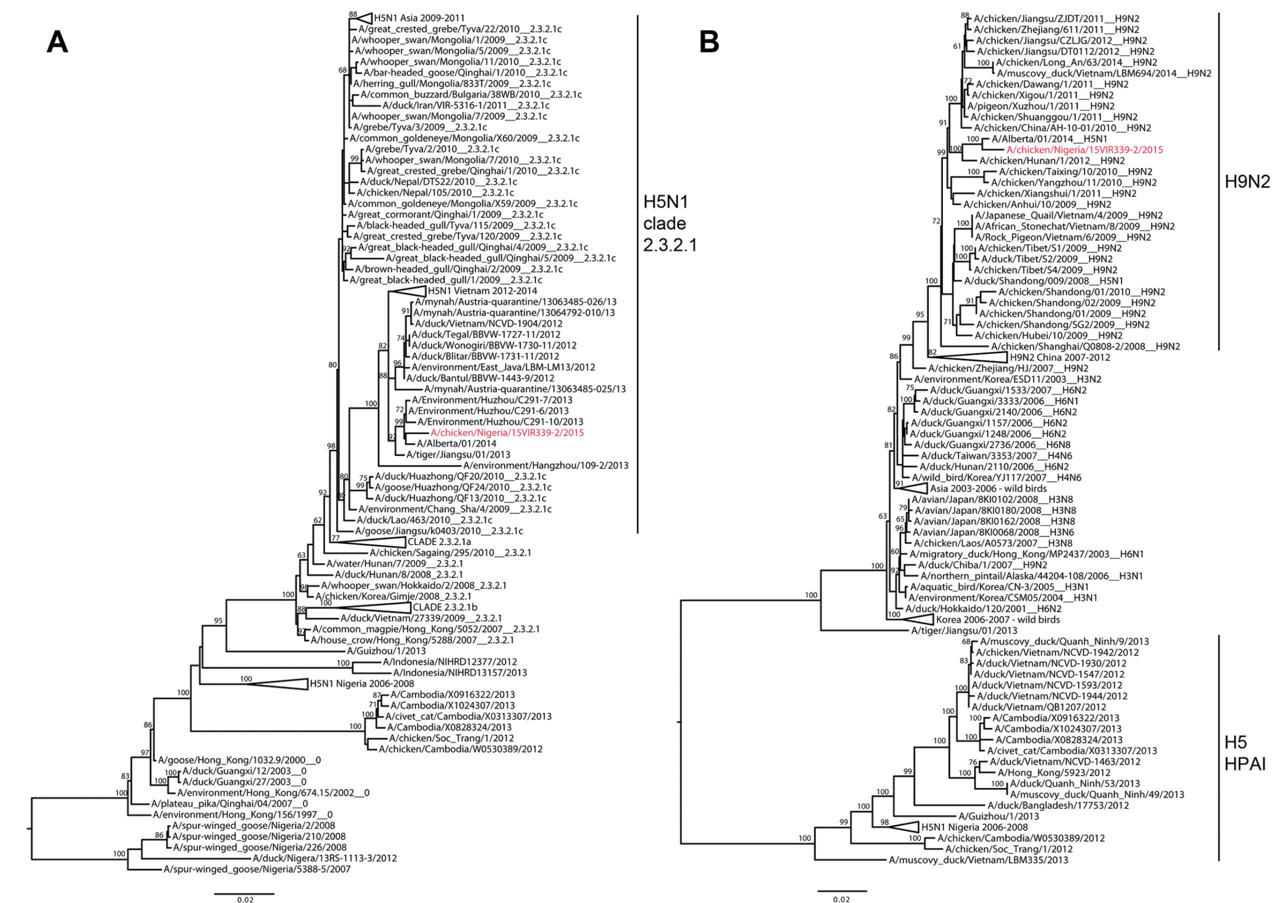

Figure. Maximum likelihood phylogenetic trees of the A) hemagglutinin and B) polymerase basic 2 gene segments of highly pathogenic avian influenza A(H5N1) virus from poultry in Nigeria, 2015 (in red). Bootstrap values (100 replicates) >60 are shown at the nodes. Scale bars indicate nucleotide substitutions per site. HPAI, highly pathogenic avian influenza.

