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2024
CDC YELLOW BOOK
Health Information for International Travel

Launch of CDC Yellow Book 2024 – A Trusted Travel Medicine Resource

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The CDC Yellow Book is available in print through Oxford University Press and online at www.cdc.gov/yellowbook.
Invasive *Nocardia* Infections across Distinct Geographic Regions, United States


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Learning Objectives

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

* Analyze the bacteriology of *Nocardia*
* Evaluate patterns of infection with *Nocardia* in the current study
* Differentiate infection sites based on different *Nocardia* species among transplant recipients
* Analyze antibiotic susceptibility and survival data in the current study of patients with *Nocardia* infection

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We reviewed invasive Nocardia infections in 3 noncontiguous geographic areas in the United States during 2011–2018. Among 268 patients with invasive nocardiosis, 48.2% were from Minnesota, 32.4% from Arizona, and 19.4% from Florida. Predominant species were N. nova complex in Minnesota (33.4%), N. cyriacigeorgica in Arizona (41.4%), and N. brasiliensis in Florida (17.3%). Transplant recipients accounted for 82/268 (30.6%) patients overall: 14 (10.9%) in Minnesota, 35 (40.2%) in Arizona, and 33 (63.5%) in Florida. Manifestations included isolated pulmonary nocardiosis among 73.2% of transplant and 84.4% of non–transplant patients and central nervous system involvement among 12.2% of transplant and 3.2% of non–transplant patients. N. farcinica (20.7%) and N. cyriacigeorgica (19.5%) were the most common isolates among transplant recipients and N. cyriacigeorgica (38.0%), N. nova complex (23.7%), and N. farcinica (16.1%) among non–transplant patients. Overall antimicrobial susceptibilities were similar across the 3 study sites.

Nocardia is a genus of aerobic, filamentous, bead-ed, gram-positive bacteria ubiquitous in the environment, especially in soil, decomposing organic matter, and water. Nocardia spp. are opportunistic pathogens in immunocompromised hosts and immunocompetent persons with chronic lung disease (1). The Centers for Disease Control and Prevention has estimated that the United States sees 500–1,000 new cases of Nocardia infection annually (2,3). Pulmonary infections acquired by inhaling aerosolized organisms account for most Nocardia-associated illness (4). Direct inoculation, which causes cutaneous infections, constitutes the second most common route of exposure (3,5). Patients with defects in cell-mediated immunity are at highest risk for infection (6,7). Other risk factors include systemic corticosteroid use, solid organ or hematopoietic stem cell transplantation, HIV infection, diabetes mellitus, and underlying malignancy being treated with chemotherapy (8). The skin, brain, and soft tissues, and to a lesser degree bones and joints or other organs, comprise the most common sites of extrapulmonary dissemination (6,9).

Geographic distribution of various Nocardia spp. and their effect on human disease has not been described. Although N. cyriacigeorgica (formerly N. asteroides drug pattern type VI) (10,11) and N. farcinica are distributed evenly throughout the United States, distribution of other Nocardia spp. varies by geographic location. N. brasiliensis is associated with tropical and subtropical environments and has a higher prevalence in the southwestern and southeastern regions of the United States (4,12). Because Mayo Clinic, headquartered in Rochester, Minnesota, USA, operates tertiary locations in the midwestern, southeastern, and southwestern United States, we had a unique opportunity to study Nocardia infections in those 3 distinct noncontiguous geographic areas.

Methods
We performed a multicenter retrospective cohort study of patients evaluated at Mayo Clinic facilities in Minnesota, Florida, and Arizona. We reviewed all culture-positive microbiologic specimens of Nocardia spp. during December 2011–November 2018. A previous study from Mayo Clinic Florida published clinical outcomes for patients with invasive nocardiosis during 1998–2018 (13); the Florida cohort for this study included all patients identified during December 2011–November 2018 from that study. We defined invasive nocardiosis diagnosis as culture-positive for Nocardia spp. together with clinical or radiographic evidence of organ involvement. We defined disseminated disease if infection was identified by culture or radiographic imaging in ≥2 noncontiguous organs, with Nocardia isolated from ≥1 site, or by a single positive blood culture.

Demographic and Clinical Data
We extracted information from electronic medical records about demographics, coexisting conditions, and antimicrobial drugs used. We classified as transplant recipients those patients who had received a solid organ or hematopoietic stem cell transplant before Nocardia infection was diagnosed. We referred to antimicrobial drugs administered soon after Nocardia spp. was isolated or detected on special stains as initial therapy. After species confirmation and antimicrobial susceptibility testing results became available, we recorded subsequent treatment as definitive therapy. Outcome data included all-cause mortality at 1 year after diagnosis of invasive nocardiosis.

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Patients received an initial diagnosis of nocardiosis, an evaluation, and treatment at 1 of 3 sites: Mayo Clinic Rochester (Minnesota), Mayo Clinic Arizona in Phoenix/Scottsdale, Arizona (Arizona), or Mayo Clinic Florida in Jacksonville, Florida (Florida). We extracted postal (ZIP) code–specific location data for all participants based on residence at the time of data extraction. Clinical outcomes for the Florida cohort include data for the December 2011–November 2018 subset of patients from the earlier Mayo Clinic study (13).

Microbiology
We extracted information on type of specimen, species, and antimicrobial susceptibilities for Nocardia isolates from the microbiology database at Mayo Clinic Laboratories (https://www.mayocliniclabs.com). We categorized species as other if <5 isolates of that species were identified or if specific species was undetermined. We identified Nocardia at the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and if necessary, 16s ribosomal RNA sequencing. Antimicrobial susceptibility testing using broth microdilution was performed at Mayo Clinic Laboratories.

Of note, 3 isolates from 2012–2015 in the study were identified as either N. asteroides (2) or N. asteroides complex (1). Because of taxonomic changes in Nocardia identification on the basis of molecular methodologies, N. asteroides complex has been reclassified into 6 taxa, with N. cyriacigeorgica (drug pattern VI) being the most common (11). Because we could not reclassify those 3 isolates, we included them in the other species category.

Statistical Analysis
We described patient demographics, coexisting conditions, disease characteristics, and outcomes for the overall cohort and for subgroups by geographic location of treatment (Minnesota, Arizona, or Florida) and by transplant status, using frequency and percentages for categorical variables and means and SDs or medians and interquartile ranges (IQRs) for continuous variables. We plotted patient residential ZIP codes and frequency of Nocardia spp. by geographic location of treatment on a map of the United States. We also described initial empiric antimicrobial therapy and subsequent treatment after identification of Nocardia spp. and antimicrobial susceptibilities were available. We performed all analyses using SAS version 9.4 (SAS Institute).

Results
Of 268 patients with invasive nocardiosis during the study period, 129 (48.2%) were enrolled at the Mayo Clinic site in Minnesota, 87 (32.4%) in Arizona, and 52 (19.4%) in Florida. We recorded data on demographics, coexisting conditions, and organs transplanted by study site (Table 1). N. nova complex, 43 (33.4%) cases, was the predominant species in Minnesota and N. cyriacigeorgica, 36 (41.4%) cases, in Arizona. In Florida, the most common species were N. brasiliensis, 9 (17.3%) cases, and N. cyriacigeorgica and N. farcina, 8 (15.4%) cases each (Table 2). Other species accounted for 12.4% of cases in Minnesota, 8% in Arizona, and 26.9% in Florida. In Arizona, N. wallacei accounted for 13.8% and N. transvalensis complex for 11.5% of isolates, but they each constituted <2% of isolates in Florida and Minnesota.

Of the 268 patients, 82 (30.6%) were transplant recipients; 70 received solid organ and 12 hematopoietic stem cell transplants. Median time from transplant to diagnosis of invasive nocardiosis was 12 months (IQR 5–54 months). Kidneys, 33 (40.2%) cases, were the most common transplanted organ; more than half (17/33, 51%) of patients underwent transplantation in Arizona. Most heart recipients (9/13, 69%) also underwent transplantation in Arizona. Most (12/13, 92%) lung recipients underwent transplantation in Florida; however, during the study period, lung transplants were not performed at Mayo Clinic Arizona. Overall mean age of all transplant recipients was 63.8 years (SD 15.1). Chronic lung disease (58.6%) and diabetes (17.9%) were 2 of the most common coexisting conditions (Table 1).

We found isolated nocardial pulmonary involvement in 84.4% of non–transplant patients and 73.2% of transplant patients. Rate of dissemination was 12.3% in the total cohort: 9.7% in non–transplant patients, 18.3% in transplant patients (Table 2). We documented central nervous system (CNS) involvement in 12.2% of transplant recipients and 3.2% of non–transplant patients. (Table 2). The most commonly identified species among transplant recipients were N. farcinica (20.7%, n = 17) and N. cyriacigeorgica (19.5%, n = 16) and among non–transplant patients, N. cyriacigeorgica (38.0%, n = 52), N. nova complex (23.7%, n = 44), and N. farcinica (16.1%, n = 30).

When we analyzed the involvement of diseased organs in transplant patients, we found that Nocardia caused isolated pulmonary disease in 60/82 (75.0%) case-patients. Isolated lung disease was the predominant manifestation for most species: N. cyriacigeorgica caused isolated lung disease in 86.7% of transplant patients and combined lung and CNS disease.
in 13.3% of cases, and *N. farcinica* resulted in isolated lung disease in 76.5% of transplant patients and disseminated disease to the lungs, CNS, and skin/soft tissue in 11.8% of patients. Similarly, *N. nova* complex caused isolated pulmonary disease in 83.3% and isolated skin/soft tissue disease in 16.7%. In contrast, *N. brasiliensis* resulted in isolated skin/soft tissue disease in 60% of transplant patients and isolated lung disease in only 20%. Only 16/268 (6.0%) of all patients and 10/82 (12.2%) transplant patients had nocardial CNS involvement; *N. farcinica* (42.8% , n = 6), *N. cyriacigeorgica* (14.3%, n = 2), and *N. wallacei* (14.3%,

### Table 1. Patient demographics and coexisting conditions by site of diagnosis and transplant status in study of invasive *Nocardia* infections across 3 distinct geographic regions, United States*

<table>
<thead>
<tr>
<th>Category</th>
<th>Site of diagnosis</th>
<th>Transplant recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MN, n = 129</td>
<td>AZ, n = 87</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, y (SD)</td>
<td>63.4 (16.3)</td>
<td>65.9 (15.0)</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>61 (47.3)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>68 (52.7)</td>
</tr>
<tr>
<td>White</td>
<td>121 (97.6)</td>
<td>71 (84.5)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0 (0.0)</td>
<td>12 (14.0)</td>
</tr>
<tr>
<td>Coexisting conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>7 (5.4)</td>
<td>18 (20.7)</td>
</tr>
<tr>
<td>Liver failure</td>
<td>4 (3.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Renal failure/dialysis</td>
<td>6 (4.7)</td>
<td>4 (4.6)</td>
</tr>
<tr>
<td>Active malignancy</td>
<td>16 (12.5)</td>
<td>12 (13.8)</td>
</tr>
<tr>
<td>Rheumatologic conditions</td>
<td>21 (16.4)</td>
<td>5 (5.7)</td>
</tr>
<tr>
<td>Chronic lung disease</td>
<td>95 (74.8)</td>
<td>34 (39.1)</td>
</tr>
<tr>
<td>Transplant recipient</td>
<td>14 (10.9)</td>
<td>35 (40.2)</td>
</tr>
<tr>
<td>Transplanted organs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2 (14.3)</td>
<td>18 (51.4)</td>
</tr>
<tr>
<td>Liver</td>
<td>2 (14.3)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>1 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
<td>2 (14.3)</td>
<td>9 (25.7)</td>
</tr>
<tr>
<td>Kidney and liver</td>
<td>1 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td>Kidney and pancreas</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSC/T</td>
<td>5 (35.7)</td>
<td>6 (17.1)</td>
</tr>
</tbody>
</table>

*Values are no. (%) patients except at indicated. AZ, Mayo Clinic Arizona, Phoenix/Scottsdale, Arizona, USA; FL, Mayo Clinic Florida, Jacksonville, Florida, USA; HSCT, hematopoietic stem cell transplant; MN, Mayo Clinic Rochester, Rochester, Minnesota, USA; NA, not applicable.† Lung transplantations not performed at Mayo Clinic Arizona during study period.

### Table 2. Disease characteristics and outcomes by site of diagnosis and transplant status in study of invasive *Nocardia* infections across 3 distinct geographic regions, United States*

<table>
<thead>
<tr>
<th>Disease characteristics</th>
<th>Site of diagnosis</th>
<th>Transplant recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MN, n = 129</td>
<td>AZ, n = 87</td>
</tr>
<tr>
<td>Diseased organ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung only</td>
<td>106 (82.2)</td>
<td>80 (92.0)</td>
</tr>
<tr>
<td>Skin/soft tissue only</td>
<td>8 (6.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Disseminated</td>
<td>15 (11.6)</td>
<td>7 (8.0)</td>
</tr>
<tr>
<td>Any CNS involvement</td>
<td>6 (4.7)</td>
<td>5 (5.7)</td>
</tr>
<tr>
<td><em>Nocardia</em> spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. abscessus</em></td>
<td>13 (10.1)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td><em>N. brevicatena/N. paucivorans</em></td>
<td>3 (2.3)</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td><em>N. cyriacigeorgica</em></td>
<td>24 (18.6)</td>
<td>36 (41.4)</td>
</tr>
<tr>
<td><em>N. farcinica</em></td>
<td>26 (20.2)</td>
<td>13 (14.9)</td>
</tr>
<tr>
<td><em>N. nova complex</em></td>
<td>43 (33.3)</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td><em>N. transvalensis complex</em></td>
<td>1 (0.8)</td>
<td>10 (11.5)</td>
</tr>
<tr>
<td><em>N. brasiliensis</em></td>
<td>2 (1.6)</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td><em>N. wallacei</em></td>
<td>0</td>
<td>12 (13.8)</td>
</tr>
<tr>
<td><em>N. veterana</em></td>
<td>1 (0.8)</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Other†</td>
<td>16 (12.4)</td>
<td>7 (8.0)</td>
</tr>
</tbody>
</table>

*Values are no. (%) patients. AZ, Mayo Clinic Arizona, Phoenix/Scottsdale, Arizona, USA; CNS, central nervous system; FL, Mayo Clinic Florida, Jacksonville, Florida, USA; MN, Mayo Clinic Rochester, Rochester, Minnesota, USA.
n = 2) were the species most often associated with CNS disease. Rates of CNS involvement among transplant patients were similar across all 3 sites: 4.7% in Minnesota, 5.7% in Arizona, and 9.6% in Florida.

Antimicrobial susceptibilities varied among the most commonly isolated Nocardia spp. (Table 3). All Nocardia isolates were 100% susceptible to linezolid. Analysis of species-specific patterns of susceptibility revealed almost no differences by geographic location in susceptibility to antimicrobials across the 3 Mayo Clinic sites. Trimethoprim/sulfamethoxazole (TMP/SMX) susceptibility was 99.5% among non-transplant patients, 96.3% among transplant recipients, and 99% for the total cohort. Exceptions among Nocardia spp. susceptibility to TMP/SMX were 94% for N. abscessus and 92.0% for those listed collectively as other species. Susceptibility to ceftriaxone ranged from 0% for N. brasiliensis to 100% for N. brevicatena/N. paucivorans. Similarly, susceptibility to imipenem varied; N. cyriacigeorgica (97%), N. nova complex (98%), and N. brevicatena/N. paucivorans (100%) were the most susceptible species (Table 3). For all antimicrobial drugs administered during initial empiric therapy, we calculated the percentage retained in subsequent treatment regimens after analysis of Nocardia antimicrobial susceptibilities (Table 4).

We also analyzed overall Nocardia susceptibility patterns in transplant and non-transplant patients. Susceptibility was 51.9% for moxifloxacin and 32.1% for ciprofloxacin in transplant patients and 34.1% for moxifloxacin and 20.2% for ciprofloxacin in non-transplant patients. Imipenem was 72.8% susceptible in transplant patients and 80.3% in non-transplant patients; linezolid was 98.8% susceptible in transplant patients and 100.0% in non-transplant patients; ceftriaxone was 32.1% susceptible in transplant patients and 43.7% in non-transplant patients. Among nocardiosis patients, 155/186 (90.6%) non-transplant patients and 66/82 (80.5%) transplant recipients survived after 1 year. Rates of all-cause mortality at 1 year after diagnosis were similar for all 3 Mayo Clinic sites and different Nocardia spp.

**Discussion**

We identified 268 patients (82 transplant recipients and 186 non-transplant recipients) with invasive nocardiosis during 2011–2018 for this retrospective cohort study. Participants were patients at 3 Mayo Clinic tertiary care and high-volume transplant sites in Minnesota, Arizona, and Florida within the United States, which provided a unique opportunity to assemble a large cohort of Nocardia infected case-patients from within the entire Mayo Clinic database.

We described microbiologic, drug susceptibility, and outcome data in patients with nocardiosis at each geographic site. Nocardia spp. varied by geographic site where diagnosis occurred, correlating with published differences in geographic distribution (2). The most common species of Nocardia isolated in Minnesota were N. nova complex, N. farcinica, and N. cyriacigeorgica. In Arizona, N. cyriacigeorgica, N. farcina, and N. wallacei were the most common species. N. brasiliensis was the most common species in Florida, which might reflect the organism’s preference for tropical climates (4). Some patient demographics and clinical characteristics differed by site and might contribute to differences among species in occurrence, susceptibilities, and clinical outcomes.