accounts of increased poultry deaths in some live bird markets in Lagos, after the birds had been moved from the north (Kano) to the south during the festive season. The identification of genetic clustering between the strains from Nigeria analyzed here and the HPAI A(H5N1) viruses originally identified in Asia suggests an unknown epidemiologic link between these regions, probably associated with human activities, migratory bird movements, or both.

Considering that this virus is an intersubtype reassortant and has already caused infection in humans, we believe that complete characterization of the strain in terms of virulence and host range is of high priority. Furthermore, because the reemergence of subtype H5N1 virus was followed by epidemiologic amplification (≈265 outbreaks in 18 states as of February 2015; T. Joannis, pers. comm., 2015) for which virus genetic characterization is not yet available, local veterinary and public health services and international organizations should take necessary measures to identify critical control points and stop circulation of this virus.

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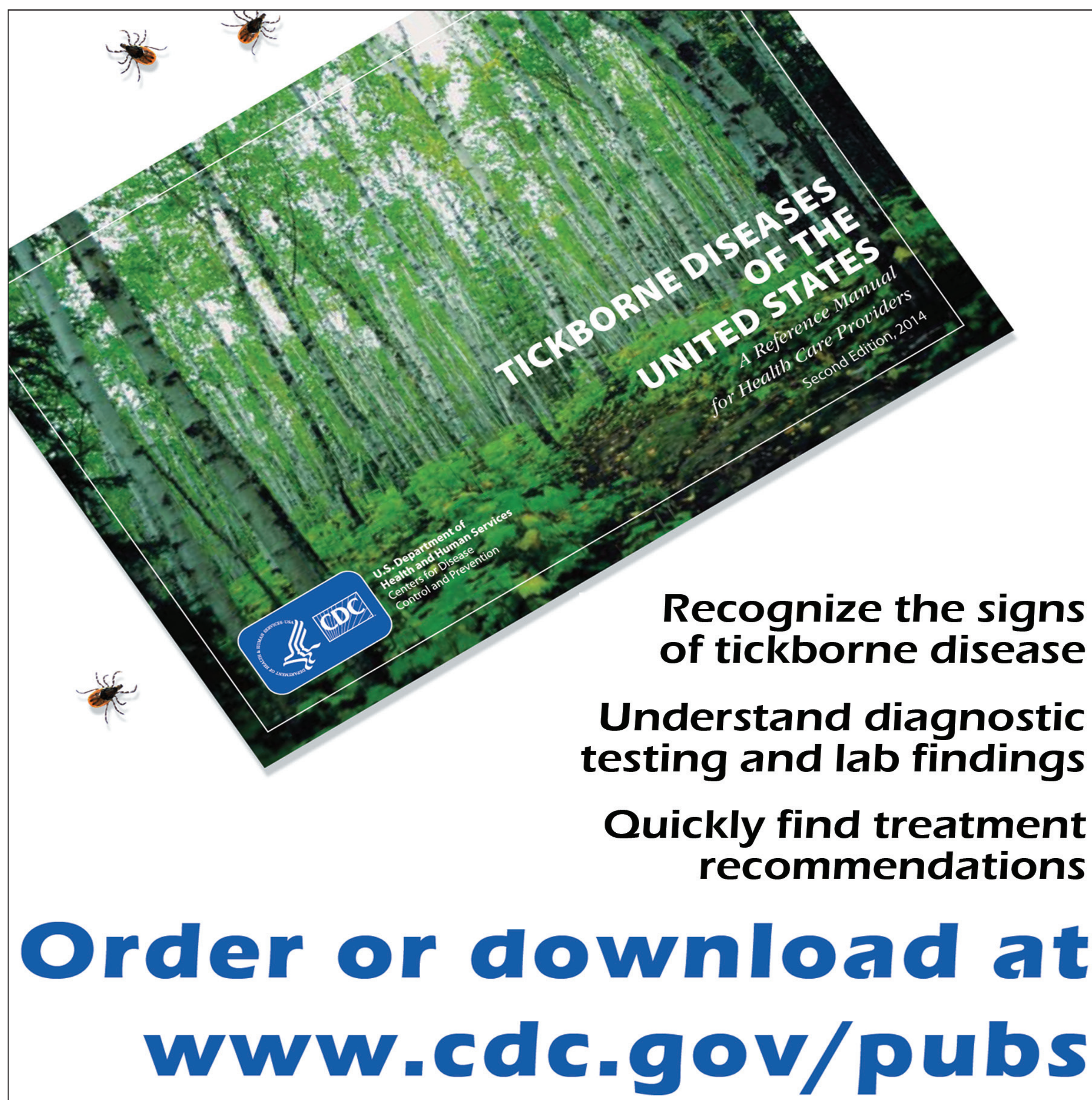

BOOKS AND MEDIA

Pulmonary Complications of HIV

Charles Feldman, Eva Polverino, and Julio A. Ramirez, editors

European Respiratory Society, Lausanne, Switzerland, 2014

ISBN: 978-1-84-984055-2
Pages: 265; Price: US $75.00 (paperback)

Pulmonary Complications of HIV summarizes current practices for diagnosing and treating common HIV-related pulmonary complications. It is a well-written, educational work that will interest anyone managing the care of HIV-infected persons. The content and amount of information packed into this easy-to-read textbook is impressive. Each chapter is well organized and well referenced, and important concepts and definitions are laid out clearly.

Since HIV/AIDS was first described, clinicians have found that the lung is the site most frequently affected and that pulmonary complications are a major cause of illness and death for HIV-infected persons. However, over the years, the discovery and use of antiretroviral therapy has increased life expectancy for HIV-infected persons, and the spectrum of infectious and noninfectious pulmonary complications has changed. For example, the incidence of opportunistic pneumonias has declined dramatically, whereas the incidence of bacterial pneumonia has not decreased proportionately. Furthermore, noninfectious complications, such as chronic obstructive pulmonary disease and lung cancer, are increasing. It is critical for anyone managing the care of HIV-infected persons to be aware of these lung complications and understand their diagnoses, possible treatments, and prevention. Pulmonary Complications of HIV does an excellent job discussing these aspects.

The authors are well-respected researchers and clinicians from throughout the world who work in the fields of pulmonary medicine and HIV-related lung diseases. The literature on HIV-related pulmonary complications is still lacking in certain areas, which most likely led to some chapters (e.g., Bronchiectasis) to be shorter and less comprehensive than others. Of the book's 19 chapters, the first 2 discuss the global epidemiology of HIV and current antiretroviral therapy guidelines, which will be useful for clinicians who might not regularly manage the care of HIV-infected patients. The third chapter discusses pulmonary immunity, a complicated topic but one the authors explain simply by emphasizing essential concepts. The next several chapters highlight various diseases and strategies for preventing them in the field, including vaccine guidelines. In addition, the authors cover a number of other key aspects to HIV care, such as pregnancy, pediatrics, and infectious and noninfectious complications, completing a thorough review of the literature. Although infectious disease specialists and others who care for HIV-infected patients might consider the first few chapters too simplistic, the latter chapters on pulmonary complications will be relevant and instructive. Pulmonologists will find that the first few chapters discuss aspects of HIV care to which they are not readily exposed and the latter chapters provide information on the epidemiology, clinical manifestations, diagnosis, and management of common complications seen in HIV-infected persons.