Isolated lung involvement was the most common manifestation of Nocardia infection at all 3 sites: 92%

### Table 3. Antimicrobial susceptibilities based on Nocardia spp. in study of invasive Nocardia infections across 3 distinct geographic regions, United States*

<table>
<thead>
<tr>
<th>Species</th>
<th>No. isolates</th>
<th>AM/CL</th>
<th>CEF</th>
<th>CTX</th>
<th>IMP</th>
<th>CIP</th>
<th>MOX</th>
<th>CLA</th>
<th>AMI</th>
<th>TOB</th>
<th>DOX</th>
<th>MIN</th>
<th>TMP/SMX</th>
<th>LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. abscessus</td>
<td>17</td>
<td>82</td>
<td>82</td>
<td>94</td>
<td>59</td>
<td>0</td>
<td>14</td>
<td>41</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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*AM/CL, amoxicillin/clavulanic acid; CEF, cefepime; CTX, ceftriaxone; IMP, imipenem; CIP, ciprofloxacin; MOX, moxifloxacin; CLA, clarithromycin; AMI, amikacin; TOB, tobramycin; DOX, doxycycline; MIN, minocycline; TMP/SMX, trimethoprim/sulfamethoxazole; LIN, linezolid.
†Other species (n<5 each): N. amicacinotolerans (1), N. asiatica (1), N. asteroides complex (3), N. beijingensis (3), N. carnea (1), N. cerradoensis (1), N. exalbida (1), N. flavosea (1), N. kuczakiae (1), N. niwae (2), N. otidiscaviarum (1), N. pseudobrasiliensis (4), N. puris (1), N. takedensis (3), N. verruculata (1), N. vinacea (1), and N. yamanashiensis (1); no species identified (9)
in Arizona, 82% in Minnesota, and 60% in Florida. Possibly the arid climate of the desert southwest in Arizona presents greater opportunity for airborne dispersal of *Nocardia* and subsequent inhalation and pulmonary infection (similar to the transmission kinetics for coccidiodiomycosis). Primary skin/soft tissue infection was present in 19.2% of cases in Florida, more common than in the other locations, likely related to a higher rate of *N. brasiliensis* infection (17.3%) in Florida; *N. brasiliensis* is known to cause isolated cutaneous infection (3,14). Patients in Florida had the highest rate of disseminated nocardiosis (21.2%). Rates of death at 1 year were similar among the 3 Mayo Clinic sites. Rates of death based on the species of *Nocardia* were also similar, which is contrary to a recent study indicating a higher death rate for *N. farcinica* (15). *N. farcinica* was most commonly associated with CNS involvement in our cohort, which is consistent with previous reports (16,17).

All *Nocardia* isolates in our study were susceptible to linezolid, in agreement with other studies (18,19). The 3 most common *Nocardia* spp. implicated in invasive infection in this study, *N. cyriacigeorgica*, *N. farcinica*, and *N. nova* complex, were 100% susceptible to TMP/SMX. Susceptibility profiles for >1,200 *Nocardia* isolates reported in another study also noted universal susceptibility to linezolid. TMP/SMX resistance was rare (2%), except among *N. pseudobrasiliensis* (31%) and *N. transvalensis* complex (19%) (20). In our study, we noted only 3.7% resistance to TMP/SMX among *Nocardia* isolates in transplant recipients, which is in agreement with other studies that have reported low overall resistance rates (21–23). However, ongoing surveillance for such trends is paramount. In patients with *Nocardia* infection, especially those receiving substantial immune-suppression treatments, using combination antimicrobial therapy before availability of susceptibility testing results is imperative. Combination therapy should always include either linezolid or TMP/SMX. Based on our susceptibility data, an appropriate second antimicrobial choice would be imipenem, which has the added advantage of excellent CNS penetration. We also calculated the percentage of each antimicrobial in our initial empiric regimen that was retained in subsequent regimens after susceptibilities to all antimicrobials were known (Table 4).

**Table 4. Choice of initial/empiric antimicrobials and subsequent antimicrobials after susceptibilities were reported in study of invasive *Nocardia* infections across 3 distinct geographic regions, United States**

| Treatment after species confirmation and antimicrobial susceptibility testing results | Initial treatment No. (%) | AM/CL | CEF | CTX | IMP | CIP | MOX | CLA | AMI | TOB | DOX | MIN | TMP | SMX | LIN | SUL | Other |
| AM/CL | 7 (2.6) | 57 | 0 | 0 | 14 | 14 | 14 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CEF | 3 (1.1) | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 33 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CTX | 22 (8.2) | 14 | 0 | 0 | 14 | 5 | 4 | 18 | 9 | 5 | 0 | 9 | 14 | 45 | 5 | 9 | 5 |
| IMP | 49 (18.3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CIP | 7 (2.6) | 14 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 14 | 34 | 14 | 0 | 0 | 0 | 0 | 0 |
| MOX | 19 (7.1) | 11 | 0 | 11 | 16 | 0 | 74 | 11 | 0 | 11 | 5 | 16 | 37 | 0 | 0 | 0 | 0 |
| CLA | 10 (3.7) | 0 | 0 | 0 | 0 | 0 | 10 | 20 | 80 | 10 | 0 | 0 | 10 | 20 | 0 | 0 | 0 |
| AMI | 8 (3.0) | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 75 | 0 | 0 | 0 | 0 |
| TOB | 2 (0.8) | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 0 | 50 | 0 | 0 | 0 | 0 |
| DOX | 15 (5.6) | 20 | 7 | 0 | 20 | 7 | 13 | 0 | 0 | 0 | 0 | 0 | 33 | 0 | 7 | 0 | 0 |
| MIN | 27 (10.1) | 11 | 0 | 7 | 7 | 4 | 30 | 7 | 0 | 0 | 4 | 37 | 52 | 0 | 0 | 0 | 0 |
| TMP/SMX | 153 (57.1) | 6 | 1 | 7 | 5 | 5 | 5 | 17 | 3 | 1 | 4 | 10 | 82 | 2 | 3 | 3 | 0 |
| LIN | 21 (7.8) | 19 | 0 | 10 | 19 | 5 | 33 | 5 | 0 | 0 | 5 | 38 | 57 | 5 | 10 | 0 | 0 |
| SUL | 4 (1.5) | 25 | 0 | 50 | 0 | 0 | 0 | 25 | 0 | 0 | 0 | 25 | 0 | 75 | 0 | 0 | 0 |
| Other | 15 (5.6) | 7 | 0 | 7 | 7 | 13 | 0 | 0 | 0 | 0 | 0 | 13 | 13 | 60 | 0 | 0 | 13 |

*Gray shaded cells show percentages of patients in which initial antimicrobial was retained as part of subsequent therapy. AM/CL, amoxicillin/clavulanic acid; CEF, cefepime; CTX, cefotaxime; IMP, imipenem; CIP, ciprofloxacin; MOX, moxifloxacin; CLA, clarithromycin; AMI, amikacin; TOB, tobramycin; DOX, doxycycline; MIN, minocycline; TMP/SMX, trimethoprim/sulfamethoxazole; LIN, linezolid; SUL, sulfa
was not associated with increased death (15). Our study found death rates after 1 year of 19.5% among transplant recipients and 9.4% among non-transplant patients, an observation concordant with findings in a larger study from Europe of nocardiosis in solid organ transplant recipients (24). However, another recent publication noted similar death rates for transplant and non-transplant recipients (13). Retrospective designs, different underlying risk factors for infection, and variable definitions used in published studies make drawing firm conclusions difficult.

Among the limitations of our study, we did not collect detailed clinical or radiographic results or match specific treatment information with outcomes. However, those data have been elaborated in previous studies, including from Mayo Clinic (13,15). The primary purposes of our study were to determine regional differences among patients with risk factors, geographic distribution of Nocardia spp., organs involved by geographic location and species of Nocardia, and differences in antimicrobial susceptibility. Second, even though patients were from distinct geographic locations, they were all primarily managed within the Mayo Clinic system. Patients from other regions and institutions were not represented. However, the substantial climatic and environmental differences among the 3 regions studied—midwestern, southeastern, and southwestern United States—led us to decide on identifying unique trends in Nocardia spp. and disease manifestations for those study sites. A larger prospective study encompassing patients and institutions from across the United States and other parts of the world could uncover additional nuances in data pertaining to both pathogens and hosts. Third, data on residential ZIP codes of patients (Figure) was collected at time of data extraction and may differ slightly from the residences of patients at time of diagnosis and treatment. Mayo Clinic’s research data platform does not store historical residential data. However, the primary geographic variable used for analysis was site of diagnosis and treatment; ZIP codes were used only to illustrate the large regional representation of patients seeking treatment.

Figure. Geographic distribution of patients for study of invasive Nocardia infections across 3 distinct geographic regions, United States. Color codes and list of Nocardia spp. represent the geographic location of diagnosis and treatment. Each circle represents a patient in the study cohort’s postal (ZIP) code of residence at the time of data extraction (2022); larger circles represent ZIP codes with more patients. Percentages for states are for the full study cohort; percentages for individual species are for that state.
treatment at Mayo Clinic sites as destination healthcare centers. It is worth noting that 88.2% of patients still resided within the region of diagnosis and treatment at time of data extraction, which further validated our conclusions about geographic distribution and variability of *Nocardia* spp. Fourth, univariate analysis of 1-year survival did not account for possible confounders in the relationship between transplant status and all-cause death. Fifth, we did not record the number of *Nocardia* isolates excluded because of lack of clinical or radiographic evidence representing potential *Nocardia* colonization. Sixth, we collected limited data on coexisting conditions and socioeconomic factors and did not record specifics of immune suppression besides organ transplantation. Finally, we recognize inherent biases associated with retrospective studies, such as selection bias and center effect.

In summary, our study provides information on differences in geographic distribution, patient characteristics, disease manifestations, and antimicrobial susceptibility patterns related to *Nocardia* spp. in non-contiguous regions of the United States with varied climatic conditions. Similar investigations of patients with invasive infections caused by pathogens of environmental origin encompassing broader geographic regions from around the world would help continue to expand knowledge about how to manage and treat such infections effectively.


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**About the Author**

Dr. Gupta obtained her MD from the University of Connecticut School and Medicine and completed internal medicine residency at Mayo Clinic in Arizona. She is a fellow in the infectious disease program at Massachusetts General Hospital/Brigham & Women’s Hospital. Her research interests include infections among critically ill populations, including transplant and intensive care patients. Dr. Vikram is a professor of medicine at the Mayo Clinic Alix School of Medicine and medical director of Transplant Infectious Diseases for the Mayo Clinic Arizona Transplant Center. His research interests pertain to immunocompromised host infections, coccidioidomycosis, and cardiovascular infections.

**References**


Nocardia Infections, United States


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etymologia revisited

Schizophyllum commune

[skiz-of′-l-am kom′-yoon]

Schizophyllum commune, or split-gill mushroom, is an environmental, wood-rotting basidiomycetous fungus. Schizophyllum is derived from “Schíza” meaning split because of the appearance of radial, centrally split, gill like folds; “commune” means common or shared ownership or ubiquitous. Swedish mycologist, Elias Magnus Fries (1794–1878), the Linnaeus of Mycology, assigned the scientific name in 1815. German mycologist Hans Kniep in 1930 discovered its sexual reproduction by consorting and recombining genomes with any one of numerous compatible mates (currently >2,800).

References


Tecovirimat Resistance in Mpox Patients, United States, 2022–2023

Todd G. Smith, Crystal M. Gigante, Nhien T. Wynn, Audrey Matheny, Whitni Davidson, Yong Yang, Rene Edgar Condori, Kyle O’Connell, Lynsey Kovar, Tracie L. Williams, Yon C. Yu, Brett W. Petersen, Nicolle Baird, David Lowe, Yu Li, Panayampalli S. Satheshkumar, Christina L. Hutson

In May 2022, an outbreak of mpox disease, caused by infection with monkeypox virus (MPXV) clade IIb (formerly West Africa clade), was identified in the United States (1). Since that time, >30,000 cases and 46 deaths associated with the outbreak have been identified in the United States. As a result of effective education, vaccination, and case identification, US cases peaked the first week of August 2022 at 459 cases per week. The United States has identified more cases than any other country in the global outbreak (2).

The FDA licensed the therapeutic agent TPOXX (SIGA Technologies, Inc., https://www.siga.com) containing the drug tecovirimat (i.e., ST-246) under the animal rule for smallpox treatment in 2018 (3). Tecovirimat has been tested extensively in cell culture (4–6) and within many orthopoxvirus (OPXV) animal models (7–15), including the nonhuman primate variola virus (VARV) model (16,17). After MPXV clade IIb emerged in 2022, Warner et al. demonstrated tecovirimat efficacy against the outbreak strain (lineage B.1) in a nonlethal mouse model (18). Although tecovirimat has shown efficacy against multiple OPXVs, researchers have noted that nucleotide alterations to the orthopoxviral F13L gene homologue leading to amino acid substitutions in the F13 protein (also known as VP37) allow for resistance (4,19). In addition, resistance emerged during use of tecovirimat in an extended treatment course of a patient with progressive vaccinia (20).

Because TPOXX is only licensed for treatment of smallpox, the Centers for Disease Control and Prevention (CDC) holds an expanded access investigational new drug protocol for treatment of nonvariola OPXV infections, including mpox. Since May 2022, at least 7,563 patients have received tecovirimat for mpox treatment in the United States; a fraction of those have been severe cases where patients have moderate to severe immunocompromise usually caused by uncontrolled HIV infection (21). To test for resistance among patients who received tecovirimat, we collected specimens from 435 patients who received tecovirimat for whom resistance was possible or suspected based on clinical data (Table 1). We genotyped
and phenotyped specimens from 68 patients and confirmed a resistant phenotype in 46 of those patients. The geographic distribution of tecovirimat resistance has conformed to the geographic distribution of the larger mpox outbreak (Figure 1). Here, we describe our investigation and findings. The activities in this report were reviewed by the Human Subjects Advisor in the National Center for Emerging and Zoonotic Diseases at the Centers for Disease Control and Prevention and determined that it does not meet the regulatory definition of research under provision 45 CFR 46.102(l); the activities fall under public health surveillance and do not require IRB review.

Methods

During the mpox outbreak, whole-genome metagenomic sequencing and, more recently, amplicon-based sequencing targeting the F13L gene have been used to screen for changes in the MPXV F13L homologue (Appendix 1, https://wwwnc.cdc.gov/EID/article/29/12/23-1146-App1.pdf). A total of 3,247 CDC-generated sequences have been screened by either passive genomic surveillance (n = 3,101) or targeted F13L sequencing (n = 146). Only genomic sequencing completed at CDC was included because the raw data were required to find minor variants. The primary outbreak strain (MPXV clade IIb lineage B.1) has a substitution, E353K, in the F13 protein that is not present in the secondary outbreak strain (lineage A.2), historical clade IIb sequences from Nigeria, or MPXV clade IIa (22). Because the E353K substitution was not previously described in other OPXV, the effect on tecovirimat phenotype was unknown.

We adapted a cytopathic effect (CPE) assay, used at CDC to test VARV sensitivity to tecovirimat, to use for MPXV as described previously (22). In brief, we used clinical specimens that were decoded but not anonymous to culture MPXV on Vero (African green monkey) cell lines (either BSC-40 or E6). We then used the isolated MPXV to infect confluent Vero E6 cell monolayers pretreated for 1 h with different concentrations of tecovirimat. We incubated infected cells for 72 h at 35°C with 6% CO₂. We fixed and stained wells with formalinized crystal violet and measured absorbance at 570 nm; intact cell monolayers having a high absorbance indicated that the drug was protective. We used the CPE assay to show that MPXV isolates with the E353K mutation remained sensitive to tecovirimat (18,22–24).

Results

In total, 130 samples from 76 patients produced sequences with amino acid changes other than E353K in the F13 protein relative to MPXV clade IIb variant B.1 reference strain (GenBank accession no. ON563414), collected in the United States in 2002 (Table 2). Isolates with amino acid substitutions D100N, D217N, D248N, D256N, and S369L identified by routine sequencing of samples from patients not treated with tecovirimat have remained sensitive to tecovirimat (18,22–24).

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<tr>
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*CDC, Centers for Disease Control and Prevention; MPXV, monkeypox virus.

Figure 1. Geographic distribution of patients with mpox who had samples received for tecovirimat resistance testing (A) and who had samples confirmed resistant (B) June 2022–July 2023, United States.
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†Fold change was calculated based on the EC_{50} of the reference strain MPXV clade Ila (U.S., 2003), which was 0.0175 µmol/L.

*All specimens belong to MPXV clade Iib lineage B.1 and contain E353K substitution in addition to the listed substitutions. All substitutions detected from a specimen are listed regardless of their proportion in the viral population. Insertions (ins) and deletions (del) were detected in addition to substitutions. EC_{50}, 50% effective concentration; MPXV, monkeypox virus.

(H238Q, Y258C, N267D, N267del, D283G, A288P, A290V, D294V, A295E, L297ins, I372N) that were previously identified in other OPXV (19,20,25–28) as resistant by phenotypic testing (Table 2). One confirmed resistance mutation, T289A, had not been described before the 2022 mpox outbreak (26). T289A resulted in up to an 8-fold increase in the 50% effective concentration when compared with the MPXV clade Ila reference strain. This position is part of the predicted tecovirimat binding site and adjacent to A288P and A290V, which both confer resistance (19). We identified 7 other amino acid substitutions (K174N, S215F, P243S, T245I, Y285H, R291K, D301del) but have not yet determined the effects of those mutations (25,26). Those mutations have been observed and tested only in combination with other resistance mutations.

Eight of 27 nonsynonymous mutations observed in F13L were GA to AA or TC to TT, which may suggest they arose through APOBEC3 editing. All the APOBEC3 motif mutations produced amino acid changes that did not affect tecovirimat resistance in culture. Resistance phenotype is currently unknown for R291K, S215F, and P243S.
For phenotype testing, we considered an isolate resistant if the increase in 50% effective concentration was ≥2-fold compared to the 2003 MPXV clade IIa reference strain. Isolates with 2-fold to 9-fold change were considered partially resistant, and isolates with ≥10-fold change were considered resistant (25). A total of 83 isolates from 41 patients were resistant, and 16 isolates from 11 patients were partially resistant. Four patients with partially resistant isolates also had ≥1 other isolate that was resistant. The clinical relevance of partially resistant and resistant isolates remains unknown.

**Discussion**

Multiple lines of evidence point to tecovirimat resistance developing during drug treatment in most patients. First, genome sequencing has revealed unique mutational profiles from different sample sites from the same patient (Figure 2, panel A), indicating different viral subpopulations were selected at different sites during treatment. Second, longitudinal sampling was investigated for 4 of the 46 patients with a resistant isolate and showed samples before tecovirimat treatment were sensitive, whereas later samples were resistant (Figure 2, panel B). An exception was found for 1 patient; T289A was detected in 58% of reads, along with minor populations of A295E (9%) and N267del (22%), from a sample the day before the patient started tecovirimat treatment. A second sample from the same patient after tecovirimat treatment showed the T289A mutation was selected (93%), and a new variant R291K was also detected (31%). In addition, N267del was detected in a cluster of cases in California with no known tecovirimat treatment (27). Whether those drug-resistant infections were acquired from another person treated with tecovirimat is unknown but is a viable hypothesis. Such rare cases show that viruses with mutations in F13L resulting in tecovirimat resistance can be transmitted from person to person.

For patients that had ≥1 specimen with confirmed tecovirimat resistance, 39/46 had HIV infection; HIV status was unknown for the remaining 7 patients. Of the 39 patients with HIV infection, 31 had a CD4+ T-cell count available; all 31 were <350 cells/μL, and 28 were <200 cells/μL. Ten of the 39 patients died (25,26), and all 10 deceased patients had CD4+ T-cell counts <200 cell/μL. In the United States, 46 deaths associated with mpox have been reported (2). In those patients, severe immunocompromise is resulting in severe disease and death as well as tecovirimat resistance.

For patients who had ≥1 specimen with confirmed tecovirimat resistance, at least 34 of 46 patients were hospitalized. No medical history was available for 6 patients, and the medical history concerning hospitalization was not clear or did not mention hospitalization for another 6 patients. Of the 46 patients, 39 patients received tecovirimat either oral or IV; 5 patients did not receive tecovirimat (27), and 2 patients’ tecovirimat status was unknown. Exact data on length of tecovirimat exposure is difficult to obtain because of possible noncompliance with oral administration and multiple rounds of treatment in which drug administration stops and starts. We estimated the average length of tecovirimat treatment using the reported start date of tecovirimat treatments. Dates were available for 28 of the 39 patients that received

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Examples of tecovirimat resistance in mpox patients, United States, 2022–2023. Patient samples were sequenced, cultured, and subjected to tecovirimat sensitivity testing in a cytopathic effect assay. A) Different samples from the same patient showed different F13 amino acid substitutions that result in different levels of resistance compared with the wild-type control (MPXV clade IIa, collected in the United States in 2003 [GenBank accession no. ON563414]). B) Samples from the same patient at different times before and after starting tecovirimat treatment in August 2022, showing sensitivity before drug treatment and increasing resistance after drug treatment. Abs<sub>570</sub> absorbance at 570 nm.
A tecovirimat-resistant phenotype was previously published for 6 patients from Los Angeles County, California, USA (25,26). The previous case reports were limited in geographic scope, whereas our study is an overview for the entire United States. The 6 patients previously reported are included in this report for completeness. The larger dataset reported herein is complementary to the previously published data and supports the conclusions of the previous reports. In addition, 1 other case report found MPXV with a tecovirimat-resistant phenotype that was linked to selection of the N267D mutation during tecovirimat treatment (29). Of note, despite detection of drug resistance from 1 anatomic site, the patient improved clinically (29). Other case reports have suspected tecovirimat resistance on the basis of deteriorating clinical status after tecovirimat treatment (30,31). Treatment with cidofovir was successful in those cases and should be considered when tecovirimat resistance is suspected.

The first limitation of our study is that the phenotype assay is culture-based, which is labor intensive and of low throughput. As of July 2023, we had phenotyped 124 specimens from 68 patients. However, the lag in testing means all the specimens that have been tested are from September 2022–April 2023, so results only give a retrospective sample of possible drug resistance. Because submission of samples for tecovirimat sensitivity is voluntary and cannot be used to inform clinical care, sampling bias may exist for certain physicians, hospitals, or states and may make it appear that certain states have more drug resistance than others (Figure 1). As genomic sequencing has increased, we have prioritized samples with predicted resistance mutations for phenotype testing. Mixed populations of cultured virus were tested to meet the need for efficiency for a public health emergency. In the future, we will begin plaque purification for selected samples to test clonal populations.

Our results confirm that tecovirimat resistance mutations are being selected in human mpox patients by tecovirimat treatment. Resistance has been confirmed in a small percentage of cases, currently <1% of the total number of patients that have received tecovirimat. Characteristics of patients with resistant isolates are very similar: uncontrolled HIV infection with very low CD4+ T-cell counts and potential for extensive tecovirimat exposure while hospitalized. The frequency of tecovirimat resistance may be higher in persons with uncontrolled HIV infection. In rare cases, a drug-resistant virus appeared to have been transmitted to another person. Genomic and phenotype testing are ongoing. Our results may be useful when considering treatment for patients that match the clinical profile we described; aggressive early dosing and combination therapy regimens could be considered in those instances (27). Results will also provide critical knowledge to potentially build a genomic assay for early detection of resistance mutations which could be used to inform clinical care decisions. For clinicians concerned about tecovirimat resistance, we encourage enrolling patients in the CDC VIRISMAP study (https://www.cdc.gov/poxvirus/mpox/clinicians/virismap.html) and the STOMP (Study of Tecovirimat for Mpox) trial (https://stomptpoxx.org).

In conclusion, we describe a large number of tecovirimat-resistant MPXV isolates from humans and provide crucial data on the amino acid changes leading to resistance in MPXV paired with clinical outcomes; these combined data may inform decisions on tecovirimat use in the future. Our findings also highlight the need for additional, well-tolerated OPXV therapeutics with different modes of action, particularly for use with immunocompromised patients.

This article was preprinted at https://www.medrxiv.org/content/10.1101/2023.05.16.23289856v1.

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Health and Mental Hygiene; Ohio Department of Health Laboratory; Pennsylvania Department of Health, Bureau of Laboratories; Rhode Island State Health Laboratories; Tennessee Division of Laboratory Services; Texas Department of State Health Services, Lab Services Section; Texas-Houston Health Department Laboratory; and Virginia Division of Consolidated Laboratory Services.

About the Author

Dr. Smith is a microbiologist in the Poxvirus and Rabies Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Infectious Diseases, Centers for Disease Control and Prevention, in Atlanta, Georgia. His primary research interest is in smallpox preparedness.

References


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**etymologia revisited**

**Picobirnavirus** [pi-ko-bur′na-vir′əs]

Picobirnavirus, the recently recognized sole genus in the family *Picobirnaviridae*, is a small (Pico, Spanish for small), bisegmented (bi, Latin for two), double-stranded RNA virus. Picobirnaviruses were initially considered to be birna-like viruses, and the name was derived from birnavirus (bisegmented RNA), but the virions are much smaller (diameter 35 nm vs. 65 nm).

Picobirnaviruses are reported in gastroenteric and respiratory infections. These infections were first described in humans and black-footed pigmy rice rats in 1988. Theafter, these infections have been reported in feces and intestinal contents from a wide variety of mammals with or without diarrhea, and in birds and reptiles worldwide.

**Sources**


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https://wwwnc.cdc.gov/eid/article/26/1/et-2601_article
Risk Factors for Enteric Pathogen Exposure among Children in Black Belt Region of Alabama, USA

Drew Capone, Toheedat Bakare, Troy Barker, Amy Hutson Chatham, Ryan Clark, Lauren Copperthwaite, Abeoseh Flemister, Riley Geason, Emery Hoos, Elizabeth Kim, Alka Manoj, Sam Pomper, Christina Samodal, Simrill Smith, Claudette Poole, Joe Brown

In support of improving patient care, this activity has been planned and implemented by Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is jointly accredited with commendation by the Accreditation Council for Continuing Medical Education (ACCME), the Accreditation Council for Pharmacy Education (ACPE), and the American Nurses Credentialing Center (ANCC), to provide continuing education for the healthcare team.

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Learning Objectives

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

• Assess characteristics of the current study examining the prevalence of enteric pathogens among children and adolescents
• Distinguish the prevalence of enteric pathogens among children and adolescents in the current study
• Compare rates of positive tests for different enteric pathogens in the current study
• Evaluate variables associated with a higher rate of positive testing for at least 1 pathogen in the current study

CME Editor

Jude Rutledge, BA, Technical Writer/Editor, Emerging Infectious Diseases. Disclosure: Jude Rutledge, BA, has no relevant financial relationships.

CME Author

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We collected stool from 488 children from 352 households living in the Black Belt region of Alabama, USA, where sanitation infrastructure is lacking. We used quantitative reverse transcription PCR to measure key pathogens in stool that may be associated with water and sanitation, as an indicator of exposure. We detected genes associated with ≥1 targets in 26% of specimens, most frequently *Clostridioides difficile* (6.6%), atypical enteropathogenic *Escherichia coli* (6.1%), and enteroaggregative *E. coli* (3.9%). We used generalized estimating equations to assess reported risk factors for detecting ≥1 pathogen in stool. However, we did observe an increased risk for pathogen detection among children living in homes with well water (adjusted risk ratio 1.7 [95% CI 1.1–2.5]) over those reporting water utility service.

Outside cities and towns served by conventional sewerage, many residents in the rural Black Belt region of Alabama, USA, have failing or inadequate sanitation infrastructure (1,2). This region was named after its rich black soils, which are typically high in clay content, limiting subsurface infiltration (3) and leading to surface discharge of domestic wastewater. Compounding those challenges is a high rate of poverty; 9 of the 10 poorest counties in Alabama are in the Black Belt region (2,4). Because common alternatives to septic systems are unaffordable (5,6), many residents use failing systems or lack systems altogether (7,8). Straight piping (i.e., direct discharge of untreated fecal wastes to the environment) of domestic wastewater is common (7).

When human fecal wastes are not safely managed, they may be transported to the environment through well-understood fecal–oral pathways (i.e., drinking water, soils, flies, food, fomites, and hands) (9,10). For households reliant on straight pipe discharge of wastewater, direct exposure to this waste may be more likely than for households served by a septic system (8). Those same households and their communities may also suffer from exposures further downstream. Inadequate treatment of fecal wastes can result in enteric pathogen transport through soil into groundwater and exposure through drinking water (e.g., well water) (11,12). Other exposures may include fecally contaminated soils (13), flies that feed on and reproduce in human feces (14,15), and contaminated food (10). Such exposures can result in infection with enteric pathogens, which is a necessary pre-condition for diarrheal disease and other sequelae, including environmental enteric dysfunction (16), growth deficits (17), cognitive impairment (18), and negative effects on the immune system (19).

Poor sanitation and persistent exposure to fecal wastes, particularly in the context of a state and nation with ample resources to address the issue (20), represents a public policy failure (7,21) affecting human health, dignity, and quality of life. Although the evidence base for public investment in sanitation on health grounds has a long history (22), the health burden attributable to poor rural sanitation in the United States remains poorly characterized, constraining the case for action. To determine the potential roles of rural sanitation improvements or other interventions in controlling disease transmission, a useful first step is estimating prevalence of enteric infections and identifying risk factors associated with them. Because of documented poor sanitation conditions in Alabama’s Black Belt region (5,7,8) and the associated potential persistence of endemic enteric infection (23–25), we conducted a cross-sectional study to assess the prevalence of stool-based enteric pathogen detection in children using molecular methods, as an indicator of previous exposure. We further sought to identify potential household-level environmental risk factors for exposure to those pathogens to understand the potential role of infrastructure in protecting public health in this underserved region.

### Methods

#### Study Site and Participants

This study was nested within a larger cross-sectional helminth surveillance study in rural Alabama (26). Participants were children 2–18 years of age living in 3 counties in the Black Belt (Lowndes, Wilcox, and Perry Counties). All children included in a household were requested to participate. The study used principles of community-based participatory research to work with stakeholders in co-creation of the project (27). Several meetings and focus groups were held with community partners and study collaborators to help guide study protocols, recruitment methods and materials, and participant enrollment. Participants were enrolled during January 2019–December 2021 (26).

We provided participants with an at-home stool collection kit. For 3 separate bowel movements, participants filled and returned 1 50-mL collection tube containing 15 mL of zinc polyvinyl alcohol (Zn-PVA) (28) with ≈15 g of stool and another tube containing 15 mL of 10% formalin with an additional 15 g of stool (Parapak; Meridian Bioscience). Participants received $150 on a prepaid debit card for their participation.
addition, participants completed a brief paper questionnaire regarding their demographic characteristics, household sanitation infrastructure, and potential exposures. Before March 2020, the questionnaire was completed at the time of enrollment; after March 2020, the questionnaire was completed by the participant at home and mailed to the study team.

**Molecular Analysis**

During January 2019–November 2020, specimens were shipped at ambient conditions to Georgia Institute of Technology (Atlanta, GA, USA); during December 2020–December 2021, specimens were shipped at ambient conditions to the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA). Upon receipt, we visually screened specimens for indicators of nonhuman origin (i.e., animal hair, dirt, color, and atypical morphology), homogenized them with sterile inoculating loops (VWR; Radnor) and stored them at 4°C for further analysis. By using the QIAamp 96 Virus QIAcube HT Kit (QIAGEN), which included a pretreatment step with Precellys SK38 bead beating tubes (Bertin Technologies) (29–31), we extracted total nucleic acids from 150 mg of the stool Zn-PVA mixture. We typically extracted specimens within 1–4 weeks of receipt (median 15 days, interquartile range 8–28 days). We analyzed extracts from specimens suspected to potentially be from nonhuman sources by using dPCR (QIAcuity 4; QIAGEN) for human mitochondrial DNA, using a previously validated method that has high sensitivity and specificity for human feces (32). Among children who submitted >1 stool specimen, we randomly selected a single replicate for extraction. We randomly selected 5% of stools for duplicate extraction and another 3% for extraction from multiple replicates. We included ≥1 extraction-negative control (33) during each day of extractions. We spiked specimens with 10^7 copies of bacteriophage MS2 and 10^6 gene copies of synthetic DNA (IDT) as extraction-positive controls. We stored extracts at −80°C until analysis.

We measured 30 enteric pathogens in specimens by using a custom TaqMan Array Card (TAC) on a Quantstudio 7 Flex (ThermoFisher) at the University of North Carolina at Chapel Hill, according to the methods described by Liu et al. (34). Targets were *Acanthamoeba* spp., adenovirus 40/41, astrovirus, *Balantidium coli*, *Blastocystis* spp., *Cystoisospora belli*, *Cyclospora cayetanensis*, *Campylobacter jejuni* or *C. coli*, *Clostridioides difficile*, *Cryptosporidium* spp., *Enteroxynoza* spp., *Enteroaggregative E. coli*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, Giardia spp., *Helicobacter pylori*, hepatitis A virus, *Shigella* spp. or enteroinvasive *E. coli*, norovirus, *Plesiomonas shigelloides*, rotavirus, *Salmonella* spp., sapovirus, SARS-CoV-2, Shiga toxin–producing *E. coli*, and *Yersinia enterocolitica*. We prepared the TAC by combining 40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems). We evaluated TAC performance by using an 8-fold dilution series (10^9–10^2 gene copies/reaction) of an engineered combined positive control developed by using methods from Kodani and Winchell 2012 (35). We used 2 plasmids (GeneArt), including 1 specifically for DNA targets. We linearized the other with a BshT1 restriction enzyme (ThermoFisher) and transcribed it (MEGAscript T7 Transcription Kit and MEGAclear Transcription Clean-Up Kit, both from ThermoFisher) to generate RNA control material, which we quantified by using a Qubit RNA HS Assay Kit on Qubit 4 Fluorometer (ThermoFisher). The linearity and efficiency for 28 of the 30 targets were within normative standards (linearity 0.97–1.0, efficiency 87%–102%) (Appendix Tables 1–3, Figure 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0780-App1.pdf). The assays for hepatitis A virus and adenovirus 40/41 did not perform well, and we excluded them from our analysis.