Pulmonary Complications of HIV does an exceptional job summarizing the major pulmonary manifestations of HIV/AIDS and discussing the progress in overall HIV treatment. Because the book itself is fairly short (265 pages), it appears to be more of a simple paperback rather than a reference textbook. Regardless, it is worthy of a spot on your bookshelf. As a clinician, educator, and researcher in the field of HIV-related lung disease, I found the book to be informative, easy to read, and a quick and simple reference to have on hand. It would be valuable to any medical trainee or clinician who manages the care of HIV-infected patients.

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The Emergence of Tropical Medicine in France

Michael A. Osborne

University of Chicago Press, Chicago, Illinois, USA

ISBN: 978-0-22-611452-1 (print); 978-0-22-611466-8 (ebook)

Pages: 312; Price: US $50.00 (print); US $7.00–$44.00 (ebook)

The idea of naval medicine as a specific and discrete art is richly illustrated in The Emergence of Tropical Medicine in France, Michael Osborne's historical account of French colonial medicine. For expanding European empires, the nineteenth century was a time when theories of tropical disease evolved as responses to distinct challenges on ships, in colonies, and in home ports. In France, a system of provincial medical schools was built by the navy

in the port cities of Brest, Rochefort-sur-Mer, Toulon, and Bordeaux. Each faculty held to and taught their own system of medical knowledge retained within the regional boundaries. As described in this book, beliefs on causality and therapeutic options remained divided among the discrete spheres among institutions. The lack of accepted curricula seems a distant reminder of the many gains made before evidence-based medicine. To enrich the perspective, most of the book's content is set in advance of the study of medical geography, which settled some longstanding misconceptions about ethnicity, location, and disease. Confusion reigned in colonial settings because of the similarity of causes implied during outbreaks of yellow fever, cholera, plague, typhus, and typhoid fever.

This book does not address the scientific advancements on infectious etiologies; rather, it provides the context for French innovation within colonial functionaries, clashing ideologies, and commercial considerations. Medical training played a pivotal role in French colonial activity, as in Madagascar in 1895, when expeditionary forces were decimated by the thousands from malaria. While the prevailing belief was that tilled and swampy land caused the illness, that belief was overturned, by persons with medical training, in favor of insect bites. Success in Madagascar, as well as other overseas colonies, depended upon knowing disease cycles and managing interaction in the human populations.

This book is a worthwhile investment for those interested in historical narratives on tropical medicine previously unavailable in the English language. Naval physicians like Charles-Adolphe Maher did remarkable studies while touring the tropics. In 1823, after having studied at Rochefort, he spent 2 years voyaging and encountered yellow fever outbreaks in Havana and Veracruz. Within the confines of his ship, Maher carefully compared the spectrum of symptoms. His conclusion on intermittent fever being a variety of malaria was far from correct, yet Mahler did initiate brave comparisons of therapies among patients, albeit with bloodletting and dietary privations. Maher's lifelong findings on medical statistics, Statistique Medicale de Rochefort, first published in 1874 and recently reprinted, recount the lively experiences of Mahler and many other persons investigating *médicine exotique*. Osborne's book provides key insight regarding influential persons who revolutionized notions of disease, recognizing their contributions as harbingers for the vast developments to follow in the twentieth century.

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Correction: Vol. 21, No. 3

The number of invasive pneumococcal disease reports was listed incorrectly in Risk Factors for Death from Invasive Pneumococcal Disease, Europe, 2010 (A. Navarro-Tornéet al.). The article has been corrected online (http://wwwnc.cdc.gov/eid/article/21/3/14-0634_article).

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ABOUT THE COVER

Portrait of the Coveted Cinchona

Byron Breedlove and Paul M. Arguin

English Victorian botanical artist Marianne North is celebrated for her meticulous attention to detail, form, and color. Her collection of 833 paintings, which portray more than 900 species of plants, comprises her life's work and is on permanent display in the Marianne North gallery at the Royal Botanic Gardens, Kew, United Kingdom. Because North painted with oils, rather than with watercolors, and because she predates color photography, her body of work offers an enduring visual record of these plant species, some of which are quite rare or extinct.