Each day of TAC analysis, we included ≥1 positive and negative control (either an extraction-negative control or a PCR-negative control). We determined quantification cycle values through manual thresholding and included comparison of each specimen’s fluorescent signal against the daily negative and positive controls (Appendix Figure 1). We categorized any target that amplified past a quantification cycle of 35 as negative to reduce the potential for false positives (34). To examine the effect of our preservation medium on the probability of detecting our targets of interest, we measured recovery of *Giardia duodenalis* and *Shigella sonnei* from stool by using different preservative conditions over a period of 8 weeks (Appendix).

**Data Analysis**

To perform Poisson regression, we used generalized estimating equations with robust SEs that accounted for clustering among children living in the same household. This method estimated unadjusted and adjusted risk ratios with 95% CIs. We created a directed acyclic graph on the basis of the variables included in the questionnaire where independent variables were biologically plausible predictors of the dependent variable, which was the detection of nucleic acids from ≥1 enteric pathogen in stool (Appendix Figure 2). Independent variables that met this criterion were
the household’s sanitation infrastructure, whether the household paid a water bill (i.e., a proxy measure indicating a connection to a water utility), reported

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<tr>
<td>No</td>
<td>67 (14)</td>
</tr>
<tr>
<td>Don’t know</td>
<td>6 (1.2)</td>
</tr>
<tr>
<td>No response</td>
<td>430 (6.1)</td>
</tr>
<tr>
<td>Household sanitation</td>
<td></td>
</tr>
<tr>
<td>Septic tank</td>
<td>207 (42)</td>
</tr>
<tr>
<td>Sewer connection</td>
<td>111 (23)</td>
</tr>
<tr>
<td>Don’t know</td>
<td>80 (16)</td>
</tr>
<tr>
<td>Straight pipe</td>
<td>56 (11)</td>
</tr>
<tr>
<td>Cesspit</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (0.1)</td>
</tr>
<tr>
<td>No response</td>
<td>31 (6.3)</td>
</tr>
<tr>
<td>Raw sewage in yard or home in past year</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>400 (82)</td>
</tr>
<tr>
<td>Yes</td>
<td>38 (7.8)</td>
</tr>
<tr>
<td>No response</td>
<td>50 (10)</td>
</tr>
<tr>
<td>History of international travel in past year</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>448 (92)</td>
</tr>
<tr>
<td>Yes</td>
<td>13 (2.7)</td>
</tr>
<tr>
<td>No response</td>
<td>27 (5.5)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>236 (48)</td>
</tr>
<tr>
<td>F</td>
<td>229 (47)</td>
</tr>
<tr>
<td>No response</td>
<td>23 (4.7)</td>
</tr>
<tr>
<td>Daily screen time, h</td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>72 (15)</td>
</tr>
<tr>
<td>2–4</td>
<td>182 (37)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>203 (42)</td>
</tr>
<tr>
<td>No response</td>
<td>31 (6.4)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>11 (4.1)</td>
</tr>
<tr>
<td>Median (interquartile range)</td>
<td>11 (8–14)</td>
</tr>
<tr>
<td>Range</td>
<td>2–18</td>
</tr>
<tr>
<td>No response</td>
<td>37 (17.6)</td>
</tr>
<tr>
<td>Ever treated for an intestinal parasite</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>418 (86)</td>
</tr>
<tr>
<td>Don’t know</td>
<td>45 (9.2)</td>
</tr>
<tr>
<td>Yes</td>
<td>12 (2.5)</td>
</tr>
<tr>
<td>No response</td>
<td>13 (2.7)</td>
</tr>
</tbody>
</table>

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

Table 1. Demographic characteristics of 488 children and water infrastructure summary based on self-administered surveys conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021*
atypical enteropathogenic E. coli (6.1% [30/488]), and enteroaggregative E. coli (3.9% [19/488]). We detected each viral, protozoan, fungal, and algae targets in <1.0% of specimens except for Calicivirus (3.7% [18/488]) and norovirus genotype group I or II (1.4% [7/488]). We observed perfect agreement in target detection among 26 specimens analyzed in duplicate (same child, same bowel movement) and 80% (12/15) agreement by 0.0037 log10/day in Zn-PVA at 4°C. The concentration of Shigella DNA we recovered from Zn-PVA decreased at ambient conditions by 0.030 log10/day and by 0.0085 log10/day in Zn-PVA at 4°C (Appendix Table 5, Figure 3).

**Discussion**

We detected various enteric pathogens in stool specimens from children living in the Black Belt of Alabama. Straight pipe sanitation (direct discharge of fecal wastes into the environment near households) was not associated with increased risk for stool pathogen detection compared with conventional sewerage. However, our

### Table 2. Prevalence of enteric pathogens in stool specimens of children in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021*

<table>
<thead>
<tr>
<th>Type and pathogen</th>
<th>Prevalence, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any ≥1 pathogen gene detected</td>
<td>127 (26)</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Clostridioides difficile</td>
<td>32 (6.6)</td>
</tr>
<tr>
<td>EPEC (atypical)</td>
<td>30 (6.1)</td>
</tr>
<tr>
<td>EAEC</td>
<td>19 (3.9)</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>11 (2.3)</td>
</tr>
<tr>
<td>EPEC (typical)</td>
<td>7 (1.4)</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>5 (1.0)</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>4 (0.8)</td>
</tr>
<tr>
<td>Plesiomonas shigelloides</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>ETEC</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Shigella or EIEC</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>Salmonella</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>STEC</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>Campylobacter jejuni or coli</td>
<td>0</td>
</tr>
<tr>
<td><strong>Fungus/algae</strong></td>
<td></td>
</tr>
<tr>
<td>Blastocystis</td>
<td>18 (3.7)</td>
</tr>
<tr>
<td>Enteroctyozoon bieneusi</td>
<td>0</td>
</tr>
<tr>
<td>Encephalitozoon intestinalis</td>
<td>0</td>
</tr>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
</tr>
<tr>
<td>Balantidium coli</td>
<td>3 (0.6)</td>
</tr>
<tr>
<td>Acanthamoeba</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Giardia spp.</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Entamoeba hystolytica</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>Cryptosporidium belli</td>
<td>0</td>
</tr>
<tr>
<td>Cyclospora cayetanensis</td>
<td>0</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>0</td>
</tr>
<tr>
<td>Entamoeba</td>
<td>0</td>
</tr>
<tr>
<td><strong>Virus</strong></td>
<td></td>
</tr>
<tr>
<td>Norovirus GI or GII</td>
<td>7 (1.4)</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>3 (0.6)</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Sapovirus</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>1 (0.2)</td>
</tr>
</tbody>
</table>

*EAEC, enteriaggregative Escherichia coli; EIEC, enteroinvasive E. coli; EPEC, enteropathogenic E. coli; ETEC, enterotoxigenic E. coli; O157:H7, genotype group I and II; STEC, Shiga toxin–producing E. coli.
finding that well water consumption was associated with an increased risk for enteric pathogen detection implicates poor sanitation in this geographic area as a possible contributor to groundwater contamination. Soils that are high in clay content undergo shrinking as they desiccate and swelling as they moisten (3). Those conditions may lead to fecal waste transport from failing septic tanks and straight pipe discharges through soils to the water table (3,38), resulting in exposures through drinking water. Previous work in the Black Belt observed an increased concentration of fecal contamination in well water compared with piped municipal water. In a cross-sectional study of randomly selected households in Hale County (bordering Perry County in the Black Belt), 20% of private wells were positive for fecal coliforms, compared with 8% of public water system specimens (12). Other studies from the region have reported fecal contamination of water supplies, possibly linked to widespread sanitation deficits (11,39,40).

We used detection of pathogens in stool as a proxy for carriage and as an unambiguous indicator of previous exposure (41), a suitable measure given the role of water and sanitation infrastructure in limiting exposures to many of the pathogens we assessed. It is important to note that detecting a pathogen in stool does not necessarily indicate the person experienced symptomatic or asymptomatic infection. For example, detecting C. difficile by PCR does not guarantee the presence of C. difficile toxin, and infection without the presence of this toxin may not result in diarrheal disease (42). Further, the relationship between carriage, infection, and disease is highly host- and pathogen-specific (43). Evidence from an international multisite study on the etiology of diarrhea in children posited that the detection of enterogregarative E. coli at low concentrations in stool appeared to be protective against diarrhea, whereas detection of pathogens such as Helicobacter pylori, Shigella, and norovirus were strongly associated with diarrhea (43). Important microbiome-mediated interactions between and among pathogens are possible, and host responses can vary.

Compared with data for children in low- and middle-income countries, the 26% combined prevalence of enteric pathogens we observed is dramatically lower than what has been previously reported (29,43). Few studies have screened populations for multiple enteric pathogens in high-income countries outside of clinical settings or from asymptomatic populations. A study of 438 children in daycare centers in Uppsala, Sweden, from 2016 tested for 21 different enteric pathogens using PCR and detected ≥1 pathogen in stool specimens from 3.7% of children (44). The pathogens they detected most frequently were C. difficile (2.5%), adenovirus 40/41 (1.6%), Campylobacter (0.7%), and norovirus (0.7%) (Appendix Table 6). A 2001 study of 1,091 asymptomatic children and adults in Australia assessed 28 pathogens and detected ≥1 pathogen in 2.6% of stool specimens, including Giardia (1.6%), Salmonella (0.4%), Cryptosporidium (0.4%), and adenovirus (0.1%) (45). Prevalence of ≥1 pathogen was higher for children <10 years of age (4.6%) compared with children 10–20 years of age (0.6%) and adults >20 years of age (1.2%). Blastocystis hominis, which the authors did not consider pathogenic and was not included in the reported 2.6% prevalence, was detected in 6.0% of stool specimens.

Our results indicate substantially higher prevalence of gut pathogens compared with those studies. However, we detected some individual pathogens less frequently than in other similar studies in the United States. Among infants in Denver, Colorado, USA, in 1990, an estimated 16% of those attending daycare and 9% of those not enrolled had Giardia duodenalis detected in stool specimens (46). In 1991, the prevalence of Cryptosporidium was 3% and G. duodenalis 7% among children attending daycare centers in Fulton County, Georgia, USA (47). Those values are higher than the 0.4% (2/488)

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**Table 3. Risk factors for detection of ≥1 enteric pathogen in stool specimens of children in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, January 2019–December 2021**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Reference</th>
<th>Exposure</th>
<th>RR (95% CI)</th>
<th>aRR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pay a water bill</td>
<td>Yes</td>
<td>No</td>
<td>1.8 (1.2–2.5)</td>
<td>1.7 (1.1–2.5)</td>
</tr>
<tr>
<td>Sanitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewer connection</td>
<td></td>
<td>Cesspit</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>3.4 (0.57–20)</td>
<td>5.2 (0.88–30)</td>
<td></td>
</tr>
<tr>
<td>Septic tank</td>
<td></td>
<td>0.89 (0.61–1.3)</td>
<td>0.95 (0.64–1.4)</td>
<td></td>
</tr>
<tr>
<td>Straight pipe</td>
<td></td>
<td>0.95 (0.55–1.6)</td>
<td>0.95 (0.55–1.7)</td>
<td></td>
</tr>
<tr>
<td>Child’s screen time</td>
<td>&lt;2 h</td>
<td>2–4 h</td>
<td>0.74 (0.48–1.1)</td>
<td>0.79 (0.51–1.2)</td>
</tr>
<tr>
<td></td>
<td>&gt;4 h</td>
<td>0.74 (0.48–1.1)</td>
<td>0.73 (0.47–1.1)</td>
<td></td>
</tr>
<tr>
<td>Child’s sex</td>
<td>Male</td>
<td>Female</td>
<td>0.89 (0.65–1.2)</td>
<td>0.89 (0.65–1.2)</td>
</tr>
<tr>
<td>International travel in past year</td>
<td>No</td>
<td>Yes</td>
<td>0.89 (0.32–2.5)</td>
<td>0.93 (0.34–2.5)</td>
</tr>
<tr>
<td>Raw sewage in home or yard in past year</td>
<td>No</td>
<td>Yes</td>
<td>1.1 (0.68–1.9)</td>
<td>1.1 (0.66–2.0)</td>
</tr>
<tr>
<td>Child’s age</td>
<td>&lt;5 y</td>
<td>5–10 y</td>
<td>0.71 (0.40–1.3)</td>
<td>0.76 (0.41–1.4)</td>
</tr>
<tr>
<td></td>
<td>&gt;10 y</td>
<td>0.82 (0.47–1.4)</td>
<td>0.90 (0.49–1.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Unadjusted RRs are from bivariate models, whereas aRRs are from full model including all covariates. aRR, adjusted risk ratio; RR, risk ratio.*
prevalence we observed for *Giardia* and the 0% prevalence for *Cryptosporidium*, although the Colorado and Georgia studies took place more than 30 years ago in different settings and populations. More recently, Tisdale et al. (48) used the TAC platform to screen adults traveling internationally from the United States and Germany to low- and middle-income countries for 22 pathogens. Similar to our results, they detected ≥1 pathogen in stool specimens from 21% of asymptomatic controls.

One limitation of this study is that logistical constraints did not enable analysis of fresh specimens. Transport and storage conditions (time, temperature, and transport media) can influence recovery of pathogen-associated nucleic acids, potentially lowering the sensitivity of molecular assays we used and possibly leading to false-negative results if DNA or RNA fell below our detection limits. Although we attempted to reduce time-to-analysis and to optimize storage conditions to preserve the stability of DNA and RNA, some loss of signal is unavoidable. We assessed Zn-PVA’s performance in preserving nucleic acids in spiked controls (Appendix). In addition, we had missing data in our surveys because of logistical difficulties imposed by the COVID-19 pandemic, such as the need for participants to complete surveys at home and mail them separately from specimens. In addition, some missing data may have been the result of hesitancy to share sanitary conditions because straight pipe discharge of domestic wastewater (8) is illegal in the study area (7). To mitigate the effect of this missing data, we used MICE and obtained similar results by using this imputation approach compared with analysis on the complete dataset. Further, we were unable to conduct household visits to confirm water and sanitation infrastructure characteristics, including those that may be additional important risk factors for exposure to key pathogens, including wastewater discharges, water source characteristics, soil types, and other environmental variables.

In conclusion, our results suggest that children in households in this region that are reliant on domestic wells may experience increased risks for enteric pathogen exposure compared with children in households with water supplied by utilities. Elevated levels of fecal contamination in groundwater (12) could be related to documented deficiencies in rural sanitation in the region, and water as a proximal exposure pathway merits further exploration. New models for infrastructure delivery and management may help expand services, given the limitations of the current paradigm of each household being fully responsible for waste management despite the potential for collective impacts on public health.

Acknowledgments
The authors gratefully acknowledge the persons and communities who participated in the study.

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About the Author
Dr. Capone is an assistant professor at Indiana University–Bloomington. He uses tools from engineering, epidemiology, and environmental microbiology in the study of public health solutions for underserved communities.

References


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Work Attendance with Acute Respiratory Illness Before and During COVID-19 Pandemic, United States, 2018–2022


Both SARS-CoV-2 and influenza virus can be transmitted by asymptomatic, presymptomatic, or symptomatic infected persons. We assessed effects on work attendance while ill before and during the COVID-19 pandemic in the United States by analyzing data collected prospectively from persons with acute respiratory illnesses enrolled in a multistate study during 2018–2022. Persons with previous hybrid work experience were significantly less likely to work onsite on the day before through the first 3 days of illness than those without that experience, an effect more pronounced during the COVID-19 pandemic than during prepandemic influenza seasons. Persons with influenza or COVID-19 were significantly less likely to work onsite than persons with other acute respiratory illnesses. Among persons with positive COVID-19 test results available by the second or third day of illness, few worked onsite. Hybrid and remote work policies might reduce workplace exposures and help reduce spread of respiratory viruses.

COVID-19 cases in the United States, first reported on January 22, 2020, began to increase in March 2020 (1). The pandemic resulted in a substantial number of employed persons being laid off or furloughed, especially during spring 2020, and increased prevalence of teleworking (2–4). Employers were advised to actively encourage employees with symptoms of any acute respiratory illness (ARI) to stay home (5).

Both SARS-CoV-2 and influenza viruses can be transmitted by infected persons who are asymptomatic, presymptomatic, or symptomatic (6,7); staying home while ill can reduce workplace virus transmission by reducing contacts between infectious and healthy persons (8). That policy is considered an everyday preventive action that should be implemented year-round, but especially during annual seasonal influenza seasons and pandemics (9).

Data collected during the early COVID-19 pandemic (March 26, 2020–November 5, 2020) showed that employed adults with previous telework experience were less likely than those without to work at the worksite (onsite) while sick (10). However, whether persons worked onsite within the early days of illness when infectiousness is higher has remained unclear (7,11,12). We aimed to assess the effects before and during the COVID-19 pandemic of employees’ previous experience with various work-location practices on work attendance patterns within the first 3 days of illness among persons with any ARI, including COVID-19 and influenza. Institutional review boards at the Centers for Disease Control and Prevention and all participating sites approved the study. The enrollees provided informed consent.

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Methods

Study Population
During November 12, 2018–June 30, 2022, the US Influenza Vaccine Effectiveness Network enrolled adults 19–64 years of age from network-affiliated sites in 7 states. During November 12, 2018–March 18, 2020, persons seeking care for an ARI with cough within 7 days of illness onset were enrolled after local influenza circulation was identified from outpatient facilities affiliated with network sites in 5 states: Michigan (Ann Arbor and Detroit); Pennsylvania (Pittsburgh); Texas (Temple and surrounding areas in central Texas); Washington (Puget Sound region); and Wisconsin (Marshfield, Wausau, and Weston). For the period October 14, 2020–June 30, 2022, case definition was broadened to include persons seeking treatment at outpatient or telehealth facilities within 10 days of illness onset with cough, fever, loss of taste or smell, or seeking clinical COVID-19 testing. Two additional sites, southern California region and Nashville, Tennessee, participated during October 2021–June 2022. For our study, we considered November 2018–March 2020 the period of prepandemic influenza seasons and October 2020–June 2022 the COVID-19 pandemic period. Detailed study methods have been published elsewhere (13–15).

Data Collection
Data were collected from patients at enrollment throughout the entire study period (November 2018–June 2022): date of illness onset, symptoms since illness began (including fever/feverishness), age, sex, race/ethnicity, education, self-rated general health status, cigarette smoking, and number of children <12 years of age living in household. Respiratory specimens were collected from all participants at enrollment and tested for influenza viruses using real-time reverse transcription PCR (rRT-PCR); during the COVID-19 period (2020–2022), specimens were also tested for SARS-CoV-2 using RT-PCR. Persons enrolled on or after January 15, 2022, were asked if they had taken an at-home rapid COVID-19 test while ill and whether the result was positive.

All participants were asked to complete a follow-up survey, either online or over the phone, 1–2 weeks after enrollment. Throughout the 4-year study period, participants were asked at follow-up whether they had fully or mostly recovered from their illness and about employment status, type of employment (hourly, salaried, or other), hours they expected to work and hours usually worked from home in a typical week, and whether the employer discouraged work-ers with influenza-like symptoms from coming to work (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/29/12/23-1070-App1.pdf). They were also asked if and where they worked on each of the first 3 days of illness (the first day being the day that symptoms started). Participants were asked about work status for the day before illness onset during November 2018–May 2019 at the Pennsylvania site and at all participating sites for the subsequent study years (Appendix Table 2). For the period November 2018–September 2021, two sites, in Washington and Wisconsin, did not collect data about work status while ill from participants who typically worked remotely before illness onset. For prepandemic influenza seasons, participants were asked at follow-up whether they worked in a healthcare setting with direct patient contact; that question was asked at enrollment during the COVID-19 pandemic period.

Definitions
To categorize work experience before illness onset for our study, we used responses to questions about the number of hours participants expected to work and usually worked from home in a typical week (Appendix Figure 1). We categorized as having only onsite experience employed persons who reported that they usually worked no hours from home. We categorized as having hybrid (both onsite and remote) experience persons who stated that hours worked from home were usually fewer than total hours they expected to work. We categorized remaining persons as having only remote experience.

We categorized daily work attendance based on whether persons scheduled to work did or did not work. We categorized persons as scheduled to work for a given day regardless of number of hours for which they were scheduled. Among persons scheduled to work, we categorized those who worked for any number of hours, even if not total hours scheduled, as having worked and remaining persons as having not worked (Appendix Figure 2). We categorized persons who reported work location for a given day as onsite only or hybrid as having worked onsite.

We classified laboratory-confirmed influenza and SARS-CoV-2 viruses on the basis of positive results from PCR tests. We categorized persons with respiratory symptoms but negative PCR test results for influenza or SARS-CoV-2 as having other ARI.

Assembly of Participants
Among participants, 61% (12,941/21,133) completed the follow-up survey within 28 days of illness onset (Appendix Figure 3). Survey completion rates were
39% for Texas, 43% for Michigan, 60% for Washington, 75% for California, 75% for Pennsylvania, 79% for Wisconsin, and 89% for Tennessee. Among those who completed the follow-up survey, 69% (8,936/12,941) worked ≥20 h/wk before their illness. After excluding persons missing information on hours usually worked from home before illness or with indeterminate or missing laboratory results, we included 91% (8,132/8,936) in the analysis.

Statistical Analysis
We used χ² testing to assess differences between frequencies of categorical variables and Wilcoxon rank-sum test to compare differences in spread and medians (16). We computed adjusted odds ratios (aOR) for each day by fitting multilevel logistic regression models to account for clustering of participants within study sites using PROC GLIMMIX in SAS version 9.4 (SAS Institute, https://www.sas.com). We ran 2 sets of regressions for employed persons who were scheduled to work. For the first set of regressions, the dependent variable was worked from home before illness or with indeterminate or missing laboratory results, we included 91% (8,132/8,936) in the analysis.

Results
During the prepandemic influenza seasons, 1,245 persons had confirmed influenza and 2,362 other ARI (Appendix Figure 4). During the COVID-19 pandemic period, 114 persons had influenza, 1,888 had COVID-19, and 2,523 had other ARI. Among persons in the study with any respiratory illness, 82.6% with influenza, 61.4% with COVID-19, and 49.6% with other ARI reported having fever.

Among all participants, 14.0% (1,139) had only remote work experience before illness onset, 18.5% (1,503) had hybrid experience, and 67.5% (5,490) had only onsite experience (Appendix Table 3). Hourly workers made up a significantly lower percentage of persons with remote-only (29.9%) or hybrid (21.8%) experience before illness onset were unlikely to work onsite while ill, we excluded them from analyses pertaining to work location.

We used a backward selection process using change in -2 log likelihood to assess model fit to determine retention of independent variables in the models and ultimately dropped age, sex, education, and number of children in the household. We then assessed interactions between remaining independent variables (Tables 1–4; Appendix Tables 7, 8, 9).

Table 1. Likelihood of working at any location among adults with COVID-19, influenza, or other acute respiratory illness who were scheduled to work, by work experience in a typical week before illness onset, United States, 2018–2022

<table>
<thead>
<tr>
<th>Period</th>
<th>Day before illness</th>
<th>Day 1 of illness</th>
<th>Day 2 of illness</th>
<th>Day 3 of illness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prepandemic influenza seasons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Work experience†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote only</td>
<td>97.5 (39/40)</td>
<td>70.5 (43/61)</td>
<td>66.7 (40/60)†</td>
<td>68.4 (39/57)†</td>
</tr>
<tr>
<td>Hybrid</td>
<td>90.6 (222/245)</td>
<td>72.6 (329/453)</td>
<td>68.0 (297/437)</td>
<td>63.3 (274/433)</td>
</tr>
<tr>
<td>Onsite only</td>
<td>92.3 (1,037/1,124)</td>
<td>69.4 (1,445/2,082)</td>
<td>51.4 (1,000/1,947)</td>
<td>48.4 (912/1,883)</td>
</tr>
<tr>
<td>Adjusted analysis§</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Work experience</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote only</td>
<td>NA</td>
<td>1.02 (0.57–1.84)</td>
<td>1.90 (1.06–3.39)</td>
<td>2.13 (1.16–3.91)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>NA</td>
<td>1.01 (0.78–1.30)</td>
<td>1.92 (1.50–2.46)</td>
<td>1.66 (1.30–2.12)</td>
</tr>
<tr>
<td>Onsite only</td>
<td>NA</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td><strong>COVID-19 pandemic period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Work experience†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote only</td>
<td>95.8 (498/520)‡</td>
<td>80.5 (495/615)‡</td>
<td>71.7 (451/629)‡</td>
<td>72.4 (449/620)‡</td>
</tr>
<tr>
<td>Hybrid</td>
<td>95.6 (540/565)‡</td>
<td>78.4 (514/656)‡</td>
<td>68.9 (451/655)‡</td>
<td>65.2 (416/638)‡</td>
</tr>
<tr>
<td>Onsite only</td>
<td>90.1 (1,490/1,653)</td>
<td>65.1 (1,242/1,907)</td>
<td>41.6 (752/1,806)</td>
<td>37.4 (666/1,782)</td>
</tr>
<tr>
<td>Adjusted analysis§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Work experience</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote only</td>
<td>NA</td>
<td>2.03 (1.58–2.59)</td>
<td>3.37 (2.68–4.23)</td>
<td>3.78 (3.00–4.77)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>NA</td>
<td>1.69 (1.34–2.13)</td>
<td>2.75 (2.22–3.42)</td>
<td>2.56 (2.06–3.19)</td>
</tr>
<tr>
<td>Onsite only</td>
<td>NA</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
</tbody>
</table>

*NA, not applicable.
†Values are not applicable (no. persons worked at any location/no. persons scheduled to work).
‡p<0.001 (comparison of 3 work experience categories for specified day and period).
§Values are adjusted odds ratio (95% CI). Dependent variable for the multilevel logistic regression models is worked at any location on a specified day of illness (0 = did not work, 1 = worked). Independent variables are work experience in a typical week before illness onset (remote only, hybrid, onsite only), study period (0 = prepandemic influenza seasons, 1 = COVID-19 pandemic period), PCR test result (0 = other acute respiratory illness, 1 = influenza or COVID-19), race/ethnicity, general health before illness, current smoker, type of employment, healthcare personnel, hours worked in a typical week before illness onset, employees discouraged from coming to work with flu-like symptoms, and study site. We excluded persons with missing information for independent variables (303 for day 1, 314 for day 2, and 279 for day 3). p<0.001 for work experience study period interaction term for days 1–3 of illness.
experience than onsite-only experience (66.6%) (p<0.001). Percentages of participants working in healthcare by location of work experience varied: 7.1% of remote-only, 15.5% of hybrid, and 25.4% of onsite-only personnel (p<0.001). Percentage of participants with at least a bachelor’s degree was significantly higher among persons with remote-only (71.3%) or hybrid (79.5%) experience than those with onsite-only experience (43.5%; p<0.001). Among 1,139 persons with remote-only experience during the study period, most (88.9%) were enrolled during the pandemic period. Among the 1,503 persons with hybrid experience, median hours worked from home in a typical week before illness onset was significantly higher during the pandemic period (16 h/wk) than during prepandemic influenza seasons (8 h/wk; p<0.001).

Approximately three fourths of participants were scheduled to work on each of the first 3 days after illness onset (Appendix Table 4). Persons with previous remote-only or hybrid experience were significantly more likely than those with only onsite experience to work at any location on the second and third days of illness (Table 1). For example, on the third day of illness during the pandemic period, the percentage who worked at any location was 72.4% for persons with remote-only experience, 65.2% for persons with hybrid experience, and 37.4% for those with onsite-only experience (p<0.001). Among all persons who worked at any location on scheduled work days, median time worked was 8 (interquartile range 8–8) hours for the day before illness and 8 (interquartile range 6–8) hours for each of the first 3 days of illness (Appendix Table 5). Analysis of the location of work showed that participants were significantly more likely to work remotely on the day before illness onset than for the first 3 days of illness during the pandemic period than prepandemic influenza seasons (Table 2). For example, on the third day of illness, 18.5% of persons worked remotely during the pandemic period, compared with 8.8% during the prepandemic influenza seasons.

Participants with hybrid experience were less likely to work onsite than persons with onsite-only experience on the day before through the first 3 days of illness (Table 3); effect magnitude was more pronounced during the pandemic period than prepandemic influenza seasons. For example, for the third day of illness, hybrid versus onsite-only aOR was greater for the pandemic (aOR 0.38, 95% CI 0.29–0.49) than the prepandemic period (aOR 0.69, 95% CI 0.54–0.87; p<0.001 for the work experience–study period interaction term). Conversely, participants were less likely to work onsite during the pandemic period than prepandemic influenza seasons and effect magnitude was more pronounced among persons with hybrid than onsite-only experience. For example, for the third day of illness, pandemic versus prepandemic aOR was greater among persons with hybrid (0.32) than onsite-only (0.59) experience (Table 3). Persons with hybrid experience were more likely to work remotely during the pandemic period than they were during the prepandemic period (Appendix Table 6). In contrast, persons with onsite-only experience were more likely not to work on scheduled-to-work days during the pandemic than during the prepan
demic period. Findings were similar even when we restricted data for the regression models to non–healthcare personnel or the sites that contributed data for all 4 study years (Appendix Tables 7, 8). Findings were also similar when we restricted the analysis to the sites with highest survey completion rates (Appendix Table 9).

### Table 2. Reported work location among adults with influenza, COVID-19, or other acute respiratory illness who were scheduled to work, United States, 2018–2022*

<table>
<thead>
<tr>
<th>Period</th>
<th>Day before illness</th>
<th>Day 1 of illness</th>
<th>Day 2 of illness</th>
<th>Day 3 of illness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prepandemic influenza seasons</strong></td>
<td>n = 1,358</td>
<td>n = 2,515</td>
<td>n = 2,372</td>
<td>n = 2,304</td>
</tr>
<tr>
<td>Work location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onsite only</td>
<td>1,161 (85.5)†</td>
<td>1,464 (58.2)†</td>
<td>1,002 (42.2)†</td>
<td>920 (39.9)†</td>
</tr>
<tr>
<td>Hybrid</td>
<td>41 (3.0)</td>
<td>75 (3.0)</td>
<td>66 (2.8)</td>
<td>52 (2.3)</td>
</tr>
<tr>
<td>Remote only</td>
<td>46 (3.4)</td>
<td>215 (8.5)</td>
<td>217 (8.2)</td>
<td>202 (8.8)</td>
</tr>
<tr>
<td>Did not work</td>
<td>110 (8.1)</td>
<td>761 (30.3)</td>
<td>1,087 (45.8)</td>
<td>1,130 (49.0)</td>
</tr>
<tr>
<td><strong>COVID-19 pandemic period</strong></td>
<td>n = 2,188</td>
<td>n = 2,509</td>
<td>n = 2,418</td>
<td>n = 2,382</td>
</tr>
<tr>
<td>Work location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onsite only</td>
<td>1,676 (76.6)</td>
<td>1,239 (49.4)</td>
<td>644 (26.6)</td>
<td>561 (23.5)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>73 (3.3)</td>
<td>83 (3.3)</td>
<td>42 (1.8)</td>
<td>43 (1.8)</td>
</tr>
<tr>
<td>Remote only</td>
<td>251 (11.5)</td>
<td>380 (15.1)</td>
<td>474 (19.6)</td>
<td>440 (18.5)</td>
</tr>
<tr>
<td>Did not work</td>
<td>188 (8.6)</td>
<td>807 (32.2)</td>
<td>1,258 (52.0)</td>
<td>1,338 (56.2)</td>
</tr>
</tbody>
</table>

*We excluded persons with only remote work experience before illness onset (560 for day before illness, 676 for day 1, 689 for day 2, and 677 for day 3) and those with missing work location (41 for day before illness, 74 for day 1, 55 for day 2, and 50 for day 3).

†p<0.001 (comparison of prepandemic to pandemic period for specified day).
Among persons with COVID-19, substantial percentages worked onsite while ill: 51.2% on day 1, 22.3% on day 2, and 14.1% on day 3 (Table 4). COVID-19–positive PCR test results were available for 1.3% (12/940) by the first day of COVID-19 illness, 10.7% (97/910) by the second day, and 23.5% (211/899) by the third day (Table 5). Persons for whom a positive COVID-19 PCR test result was available by the second day of illness were significantly less likely to work onsite on that day than those whose positive PCR result was available on the third day or later (5.2% vs. 25.0%; p<0.001) (Table 5). Persons for whom a positive PCR test result was available by the third day of illness were significantly less likely to work onsite on that day than those whose positive PCR result was available later than the third day of illness (4.7% vs. 17.2%; p<0.001). Among persons for whom positive PCR test results were available after the second or third day of illness, the percentage who worked onsite was slightly higher when we excluded persons with COVID-19–positive at-home test results by the second or third day of illness (Appendix Table 12).
Discussion
During both prepandemic and pandemic periods, adults with remote-only or hybrid experience were more likely to work within the first 3 days of illness compared with those with onsite-only experience. It is notable, however, that persons with hybrid experience were significantly less likely to work onsite on the day before illness through the first 3 days of illness than those with only onsite experience. The effect magnitude of hybrid compared with onsite-only experience on working onsite while ill was more pronounced for the pandemic period than for the prepandemic period. Persons with influenza or COVID-19 were significantly less likely to work onsite on the second and third days of illness than were persons with only onsite experience. Those with only onsite experience has been reported in studies conducted during the 2017–2018 influenza season and during the early part of the COVID-19 pandemic (March–November 2020) (10,17). Remote-only or hybrid experience before illness can enable persons to work remotely if they are well enough, instead of taking sick days.

It is possible that persons without experience working from home were more likely to work in occupations in which remote-only or hybrid work is less feasible and, therefore, workers are less likely to have the option or incentive to work remotely. Those workers might include persons with jobs in hospitality and leisure, transportation, utilities, construction, production, and agriculture (18,19).

Employers were required to provide paid sick leave to workers with COVID-19 during the pandemic (20). It is unlikely that persons with only onsite experience worked less than persons with hybrid experience after testing SARS-CoV-2–positive because they received paid sick leave. This pattern of persons with only onsite experience working less than persons with hybrid experience was also observed during the prepandemic influenza seasons.