North did not take lessons in oil painting until 1867 or begin her odyssey to paint flora from around the world until 3 years later when she was 40 years of age, which makes the quality and volume of her work more remarkable. After exhibiting her paintings in a London gallery in 1879, North wrote to Kew director Sir Joseph Hooker, offering to build a gallery if he would agree to display her life's work there. He consented, and North then devoted a year arranging her paintings inside her eponymous gallery before its public opening in 1882. Her paintings still hang in this gallery, which was faithfully restored to its original character in 2009.

Earlier in her life, North had traveled broadly with her father Frederick North, who had been a member of parliament for Hastings. Following his death in 1869, her substantial inheritance and many political connections allowed her free rein to travel and pursue her passion for painting. During the next 13 years, she visited 16 countries on 6 continents, including Brazil, Japan, Singapore, Sri Lanka, and South Africa. Following a recommendation of Charles Darwin, a friend both of her late father and Hooker, North also explored Australia, New Zealand, and Tasmania.

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Marianne North (1830–1890), Foliage, Flowers, and Seed-vessels of a Peruvian Bark Tree, 1870s. Oil on card. 20 × 11.5 in/50.8 × 29.2 cm © Royal Botanic Gardens Kew.

North was not interested in mingling with political leaders or ambassadors or splurging on indulgences. Strange, even unwelcoming terrain teeming with botanical specimens beckoned her. She preferred to paint images of her specimens where they naturally grew. This free spirit favored solitary travel—she was known to elude travel companions or guides assigned to her—and did not mind simple, primitive accommodations that enabled her to be close to local flora. She did, however, consider a supply of paper and oil paints to be indispensable. In her autobiography, she notes that painting for her was "a vice like dram-drinking, almost impossible to leave off once it gets possession of one."

North painted this month's cover image, "Foliage, Flowers, and Seed-vessels of a Peruvian Bark Tree," while traveling in South America during the early 1870s. North's graceful painting captures many key facets of her specimen. In the center, a small branch is clustered with white flowers thrusting about a number of examples of this specimen's oval leaves. Her palette of greens, reds, and browns amply juxtaposes the leaves in various stages from shiny new growth to older leaves. Many of the coveted cinchona seeds cling to twigs to the right of the flowers. A small branch in the upper right corner provides our only close look at the much-valued and bitter tasting bark. A tree-covered ridge juts above the lower forest, and clouds and mist swirl across the sky and down the ridge, showing the lushness of this tree's natural habitat.

The Peruvian bark tree, also known as the Jesuit tree or the fever tree, is a cinchona[1] of the family Rubiaceae, native to the western forests of the South American Andes. Its bark produces several alkaloids, including quinine, which has potent antimalarial properties, and quinidine, which has antiarrhythmic properties. The medicinal properties of the cinchona tree are thought to have been discovered by the Quechua, indigenous people from Peru and Bolivia. After the Jesuits learned about cinchona and brought it to Europe, its bark was widely used there to treat fevers starting in the 17th century. Not long after French scientists Pierre Joseph Pelletier and Joseph Bienaimé Caventou isolated quinine from cinchona bark in 1820, the governments of Bolivia, Columbia, Ecuador, and Peru unsuccessfully attempted to embargo the exportation of cinchona seeds, seedlings, or trees.