Persons with previous hybrid experience were less likely to work onsite the day before illness onset through the first 3 days of illness than persons with only onsite experience, thus reducing the likelihood of workplace exposures to respiratory viruses. A study conducted during the 2017–18 influenza season to those mentioned above.

Table 4. Likelihood of working onsite among adults who were scheduled to work, by PCR test result, United States, 2018–2022*

<table>
<thead>
<tr>
<th>Category</th>
<th>Day before illness, n =</th>
<th>Day 1 of illness, n =</th>
<th>Day 2 of illness, n =</th>
<th>Day 3 of illness, n =</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepandemic influenza seasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza</td>
<td>3,489</td>
<td>4,959</td>
<td>4,720</td>
<td>4,619</td>
</tr>
<tr>
<td>Other ARI</td>
<td>88.8 (443/499)</td>
<td>59.3 (504/850)</td>
<td>34.1 (285/835)†</td>
<td>28.2 (236/837)†</td>
</tr>
<tr>
<td>aOR (95% CI)‡</td>
<td>1.01 (0.70–1.46)</td>
<td>0.92 (0.77–1.10)</td>
<td>0.51 (0.43–0.61)</td>
<td>0.39 (0.32–0.47)</td>
</tr>
<tr>
<td>COVID-19 pandemic period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COVID-19§</td>
<td>78.7 (681/865)</td>
<td>51.2 (522/1,020)</td>
<td>22.3 (220/986)†</td>
<td>14.1 (137/974)†</td>
</tr>
<tr>
<td>Other ARI</td>
<td>81.4 (1,031/1,266)</td>
<td>53.9 (768/1,424)</td>
<td>33.0 (450/1,362)</td>
<td>33.7 (452/1,341)</td>
</tr>
<tr>
<td>aOR (95% CI)‡</td>
<td>0.80 (0.63–1.01)</td>
<td>0.92 (0.77–1.09)</td>
<td>0.59 (0.49–0.73)</td>
<td>0.31 (0.25–0.39)</td>
</tr>
<tr>
<td>Influenza or COVID-19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COVID-19 pandemic period</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza</td>
<td>78.7 (681/865)†</td>
<td>51.2 (522/1,020)†</td>
<td>22.3 (220/986)†</td>
<td>14.1 (137/974)†</td>
</tr>
<tr>
<td>Preparative influenza seasons</td>
<td>88.8 (443/499)</td>
<td>59.3 (504/850)</td>
<td>34.1 (285/835)†</td>
<td>28.2 (236/837)†</td>
</tr>
<tr>
<td>aOR (95% CI)‡</td>
<td>0.53 (0.37–0.75)</td>
<td>0.84 (0.69–1.03)</td>
<td>0.65 (0.52–0.81)</td>
<td>0.45 (0.35–0.58)</td>
</tr>
<tr>
<td>Other ARI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COVID-19 pandemic period</td>
<td>81.4 (1,031/1,266)†</td>
<td>53.9 (768/1,424)†</td>
<td>33.0 (450/1,362)†</td>
<td>33.7 (452/1,341)†</td>
</tr>
<tr>
<td>Preparative influenza seasons</td>
<td>88.4 (785/859)</td>
<td>62.2 (1,035/1,665)</td>
<td>50.9 (783/1,537)</td>
<td>50.2 (736/1,467)</td>
</tr>
<tr>
<td>aOR (95% CI)‡</td>
<td>0.67 (0.51–0.89)</td>
<td>0.84 (0.72–0.99)</td>
<td>0.56 (0.47–0.66)</td>
<td>0.56 (0.48–0.67)</td>
</tr>
</tbody>
</table>

*Values are percentage (no. persons worked onsite/no. persons scheduled to work) except as indicated. Worked onsite represents onsite only or hybrid work location. We excluded persons with influenza during the COVID-19 pandemic period (57 days before illness, 65 for day 1, 70 for day 2, and 67 for day 3); persons with only remote work experience before illness onset period (560 for day before illness, 676 for day 1, 689 for day 2, and 677 for day 3); persons with missing work location (41 for day before illness, 74 for day 1, 55 for day 2, and 50 for day 3); ARI, acute respiratory illness; aOR, adjusted odds ratio.

†p<0.001 (comparison of % working onsite for specified day).
‡Dependent variable in the multilevel logistic regression models is worked onsite on a specified day (0 = did not work or worked remotely only, 1 = worked onsite [onsite only or hybrid]). Independent variables are work experience in a typical week before illness onset (0 = onsite only, 1 = hybrid), study period (0 = prepandemic influenza seasons, 1 = COVID-19 pandemic period), PCR test result (0 = other acute respiratory illness, 1 = influenza or COVID-19), race/ethnicity, general health before illness, current smoker, type of employment, healthcare personnel, hours worked in a typical week before illness, employees discouraged from coming to work with flu-like symptoms, and study site. We excluded persons with missing information for independent variables (170 for day before, 237 for day 1, 247 for day 2, and 216 for day 3) in addition to those mentioned above.
§Among persons with COVID-19, healthcare personnel were significantly more likely to work onsite than nonhealthcare personnel on the day before illness (85.9% vs 76.7%, p<0.01) and the first day of illness (58.4% vs 49.2%, p<0.05), but not on the second (22.0% vs. 22.3%) and third (11.7% vs. 14.7%) days of illness.
concorred with that finding, but the study did not examine the likelihood of working onsite on the day before illness (17). A study conducted during the early part of the COVID-19 pandemic found that persons with hybrid experience were less likely to work onsite while ill than were persons with only onsite experience (10), an effect more pronounced during the pandemic than the prepandemic period. That difference may have been because of the greater prevalence of telework regardless of Illness status during the pandemic (3,4). During the pandemic period, intense public health messaging to stay home when ill, employer policies discouraging or prohibiting employees with ARI symptoms from working onsite, and provision of flexible paid leave for persons with COVID-19 illness may have contributed to the greater effect (5,20).

Persons with laboratory-confirmed influenza or COVID-19 were significantly less likely than persons with other ARI to work onsite on the second and third days of illness. Previous studies have reported similar findings but did not assess the likelihood of working onsite on each of the first 3 days of illness (10,17). Those findings might be attributable to more severe manifestations of illness in persons with influenza or COVID-19 (15). The finding that the likelihood of working onsite was similar in persons with influenza or COVID-19 compared with persons with other ARI on the first day of illness, as well as the greater likelihood of working onsite on the first day of illness compared with the second or third day of illness, might have been because illness had begun when participants were already at work. For persons ill with COVID-19, having positive PCR test results by the second or third day of illness might have reduced the likelihood of working onsite for several reasons, including being concerned for coworkers, being advised to isolate by case investigators, having employers discourage or prohibit persons with COVID-19 from entering the worksite, and having employers provide flexible sick leave. However, COVID-19-positive PCR test results were available for only a small proportion of persons within the first 3 days of illness because of the lag between illness onset and seeking medical care. At-home rapid COVID-19 tests may enable early testing for persons with symptoms of ARI. Use of at-home tests among persons with COVID-19-like illness in the United States increased from 6% during August 23–December 11, 2021, to 20% during December 19, 2021–March 12, 2022 (21).

Strengths of our study were that we included data from ≈8,000 persons over a 4-year study period that encompassed both prepandemic and pandemic periods. We obtained respiratory specimens that enabled laboratory confirmation of influenza and SARS-CoV-2. Also, we assessed work attendance within the presymptomatic phase, when persons can be infectious, and the first 3 days of illness, when infectiousness is greatest. One limitation of the study was that 39% of participants did not complete the follow-up survey. However, findings were similar when we restricted the analysis to the sites with the highest survey completion rates. Second, we assessed the proportion of employees who worked at any location within the first 3 days of illness as an indicator of maintenance of workflow. We did not assess how illness may have diminished productivity among persons working while ill versus those working while well. Third, we assessed work attendance only among persons with medically attended ARI. Findings may not be generalizable to persons who were asymptomatic or who did not seek medical care.

Future research should ascertain productivity in persons who work while ill with influenza or COVID-19. In addition, an assessment of the likelihood of working onsite among persons with ARI who do not seek medical care is needed. Research is also needed.

Table 5. Likelihood of working onsite among adults with COVID-19 illness who were scheduled to work, by day when COVID-19–positive PCR test result was available, United States, October 2020–June 2022*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheduled to work on day 1 of COVID-19 illness†</td>
<td>50.0 (6/12)</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available on day 1 of illness</td>
<td>52.1 (483/928)</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available after day 1 of illness</td>
<td>52.1 (483/928)</td>
</tr>
<tr>
<td>Scheduled to work on day 2 of COVID-19 illness‡</td>
<td>5.2 (5/97)§</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available on day 1 or 2 of illness</td>
<td>25.0 (203/813)</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available after day 1 of illness</td>
<td>25.0 (203/813)</td>
</tr>
<tr>
<td>Scheduled to work on day 3 of COVID-19 illness¶</td>
<td>4.7 (10/211)¶</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available on day 1, 2, or 3 of illness</td>
<td>17.2 (118/688)</td>
</tr>
<tr>
<td>COVID-19–positive PCR result available after day 3 of illness</td>
<td>17.2 (118/688)</td>
</tr>
</tbody>
</table>

*Day of illness when COVID-19 positive PCR test result was available was computed by comparing the date of illness onset with the date that COVID-19–positive PCR test result was available. Values represent % worked onsite (no. worked onsite/no. scheduled to work). Worked onsite represents onsite only or hybrid work location. Analysis was restricted to persons with COVID-19 as shown in Table 4.
†Unknown when COVID-19 positive PCR result was available = 80 persons.
‡Unknown when COVID-19 positive PCR result was available = 76 persons.
§p<0.001.
¶Unknown when COVID-19 positive PCR result was available = 75 persons.
on how type of occupation and other workplace policies affect work attendance of sick employees.

In conclusion, working-age adults continue to be at risk for severe COVID-19 (22). Our study findings show that hybrid work experience before illness onset might give workers the opportunity to continue working but also reduce time worked onsite early in illness, when infectiousness is high. When feasible for a given occupation, employers should consider hybrid and remote work policies that might reduce likelihood of workplace exposures to influenza and SARS-CoV-2 viruses. Such work policies could minimize interaction with infectious persons in workplaces during both the presymptomatic and symptomatic phases of illness and help reduce spread of respiratory viruses.

Acknowledgments

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**July 2023**

**Fungal Infections**

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- Nationwide Outbreak of *Candida auris* Infections Driven by COVID-19 Hospitalizations, Israel, 2021–2022
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To revisit the July 2023 issue, go to: https://wwwnc.cdc.gov/eid/articles/issue/29/7/table-of-contents
Since October 2021, outbreaks of highly pathogenic avian influenza (HPAI) A(H5N1) virus belonging to A/Goose/Guangdong/1/1996 lineage H5 clade 2.3.4.4b have been reported throughout Europe (1). Transatlantic spread of HPAI H5N1 virus with genetic similarity to Eurasian lineages was detected in the United States in December 2021 and has subsequently spread throughout the continental United States in wild birds and domestic poultry (2–6).

In addition to disease outbreaks in domestic poultry, currently circulating HPAI H5N1 virus is persisting in wild bird reservoirs, with multiple reports of spillover into and clinical infection in various mammal species in countries in Europe during 2021 (1,3,7). We report a case series on the pathology of natural infections with HPAI H5N1 virus in terrestrial wild mammals in the United States concurrent with high levels of circulating HPAI viruses in wild birds during the spring and summer of 2022.
Materials and Methods
Case inclusion criteria were confirmed positivity for HPAI H5N1 virus infection by real-time reverse transcription PCR (rRT-PCR) or, in 3 cases, by having consistent lesions, positive avian influenza virus immunohistochemistry (IHC) results, and being part of a litter with other animals confirmed positive by rRT-PCR. The cases represent opportunistic samples from wild mammals reported ill or dead by citizens and submitted to state wildlife agencies or veterinary diagnostic laboratories for diagnostic purposes. Clinical observations were recorded by citizens, wildlife professionals, rehabilitators, or veterinary professionals. Except for 2 red foxes, for which only antemortem samples were available, all animals had postmortem examinations performed by veterinarians or pathologists. Postmortem samples included swab specimens in viral transport medium, tissue samples stored refrigerated or frozen, and tissues fixed in 10% neutral-buffered formalin.

We used the standardized protocols for the National Animal Health Laboratory Network for HPAI virus rRT-PCR testing on a variety of sample types. We extracted total nucleic acids from samples by using the KingFisher Flex or KingFisher Purification System platforms and MagMAX-96 Viral RNA Isolation Kit or MagMAX Pathogen RNA/DNA Kit (Life Technologies, https://www.thermofisher.com) according to the manufacturer’s protocol. We also performed a general influenza A virus (IAV) rRT-PCR targeting the conserved region of the IAV matrix gene (8,9). We performed further subtyping by using 2 IAV H5 subtyping assays: the avian influenza H5 subtype rRT-PCR targeting the hemagglutinin gene for the North American, Eurasian, and Mexican lineage of avian influenza (8–11) and an H5 2.3.4.4b-specific rRT-PCR developed in collaboration with the US Department of Agriculture Southeast Research Laboratory (SEPRL; Real-Time RT-PCR Assay for the Detection of Goose/Guangdong lineage Influenza A subtype H5, clade 2.3.4.4; NVSL-WI-1732).

Influenza A–positive samples were sent to the National Veterinary Services Laboratories (NVSL) in Ames, Iowa for confirmation and characterization. Testing at NVSL included an H5 clade 2.3.4.4 pathotyping assay and an assay targeting neuraminidase 1 (N1) for rapid pathotyping (SEPRL; Real-Time RT-PCR Assay for Pathotyping Goose/Guangdong lineage Influenza A subtype H5, clade 2.3.4.4; NVSL-WI-1767) and neuraminidase subtyping (SEPRL; Real-Time RT-PCR Assay for the Detection of Eurasian-lineage Influenza A Subtype N1; NVSL-WI-1768). Influenza A viruses were sequenced directly as previously described (4). We used RAxML (https://github.com/amkozlov/raxml-ng) to generate phylogenetic trees and created tables of single-nucleotide polymorphisms (SNPs) by using the vSNP pipeline (https://github.com/USDA-VS/vSNP) with a reference composed of 6 segments from an H5N1 2.3.4.4b clade virus and 2 segments from North America–origin wild bird viruses (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0464-App1.pdf). Routine detection of mutations associated with mammalian adaptation at the consensus level uses the designated SNP functionality of the vSNP pipeline, generating an SNP table of all sequences with mutations at the assigned position. The included locations are in the polymerase basic 2 (PB2) gene: position 1894 for E627K, position 2116 for D701N, and position 826 for T271A. We used a search of the variant call files generated by the vSNP pipeline to generate a list of both consensus and subconsensus level presence of the designated variants. Genotypes were assigned according to the scheme described in Youk et al. (3), using designated SNPs in the vSNP pipeline, as well as the GenoFLU tool (https://github.com/USDA-VS/GenoFLU).

In some cases, additional ancillary testing was performed. Formalin-fixed tissues were processed for histopathologic analysis and evaluated by veterinary pathologists at multiple institutions. A subset of tissues from some animals was processed for monoclonal IHC analysis for IAV, canine distemper virus antigen, or both. All ancillary testing procedures were performed according to validated procedures at federal and state diagnostic laboratories accredited by the American Association of Veterinary Laboratory Diagnosticians.

Results
During April 1–July 21, 2022, HPAI virus was detected in 67 wild mammals from 10 states: Alaska, Idaho, Iowa, Michigan, Minnesota, New York, North Dakota, Utah, Washington, and Wisconsin (Figure 1). Species identified were 50 red foxes (Vulpes vulpes), 6 striped skunks (Mephitis mephitis), 4 raccoons (Procyon lotor), 2 bobcats (Lynx rufus), 2 Virginia opossums (Didelphis virginiana), 1 coyote (Canis latrans), 1 fisher (Pekania pennanti), and 1 gray fox (Urocyon cinereoargenteus) (Appendix Table 2).

Based on weight and dentition, all red foxes, striped skunks, opossums, 3 of 4 raccoons, and the coyote were juveniles, and 1 raccoon, both bobcats, the fisher, and the gray fox were adults (12). Within species with >4 animals, no sex predilections were apparent (Appendix Table 2). Six pairs of red foxes...
and 2 opossums were known or strongly suspected to be littermates, and skunks 1–5 comprised 2 litters that were comingled at a rehabilitation center.

Nine animals were found dead. Of those found alive, 13 were euthanized in the field and 44 were under the care of a wildlife rehabilitator or veterinarian (Appendix Table 2). Of those 44 animals, 2 red foxes remained alive and were clinically normal before release, 13 died, and the remainder were euthanized. The outcome was not reported for 1 animal.

Among the 58 animals found alive, most (n = 54) had neurologic abnormalities, comprising seizures (n = 29), ataxia (n = 23), tremors (n = 17), lack of fear of humans (n = 7), vocalization (n = 5), circling (n = 4), blindness (n = 3), torticollis (n = 2), nystagmus (n = 1), and grimace (n = 1) (Appendix Table 3). Less frequently recorded abnormalities were lethargy (n = 28), fever (n = 7), diarrhea (n = 2), unconsciousness (n = 2), recumbence (n = 1), paralysis (n = 1), and vomiting (n = 1). Dyspnea was reported in 2 skunks, 1 bobcat, and 1 red fox.

Gross postmortem observations were recorded in 58 animals. Most animals were in fair to good nutritional condition (n = 39). Lung lesions were consistently observed (n = 49) and included congestion (n = 42), edema (n = 22), failure to collapse (n = 18), hemorrhage (n = 18), and pleural effusion (n = 6) (Figure 2, panel A). The most common brain lesions were hemorrhage (n = 11) and congestion (n = 7). Other lesions included pallor (n = 8), congestion (n = 7), enlargement (n = 6), and hemorrhage (n = 1) in the liver, as well as congestion (n = 4) and cortical hemorrhage (n = 1) in the kidney. Pericardial effusion (n = 3), petechia (n = 2), and myocardial pallor (n = 2) were infrequently noted in the heart (Figure 2, panel B). In the gastrointestinal tract, nematode parasitism was relatively common (n = 15), but other rarely observed lesions included congestion, hemorrhage, and loose feces. Three red foxes had gastric contents that included feathers (Figure 2, panel C). Three red foxes had mild ocular discharge. The gray fox had severe hemorrhage in all body cavities.

Histopathologic findings were recorded for 55 animals (Table 1; Appendix Table 4). Of 54 animals with ≥1 section of brain suitable for histopathologic evaluation, all but 2 skunks and the gray fox had...
brain lesions. The most consistently affected brain region was the frontal lobe (16 animals of 16 tested, 100%), followed by the cerebral cortex (46/53, 87%), brainstem (22/29, 76%), thalamus (20/29, 69%), hippocampus (17/29, 59%), and cerebellum (12/33, 36%). Brain lesions had a multifocal and random distribution, affected gray and white matter, and sometimes exhibited a periventricular distribution. Lesions primarily consisted of regions of malacia and inflammation (Figure 3, panel A). Acidophilic neuronal necrosis was prominent and often associated with satellitosis (Figure 3, panel B) or karyorrhectic debris (Figure 3, panel C). Laminar neuronal necrosis within the hippocampus was striking in some cases (Figure 3, panel E). Alveolar septa were thickened by neutrophils, macrophages, perivascular lymphocytes and plasma cells, and aggregates of fibrin. Type II pneumocyte hyperplasia was rare, and few animals had bronchopneumonia. Most lung lesions were acute, except in 1 fox and 1 bobcat. Concurrent lungworm parasitism was noted in 3 raccoons, 1 bobcat, and the fisher.

Multifocal myocardial necrosis, most commonly affecting the left ventricular wall, was present in roughly half of the animals (n = 29). Mineralization of affected cardiomyocytes was often noted (Figure 3F). Mild lymphoplasmacytic or histiocytic infiltrates and regions of fibrosis were infrequently reported in conjunction with myocardial necrosis. No heart lesions were observed in the striped skunks, the opossum joeys, or the gray fox.

Lymphoid depletion was observed in 28 animals and was most consistent in the striped skunks and raccoons. Lymphoid necrosis was prominent in the spleen (n = 5), lymph nodes (n = 4), and Peyer’s patches of the ileum (n = 1) of the striped skunks.
and in the thymus and intestinal Peyer’s patches of 1 raccoon.

Foci of inflammation or necrosis were rarely observed in the kidney, tongue, and gastrointestinal tract in the red fox kits. A focal area of acute pancreatic necrosis and multifocal lymphoplasmacytic pancreatitis were noted in 1 red fox each. With the exception of a few small clusters of lymphocytes and plasma cells within the photoreceptor layer of the retina of 1 red fox, no lesions were reported in any of the examined eyes from other red foxes, including the 3 red foxes reported as clinically blind. The 1 gray fox had no microscopic lesions.

We performed IHC analysis for avian influenza antigen on a variety of tissues in 29 animals (Table 1). Of the 13 red foxes with immunoreactivity in the brain, positively staining neurons were detected in the cerebral cortex (n = 13), thalamus (n = 6), hippocampus (n = 3), and brainstem (n = 1) (Figure 3, panel G).

Immunoreactivity within lung tissue was limited to scattered mild staining of epithelial cells and interstitial macrophages (Figure 3, panel H) in 2 of 15 red foxes, both striped skunks and the 1 raccoon. One of 2 opossums had strong immunoreactivity within pneumocytes and to a lesser extent alveolar macrophages and bronchial and bronchiolar epithelium. Neither bobcat had immunoreactivity in the lung.

Three of 5 red foxes and the 1 raccoon that had IHC analysis performed on heart showed immunoreactivity of scattered cardiac myofibers and interstitial macrophages surrounding foci of necrosis. Immunoreactivity within hepatocytes surrounding necrotic foci was present in all 5 striped skunks, whereas only 1 of 3 red foxes tested had immunoreactivity in the liver.

A variety of sample types from 64 animals were tested by rRT-PCR in National Animal Health Laboratory Network laboratories using several assays that can detect IAV (Table 1; Appendix Table 5). With 1 exception (assay performed on formalin-fixed, paraffin-embedded brain tissue), all animals with nonnegative rRT-PCR results had ≥1 confirmed positive at NVSL. Most samples were uniformly positive using the general IAV, H5 subtype, and H5 clade 2.3.4.4b subtype rRT-PCRs, when performed (Appendix Table 5). Of 35 animals with ≥2 sample types tested, brain samples frequently had the strongest amplification signal (n = 21). Pooled nasal and oropharyngeal swab specimens had greater detection rates than did nasal or oropharyngeal swab specimens.

Full genome sequence data were obtained directly from 77 samples representing 48 animals across 7 species from 10 states. Sequences were deposited in GISAID (https://gisaid.org) (Appendix Table 6).

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**Figure 3.** Histopathology of lesions in red foxes naturally infected with highly pathogenic avian influenza virus, United States. A) Throughout the brain, there are multifocal regions of necrosis and hypercellularity. Original magnification ×4. B) Within the gray matter, there is prominent neuronal necrosis (arrowheads), satellitosis (arrow), and reactive astrocytes. A vessel is surrounded by lymphocytes and plasma cells. Original magnification ×40. C) In areas of necrosis within the brain, there is often abundant, stippled, basophilic karyorrhectic debris. Original magnification ×40. D) Within the hippocampus, there are numerous shrunken, angular, and acidophilic (necrotic) neurons in a laminar pattern. Original magnification ×20. E) Within the lung, there is diffuse vascular congestion. Alveoli contain fibrin, hemorrhage, and edema fluid. Original magnification ×20. F) Regions of cardiomyocyte necrosis in the heart are often mineralized. Original magnification ×20. Panels A–F, hematoxylin and eosin stain. G) Within the brain, there is positive nuclear and cytoplasmic staining of neuron cell bodies and processes. Avian influenza virus monoclonal immunohistochemical analysis. Original magnification ×40. H) Scattered positive nuclear and cytoplasmic staining of bronchiolar epithelial cells and interstitial macrophages in the lung. Monoclonal immunohistochemical analysis of influenza A virus nucleoprotein. Original magnification ×40.
Among the 48 animals, 9 different H5N1 genotypes were identified according to the specific combinations of Eurasian and North American gene segments (Table 2). All but 1 of these genotypes have at least North American polymerase basic 1 and nucleoprotein genes and represent reassortments of the Newfoundland-like H5N1 2.3.4.4b virus initially introduced into the Americas by the Atlantic Flyway with North American wild bird–origin influenza viruses (2,3). The 1 unreassorted virus from a red fox in Alaska represents a separate introduction of the H5N1 2.3.4.4b into the Americas based on phylogenetic analysis and high sequence identity to Asian-origin H5N1 viruses across all 8 segments (a Japan-like virus likely introduced via the Bering Strait) (13). The PB2 E627K substitution previously associated with mammalian adaptation (14) was identified at the consensus level in three animals as well as at the subconsensus level (mixed population) in 3 additional animals (Table 2). The PB2 D701N mutation was identified at the consensus level in 1 animal and at the subconsensus level in 3 animals (Table 2). The PB2 T271A mutation was not detected at consensus or subconsensus levels in any animal. Detections of PB2 E627K and D701N mutations are diverse in their geographic locations and genotypes. Analysis of all sequences indicated infections resulted from regional spillovers from wild birds, as shown by a subset of genotypes B1.2 and B3.2 (Appendix Figure) and their phylogenetic trees (Figure 4).

Ancillary testing for other viruses was performed on many of these animals and concurrent viral infections were identified in 4 animals (Appendix Table 7). One red fox had concurrent Escherichia coli enteritis and septicemia. No anticoagulant rodenticides were detected in a liver sample from the gray fox.

**Discussion**

This case series highlights multiple detections of HPAI virus Eurasian lineage H5 clade 2.3.4.4b in wild terrestrial mammals in the United States. Our findings build on previous reports of natural infections with HPAI virus in red foxes from the Netherlands in 2021 (15) and add to the global list of species susceptible to H5N1 HPAI virus (16).

Red foxes are the most represented species in this report. Intrinsic factors related to exposure and infection risk could explain this finding, including opportunistic dietary preferences, likelihood of sharing environments with infected birds, abundance of immunologically naive animals present during the onset of the avian outbreak, and potentially increased susceptibility to infection in this species. Many of the red foxes were found in urban or periurban environments, and extrinsic factors, such as human interest in the highly visible animals, might have led to increased public reporting. Raccoons, skunks, opossums, and coyotes are also generalist mesopredators frequently encountered in urban and periurban areas (17), and reasons why these species were less represented are unclear. Although serologic evidence of exposure to AIVs has been documented in many wild mammal species, few experiments have investigated the susceptibility of wild mammals to these viruses, and even fewer specifically to HPAI H5N1 virus (18). Additional studies on the susceptibility of mammal species to infection with the currently circulating strains of HPAI H5N1 virus might be warranted, especially in light of the unprecedented reassortment of the Newfoundland-like virus with North American wild bird origin influenza viruses (3).

Neurologic signs were the primary clinical observation in this report, consistent with reports of HPAI H5N1 infections in mammals in Europe (7,15) and in infected birds of prey (19,20). Although widespread lesions and viral detection from multiple tissues are consistent with systemic infections in those mammals, necrotizing and nonsuppurative meningoencephalitis and acute interstitial pneumonia were the primary microscopic lesions, followed by myocardial necrosis, hepatic necrosis, and lymphoid depletion.

**Table 2.** Genotype analysis for complete sequences of H5N1 highly pathogenic avian influenza virus from mammal species, including substitutions in the PB2 gene at the consensus and subconsensus level, grouped by species, United States, 2022*

<table>
<thead>
<tr>
<th>Animal</th>
<th>A3</th>
<th>B1.1</th>
<th>B1.2</th>
<th>B2</th>
<th>B3.1</th>
<th>B3.2</th>
<th>B4</th>
<th>Minor 02</th>
<th>Minor 15</th>
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<td>6 E627K (1), D701N† (1)</td>
<td>8 E627K† (2), D701N† (1)</td>
<td>4 D701N (1)</td>
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<td>2</td>
<td>2</td>
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*Values are no. detections of PB2 substitutions. PB2, polymerase basic protein 2.
†Substitutions were detected at the PB2 subconsensus level (mixed); those without a dagger were detected at the consensus level.
Those findings are also consistent with lesion distribution in previous reports of natural and experimental HPAI virus infections in mammals (7,15,21,22) and raptors (19,20,23).

Brain and heart lesions were absent or mild in the striped skunks in this study; hepatic necrosis, lymphoid necrosis, and interstitial pneumonia were the predominant findings, corresponding to the distribution of virus as detected by IHC analysis and rRT-PCR. A similar distribution of lesions has been reported in domestic cats naturally infected with HPAI H5N1 virus (24). Therefore, the typical constellation of lesions associated with HPAI virus infection cannot be assumed to be uniform across mammalian families.

Consistent with the brain being the most frequently and strongly positive sample for rRT-PCR detection in most species in this study, 17 of 22 brains tested by IHC analysis had detectable immunoreactivity. Conversely, immunoreactivity within the lungs was rare and scattered in most species, despite the severity of gross and microscopic lung lesions. The cause of this discrepancy is unclear but might relate to early pulmonary clearance of the virus or cytokine-induced pulmonary injury, which has been reported in experimental infection trials in laboratory mammals (25).

rRT-PCR was the most sensitive method for IAV detection in this study, and there were only 4 cases in which HPAI virus was detected by rRT-PCR but tissues lacked immunoreactivity. In 2 red foxes that were either weakly positive by rRT-PCR or positive on nasal swab specimen only, lack of tissue immunoreactivity was probably caused by absence or limited amounts of HPAI virus in tissues. Detecting HPAI virus in these 2 foxes might have been clinically incidental because both foxes had concurrent infections that could have caused additional illness. Similarly, the 1 gray fox had no microscopic lesions and was weakly positive on pooled nasal and oropharyngeal swab specimens only, and this detection could have represented early or subclinical infection or simply mucosal carriage of HPAI virus. In our study, oropharyngeal swab specimens were generally more sensitive for the detection of HPAI virus in foxes than were nasal or intestinal/rectal swab specimens, consistent with higher oropharyngeal viral shedding reported in experimental infections of red foxes (21).
Most clinically ill mammals in this study died or were euthanized because of disease progression, similar to outcomes reported in other natural infections with HPAI H5N1 virus in mammals (7,15,24,26,27). However, clinical resolution of neurologic signs was documented in 1 juvenile red fox.

Adult red foxes, striped skunks, coyotes, and Virginia opossums infected with HPAI virus were not identified in this study. Reperant et al. did not observe clinical signs in 6- to 10-month-old foxes experimentally infected with HPAI virus (21). Reasons that clinical infections have been primarily restricted to young mammals are unknown but could include naive immune systems in juveniles, different exposure risks across age groups, and behavioral differences that might make ill adult mammals less likely to be encountered. Both bobcats were co-infected with parvovirus, which may have contributed to the development of clinical disease caused by HPAI virus infection in these adults.

Ingestion of birds infected with HPAI virus is presumed to be the most likely source of infection in wild mammals, and 3 red foxes in this study had evidence of bird ingestion. Wild birds, including waterfowl, are a typical or occasional component of the natural diet in those species, and infection following ingestion of HPAI virus–positive birds has been confirmed in multiple mammals (21,26–28) and in raptors and scavenging birds (19,20,23). Clear evidence of mammal-to-mammal transmission was not apparent in these cases. However, horizontal transmission of HPAI H5N1 virus has been documented in experimentally infected domestic cats (27) and ferrets (29), and transmission from an infected parent or conspecific cannot be ruled out as a potential source of infection in some of the mammals in this study, particularly within affected littersmates.

The scattered geographic and temporal distribution of the HPAI virus–infected mammals in this study suggests that these infections represent sporadic spillover events into individual animals sharing the landscape with HPAI virus–infected wild birds. This theory is supported by sequencing data from mammalian samples, confirming the presence of several different genotypes that have also been documented emerging and circulating in the United States in wild birds (3). In mammals, sustained deaths caused by HPAI H5N1 virus have thus far only been reported in seals (30), and methods of transmission responsible for the outbreak remain unclear. The PB2 gene E627K substitution that has been associated with mammal adaptation (14) and another PB2 mutation of concern D701N were identified in 10 (21%) of 48 mammals in this study, and a similar rate of mutations has been detected in HPAI virus–infected wild mammals in Canada (31). Continued vigilance is warranted as ongoing spillover of avian influenza viruses into mammalian hosts could potentially result in further reassortment or adaptation of these viruses to broader host ranges (32,33).

In summary, we demonstrate that multiple North America wild terrestrial mammal species are susceptible to natural infection with HPAI H5N1 virus of Eurasian lineage goose/Guangdong H5 clade 2.3.4.4b, probably by ingestion of infected wild birds. Neurologic signs were the primary clinical manifestation, and HPAI virus infection warrants consideration as a differential diagnosis along with more common causes of neurologic disease in wild mammals. Given the ongoing nature of the HPAI virus outbreak and the detection of genetic substitutions concerning for mammalian adaptation, surveillance for HPAI virus in wild mammals would contribute to a better understanding of the distribution and evolution of these viruses in free-ranging wildlife.

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US Geological Survey data can be found at https://doi.org/10.5066/P9KAJA8J (34).

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References


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June 2023

Poxvirus Infections

- Association of Persistent Symptoms after Lyme Neuroborreliosis and Increased Levels of Interferon-α in Blood
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Safe water and sanitation are fundamental to public health (1, 2). Breakdowns in these systems lead to disease and, in temperate and tropical climates, soil-transmitted helminthiases (STH). STH are parasitic infections adversely affecting health, particularly in children and pregnant women, by contributing to anemia and malnutrition (3). An estimated 1 billion persons are infected with STHs worldwide, largely in low- and middle-income countries (3). Although the wastewater infrastructure need in such countries is well-reported, underserved communities in the United States may also lack basic services, including effective sanitation (4–6). In 2011, the United Nations’ special rapporteur on the human right to safe drinking water and sanitation reported many failures in the United States (7), including in Alabama’s Black Belt region (8), where many households lack effective wastewater systems (9, 10). The Black Belt region, named for its rich black soils, is characterized by extreme poverty, poor health outcomes, limited healthcare access, limited economic opportunities, and other challenges (11, 12).