Smuggled seeds enabled Europeans to establish cinchona plantations in Southeast Asia, and the Dutch soon held a monopoly on supplies. In 1942, Japan gained control of the cinchona trees cultivated for quinine in parts of Asia, and Germany captured the quinine reserves in Amsterdam. Also in 1942, confronted by advancing Japanese troops, Colonel Arthur Fischer boarded the last plane to leave Mindanao, the second largest island in the Philippines, with a tin can filled with cinchona seeds. These seeds were used to establish plantations in Costa Rica and Ecuador, but those plantings were too late to benefit the war effort. The scarcity of quinine during the war led to the development of alternate antimalarial drugs, some of which are still in use today. During the 1960s, several strains of the malarial parasite *Plasmodium falciparum* developed resistance to some synthetic drugs, particularly chloroquine. The parasite remained sensitive, however, to quinine, leading to a resurgence of its use, despite potential side effects from large doses.

The World Health Organization (WHO) documents that 198 million cases of malaria occurred globally in 2013 (uncertainty range 124–283 million) and that malaria was responsible for 584,000 deaths (uncertainty range 367,000–755,000). WHO further reported that in 2014, malaria transmission was ongoing in 97 countries and territories and ≈3.3 billion persons remained at risk for malaria. However, recent increases in resources, political will, and commitment have led to great improvements in malaria control in many parts of the malaria-endemic world. These efforts must be sustained to ensure progress toward malaria elimination and ultimately eradication.

[1]A common story about the discovery of the fever-reducing properties of this tree's bark centers on the wife of a Viceroy of Peru, Countess Ana of Chinchonm who contracted malaria in 1638 and was reputedly cured of fever by a powder made from cinchona bark. Despite its popularity, evidence does not support its veracity. Regardless, Linnaeus named the genus *Cinchona* in her honor but with a misspelling that in 1866 the International Botanical Congress opted to retain.

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EMERGING INFECTIOUS DISEASES®

Upcoming Issue

Drivers of Emerging Infectious Disease Events as a Framework for Digital Detection

Health Care–Associated Infection Outbreak Investigations in Outpatient Settings, Los Angeles County, California, USA, 2000–2012

Escherichia coli O157 Outbreaks in the United States, 2003–2012

Differentiation of Acute Q Fever from Other Infections, the Netherlands

Community-based Outbreak of *Neisseria meningitidis* Serogroup C in Men who Have Sex with Men, New York, New York, USA, 2010–2013

Risk for Mycobacterial Disease among Patients with Rheumatoid Arthritis, Taiwan, 2001–2011

Phylogeography of Influenza A(H3N2) Virus in Peru, 2010–2012

Extensive Nosocomial Transmission of Measles Originating in Cruise Ship Passenger, Sardinia, Italy, 2014

Smallpox Vaccination of Laboratory Workers at US Variola Testing Sites

Infections with *Candidatus* Neoehrlichia mikurensis and Cytokine Responses in 2 Persons Bitten by Ticks, Sweden

Occupational Exposure to Dromedaries and Risk for MERS-CoV Infection, Qatar, 2013–2014

Genome of Emerging Norovirus GII.17, United States, 2014

Seasonal Patterns of Buruli Ulcer Incidence, Central Africa, 2002–2012

Human–Bat Interactions in Rural West Africa

Enterovirus A71 Meningoencephalitis Outbreak, Rostov-on-Don, Russia, 2013

Detection and Full-Length Genome Characterization of Novel Canine Vesiviruses

Measles Vaccination Coverage and Cases among Vaccinated Persons

Rickettsia felis Infections, Bangladesh, 2012–2014

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 10–21, 2015
14th International Dengue Course
Havana, Cuba
http://instituciones.sld.cu/ipk/14thdenguecourse

August 24-26, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA
http://www.iceid.org/

August 29–September 2, 2015
IDBR
20th Annual Infectious Disease Board Review Course
McLean, VA, USA
http://smhs.gwu.edu/cehp/activities/courses/idbr

September 17–21, 2015
ICAAC
Interscience Conference on Antimicrobial Agents and Chemotherapy
San Diego, CA, USA

October 25–29, 2015
ASTMH
American Society of Tropical Medicine and Hygiene
64th Annual Meeting
Philadelphia, PA, USA
info@astmh.org

February 8–10, 2016
ASM Biodefense and Emerging Diseases Research Meeting
Arlington, VA, USA
biodefense@asmusa.org

March 2–5, 2016
ISID
17th International Congress on Infectious Diseases
Hyderabad, India

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.

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Article Title

Disseminated Infections with *Talaromyces marneffei* in Non-AIDS Patients Given Monoclonal Antibodies against CD20 and Kinase Inhibitors

CME Questions

1. According to the case series report by Chan and colleagues, which of the following statements about the clinical and epidemiologic characteristics of *Talaromyces marneffei* infection is correct?

A. *T. marneffei infection* is the most important pathogenic thermal dimorphic fungus causing systemic mycosis in Southeast Asia

B. *T. marneffei* infection is typically limited to the gastrointestinal and/or urinary tract

C. *T. marneffei* infection is usually self-limited with a good prognosis for recovery

D. *T. marneffei* infection is common among non-AIDS hematology patients

2. Your patient is a 57-year-old Chinese man with acute myeloid leukemia and fever. According to the case series report by Chan and colleagues, which of the following statements about the recent emergence of disseminated *T. marneffei* infection in non-AIDS patients with hematologic malignant neoplasms treated with targeted therapies is correct?

A. The appearance of 4 cases in the past 2 years is the result of a change in the methodologies of a laboratory diagnosis of *T. marneffei* infection

B. The appearance of 4 cases in the past 2 years is the result of a dramatic increase in the number of hematology patients at the investigators' hospital

C. Recent emergence of disseminated *T. marneffei* infection is most likely because of overall immunosuppression

D. Recent emergence of disseminated *T. marneffei* infection is most likely because of targeted therapies, such as anti-CD20 monoclonal antibodies and kinase inhibitors

3. According to the case series report by Chan and colleagues, which of the following statements about possible mechanisms of action underlying disseminated *T. marneffei* infection in non-AIDS patients with hematologic malignant neoplasms treated with targeted therapies would most likely be accurate?

A. Rituximab and obinutuzumab used in 2 cases are anti-CD20 monoclonal antibodies that predominantly target T cells

B. *T. marneffei* infection is usually seen at CD4+ counts of more than 300/µL

C. Patients with B-cell dysfunction may have impaired production of neutralizing antibodies against key virulence factors of *T. marneffei*

D. The investigators do not suggest any role of cytokine-producing B-cells

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Parechovirus Genotype 3 Outbreak among Infants, New South Wales, Australia, 2013–2014

CME Questions

1. Your patient is an 18-month-old Australian boy presenting with fever and presumed sepsis. According to the surveillance report by Cumming and colleagues, which of the following statements about the clinical and epidemiologic features of a human parechovirus genotype 3 (HPeV3) outbreak among Australian infants is correct?

A. Between October 2013 and February 2014, a total of 183 cases of HPeV3 were identified in NSW infants
B. Three-quarters of affected infants were girls
C. Half of affected infants required hospitalization
D. Common symptoms were diarrhea, sleepiness, and cough

2. According to the surveillance report by Cumming and colleagues, which of the following statements about the presentation of the HPeV3 outbreak in Australia compared with that in the northern hemisphere is correct?

A. The Australian outbreak affected slightly younger infants
B. The Australian outbreak had a more even gender split
C. Frequency of skin rash was lower in the Australian outbreak
D. Peak number of cases was later than documented in the northern hemisphere

3. According to the surveillance report by Cumming and colleagues, which of the following statements about the efficacy of active surveillance in detecting and monitoring the HPeV3 outbreak among Australian infants would most likely be accurate?

A. Syndromic surveillance was not useful for outbreak monitoring
B. Public health response had no apparent effect on infant length of stay
C. Awareness-raising communication strategies were ineffective
D. Active surveillance is resource intensive but helped to define the infection and link it with a syndromic surveillance indicator

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

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Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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