Necator americanus hookworms were prevalent in Alabama until the mid-1900s (13). In 1929, the highest prevalence (26%–75%) was observed in coastal counties with sandy soils, whereas counties in northern Alabama had much lower prevalence (1%–5%). A moderate prevalence was noted in the Black Belt counties (Lowndes [24%], Wilcox [44%], and Perry [45%]) (14). Large-scale public health efforts, supported by the Rockefeller Sanitary Commission and the state and local health departments, attempted to eradicate hookworm. A survey of 13 Alabama counties in 1937, and again in 1951, showed a decrease in prevalence from 37% to 17% among school age children (15). However, few systematically collected data have been available since the 1950s.

In the early 1990s, rural healthcare providers in the Alabama Black Belt continued to empirically treat children for STH, but microscopically confirmed cases of hookworm were not reported (16). Subsequently, hookworm in Alabama received limited attention until a study published in 2017 reported an analysis of 55 stool samples in which 19 (35%) were positive by qPCR for N. americanus hookworms and 4 (7.2%) were positive for Strongyloides stercoralis roundworms, from a cohort of mostly adults living with poor sanitation conditions in Lowndes County (17). Those results, combined with reports of widespread wastewater sanitation failures, raised the possibility of continued STH transmission in the southeastern United States. Our study objective was

We conducted a cross-sectional study to determine the prevalence of soil-transmitted helminthiases (STH) in areas of rural Alabama, USA, that have sanitation deficits. We enrolled 777 children; 704 submitted stool specimens and 227 a dried blood spot sample. We microscopically examined stool specimens from all 704 children by using Mini-FLOTAC for helminth eggs. We tested a subset by using molecular techniques: real-time PCR analysis for 5 STH species, TaqMan Array Cards for enteric helminths, and digital PCR for Necator americanus hookworm. We analyzed dried blood spots for Strongyloides stercoralis and Toxocara spp. roundworms by using serologic testing. Despite 12% of our cohort reporting living in homes that directly discharge untreated domestic wastewater, stool testing for STH was negative; however, 5% of dried blood spots were positive for Toxocara spp. roundworms. Survey data suggests substantial numbers of children in this region may be exposed to raw sewage, which is itself a major public health concern.

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to estimate the prevalence of STH among children in rural Alabama.

Methods

Study Design and Setting
We calculated sample size by using a prevalence range of 3%–30% on the basis of recent published reports (16,17). By using an estimated prevalence of 3% (the theoretically lowest prevalence to support ongoing transmission) (18,19) with an infinite population size and a precision of 1.5%, we determined that a sample size of 497 was needed, giving 95% binomial exact CIs of 1.7%–4.9% with 15 observed events.

We selected 3 counties in the Alabama Black Belt as the study site because of previously reported STHs in Lowndes County (17), community concerns regarding water and sanitation in Wilcox County (20), and the longstanding failure of the sewer treatment facility in Perry County (21). We invited residents of those counties to enroll their children through several recruitment strategies, including word-of-mouth by trusted community leaders, flyer distribution, and advertisements in local newspapers, social media, and radio. Any child 2–18 years of age who had resided for ≥1 year within the study region were eligible to enroll; however, we used community partners to help identify households most at risk on the basis of levels of poverty, known housing clusters without functioning sanitation, or living close to the failing sewer facility. Enrollment occurred during December 2019–August 2022.

Survey
We obtained informed consent from guardians and assent of children ≥7 years of age and administered a short paper survey. We collected demographic data, contact information, and preference for treatment by the project physician or personal physician (if an infection was found). The survey asked about possible risk factors for infections, including household sanitation type, home sewage contamination, domestic animal exposure, well-water consumption, home-grown produce consumption, international travel history, and exposure-limiting behaviors such as screen time. The survey also assessed prior treatment for STH.

Sample Collection
During December 12, 2019–March 31, 2020, we obtained finger-prick blood samples on dried blood spot cards (PerkinElmer, https://www.perkinelmer.com) that were shipped to the Centers for Disease Control and Prevention (CDC), Center for Global Health, Division of Parasitic Diseases and Malaria, for multiplex serologic antibody detection for *Strongyloides stercoralis* and *Toxocara* spp. (22). We gave families at-home stool collection kits and asked them to deliver self-collected stool specimens for shipment to the University of North Carolina Chapel Hill (UNC) for analysis. During April 1, 2020–August 10, 2022, because of the COVID-19 pandemic, we stopped collecting finger-prick blood samples and asked participants to mail the self-collected stool specimens directly to the UNC laboratory in prepaid packaging. We asked participants to collect stools from 3 separate bowel movements on separate days, then fill two 50-mL collection tubes each with 15 g of stool (1 containing 15 mL of 10% formalin and another containing 15 mL of zinc polyvinyl alcohol [Zn-PVA] [Parapak; Meridian Bioscience, https://www.meridianbioscience.com]). This method enabled preservation of stool specimens at ambient temperature for transportation to the laboratory. We offered participants monetary stipends on receipt of adequate stool specimens ($25 for the first specimen, $50 for the second, and $75 for the third).

Microscopic Analysis
Upon receiving the specimens at the UNC laboratory, we homogenized the specimens by using sterile inoculating loops (VWR, https://us.vwr.com). We stored formalin-preserved stools at ambient temperature and stored Zn-PVA–preserved stools at 4°C. Trained laboratory technicians used the mini-FLOTAC method (23) to identify and enumerate helminth eggs from formalin-preserved samples (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0751-App1.pdf). In brief, we homogenized 4 grams of the stool-formalin mixture with 36 mL of sodium nitrate (VWR) solution (specific gravity 1.25) in a fill-FLOTAC and then dispensed them into 3 mini-FLOTAC disks. After 10 minutes, we turned and read the disks at 100× magnification by using a trinocular light microscope (VWR). The theoretical limit of detection of this method was 3.3 eggs/g (24). We photographed suspected eggs by using a mounted camera (Motic, https://www.motic.com) and sent images to CDC’s DPDx telediagnosis service (https://www.cdc.gov/dpdx/index.html) for morphologic confirmation.

Molecular Analysis by TaqMan Array Card and Digital PCR
After homogenization, we extracted nucleic acids from 150 mg of selected Zn-PVA preserved stool by using the QIAamp 96 Virus QIAcube HT Kit (QIAGEN, https://www.qiagen.com), which included a pretreatment step using Precellys SK38 bead beating.
tubes (Bertin Technologies, https://www.bertin-technologies.com) (25–27). We typically extracted samples within 1–4 weeks of collection (median 15 days, interquartile range [IQR] 8–28 days, range 1–405 days); we extracted 92% of samples within 8 weeks. Among children who submitted >1 stool specimen, we randomly selected a single replicate for extraction. We randomly selected ≈5% of stools for duplicate extraction and another 3% for extraction from multiple replicates. We included ≥1 extraction-negative control (28) during each day of extractions and spiked samples with 10^4 copies of phage MS2 and 10^6 gene copies of synthetic DNA (IDT, https://www.idtdna.com) as extraction-positive controls. We stored extracts at −80°C until analysis. We assessed extracts from specimens suspected to potentially be from nonhuman sources by using digital PCR (dPCR) (QIAcuitiy 4; QIAGEN) for human mitochondrial DNA (29).

At the UNC laboratory, we analyzed nucleic acids for 7 helminths by using a custom TaqMan Array Card (TAC) on a Quantstudio 7 Flex (ThermoFisher Scientific, https://www.thermofisher.com), following the methods described in Liu et al. (30) The targets included were *Anclylostoma duodenale*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *N. americanus*, *Rodentolepsis (Hymenolepsis) nana*, *S. stercoralis*, and *Trichuris trichiura*. We prepared the TAC by combining 40 µL of template with 60 µL of AgPath-ID One-Step RT-PCR Reagents (ThermoFisher Scientific). We evaluated the TAC performance by using an 8-fold dilution series (10^0–10^8 gene copies per reaction) of an engineered combined positive control that was developed using the methods from Kodani and Winchell (31). Linearity and efficiency for the six targets were within normative standards (linearity 0.99–1.0, efficiency 95%–100%) (Appendix Tables 1, 2, Figure 1). Each day of TAC analysis, we ran ≥1 positive and negative (either an extraction-negative control or a PCR-negative control). We determined quantification cycle values by manual thresholding and included comparison of each specimen’s fluorescent signal against the daily negative and positive controls (Appendix Figure 2). We categorized any target that amplified past a quantification cycle of 35 as negative to reduce the potential for false positives (30).

In addition, we analyzed nucleic acids available from children living in Lowndes and Wilcox counties for *N. americanus* DNA by using dPCR because of its higher sensitivity (Appendix Tables 3, 4, Figure 2). We prepared reactions with QIAcuitiy Probe Mastermix (QIAGEN) by using 200 nM forward and reverse primers, 800 nM probe, and 4 µL of template. Thermocycling conditions were 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 55°C for 60 s. We included ≥1 positive and negative control on each dPCR nanoplate. We set the threshold manually between the bands of the positive and negative controls. We classified specimens with <3 positive partitions as negative (Appendix Table 4).

**Molecular Analysis by Multiparallel Quantitative PCR**

We aliquoted 2 mL of Zn-PVA stool samples into sterile cryovials, stored them at 4°C, and shipped them to CDC’s Division of Parasitic Diseases and Malaria for qPCR analysis. We removed the preservative and extracted DNA from 500 mg stool by using either DNeasy PowerSoil Kit or DNeasy PowerSoil Pro Kit (QIAGEN). Eggs were broken up through bead beating in FastPrep-24 homogenizer (MP Biomedicals, https://www.mpbio.com) for 3 min at 6.5 m/s. We performed the DNA extraction procedure in the QIAcube automated nucleic acid purification system (QIAGEN) following the manufacturer’s instructions. We quality control tested DNA extracts for presence of potential amplification inhibitors by using a human cytomegalovirus gene qPCR (32). We tested DNA samples without inhibition by using multiparallel qPCR assays specific for *N. americanus*, *A. duodenale*, *T. trichiura*, *S. stercoralis* (33), and *A. lumbricoides* (34). We performed qPCR reactions in a total volume of 25 µL, consisting of 250 nM of each primer, 125 nM of probe (Platinum Quantitative PCR SuperMix-UDG w/ROX; ThermoFisher Scientific), and 2 µL of DNA template. Each qPCR run was accompanied by positive (genomic DNA from STH worms) and negative (water and DNA extracted from STH-free feces) amplification controls. We performed the qPCR on an AriaMx Real-Time PCR System (Agilent, https://www.agilent.com) with the following cycling conditions: 50°C for 2 min, 95°C for 2 min, then 40 cycles of 95°C for 15 s and 59°C for 60 s.

**Antibody Detection for Toxocara spp. and S. stercoralis**

We performed detection of antibodies against *Toxocara* spp. and *S. stercoralis* on dried blood spots by using Luminex assay as previously described (35,36). In brief, we placed the dried blood spots in 0.25 mL of elution buffer at 4°C overnight. We allowed antibodies in the eluate to bind to recombinant antigens *T. canis* C-type lectin and 31 kDa third stage *S. stercoralis* larval antigen coupled to beads. We detected bound antibodies by using R-phycoerythrin reporter (ThermoFisher) in a MAGPIX reader with xPONENT software (ThermoFisher). We considered samples positive at >8 median fluorescence intensity for *S. stercoralis* and 23.1 median fluorescence intensity for
Toxocara spp. We determined cutoff points by testing sets of defined positive, negative, and cross-reactive serum samples and analyzing the results by receiver operating characteristics curve. We logged in data from case report forms and laboratory results into REDCap (https://www.project-redcap.org) and analyzed the data by using SAS version 9.4 (SAS Institute Inc., https://www.sas.com). We estimated combined sensitivity resulting from multiple microscopic and molecular assays by using surrogate canine hookworm (Ancylostoma caninum) (Appendix Table 5, Figures 3, 4).

Ethics Considerations
This study was approved by the institutional review boards of the University of Alabama at Birmingham (approval no. 30002219), Georgia Institute of Technology (approval no. H19021), and UNC (approval no. 20–3212). The study was reviewed by CDC and conducted consistent with applicable federal laws and policy.

Results
We enrolled 777 eligible participants from 442 unique households, representing ≥10% of the children living in the study area (Tables 1, 2; Figure 1). The higher density of enrollment overlapped with higher density of households. Of enrolled children, 93 (12%) reported living in homes with a straight pipe, discharging untreated sewage in the yard or nearby (Figure 2). A total of 227 participants submitted dried blood spot samples, and 704 participants submitted stool samples; 676 children submitted ≥3 separate stool samples. For 169 participants, we collected both blood and stool samples.

Antibody Detection on Dried Blood Spots
Of the 227 dried blood spots analyzed, 8 were inconclusive because of insufficient sample and 11 tested positive for Toxocara antibodies, resulting in a positive exposure rate of 5%. None were positive for S. stercoralis antibodies.

Control Sample Results for Molecular Detection Methods
For PCR run on the TAC platform at the UNC laboratory, the extraction-positive control consistently amplified (median cycle threshold 18), indicating no inhibition present. We observed no contamination among extraction-negative controls (n = 19) or PCR-negative controls (n = 2), and our PCR-positive controls (n = 30) exhibited the expected amplification for all targets (Appendix Table 2). We observed no contamination among any template controls (n = 16) for dPCR, and positive controls exhibited positive partitions (n = 14) (Appendix Table 4). At CDC, 11 DNA extracts (0.6%) showed amplification inhibition and were thus excluded from further testing.

Microscopic Examination and Molecular Results
We observed no STH eggs through microscopic examination on any stool sample received from the 704 eligible children who submitted stool samples to the UNC laboratory. Aliquots from samples with sufficient volume (1,803 stools from 625 children) were also tested at the CDC by multiparallel qPCR assays specific for N. americanus, A. duodenoale, T. trichura, S. stercoralis, and A. lumbricoides; all results were negative. We randomly selected a subset of samples for additional testing by 2 different molecular methods at the UNC laboratory; we analyzed 1 stool each from 488 children on TAC and 265 on dPCR. We observed E. vermicularis eggs in stool from 2 children (0.28% [2/704]) by microscopic examination and detected E. vermicularis DNA in 2 samples (0.41% [2/488]) by dPCR. We did not detect DNA from A. duodenoale, A. lumbricoides, H. nana, N. americanus, S. stercoralis, or T. trichura by using the TAC platform, and we did not detect DNA from N. americanus by using dPCR.

Combined Sensitivity
In recovery experiments using canine hookworm (Ancylostoma caninum) (Appendix), for 10% formalin at ambient temperature we observed a 0.005 log₁₀ reduction in egg count per day; for Zn-PVA at ambient temperature we observed a 0.033 log₁₀ in gene copies per day, and at 4°C we observed a 0.015 log₁₀ reduction in gene copies per day (Appendix Figures 3, 4). A 2-week gap typically occurred from sample collection to receipt at the laboratory (median 14 days, IQR 11–21 days); we extracted DNA approximately 2 weeks later (median 15 days, IQR 8–28 days), and we usually performed mini-FLOTAC within 2 weeks of receipt (median 13 days, IQR 4–28 days). The 95% limits of detection were 4.0 gene copies/µL template for the N. americanus qPCR assay and 0.43 gene copies/µL template for the dPCR assay (Appendix Figure 1). In addition, we estimated that a single undeveloped A. caninum ova on average contained 2,220 gene copies of our target sequence (Appendix Figure 4).

Considering this time-dependent reduction in targets (i.e., eggs and DNA), we calculated the estimated sensitivity by assay and the combined sensitivity for a single child shedding 1–100 eggs/g of stool (Table 3). We estimated 100% combined sensitivity to detect hookworm eggs at a concentration of 7 eggs/g (accounting for recovery), which is at the low end of a
Table 1. Characteristics of 777 participants based on self-administered surveys conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*  

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
<th>No. missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean (SD), median (range)†</td>
<td>10.6 (4.4), 11 (2–18)</td>
<td>7</td>
</tr>
<tr>
<td>Years living in current house, mean (SD), median (range)</td>
<td>8.1 (4.8), 8.0 (0–18.0)</td>
<td>18</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>393 (50.8)</td>
<td>4</td>
</tr>
<tr>
<td>M</td>
<td>380 (49.2)</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black or African American</td>
<td>734 (95.2)</td>
<td>6</td>
</tr>
<tr>
<td>White</td>
<td>21 (2.7)</td>
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</tr>
<tr>
<td>Unknown</td>
<td>2 (0.3)</td>
<td></td>
</tr>
<tr>
<td>Prefer not to answer</td>
<td>14 (1.8)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
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<td></td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>11 (1.6)</td>
<td>89</td>
</tr>
<tr>
<td>Not Hispanic or Latino</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Prefer not to answer</td>
<td>36 (5.2)</td>
<td></td>
</tr>
<tr>
<td>County of residence</td>
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<td></td>
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<tr>
<td>Wilcox</td>
<td>352 (45.3)</td>
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<tr>
<td>Lowndes</td>
<td>132 (17.0)</td>
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<tr>
<td>Perry</td>
<td>293 (37.7)</td>
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</tr>
<tr>
<td>Animals</td>
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<td></td>
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<tr>
<td>Dogs</td>
<td>331 (43.3)</td>
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</tr>
<tr>
<td>Cats</td>
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<tr>
<td>Pigs</td>
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<tr>
<td>None</td>
<td>380 (49.7)</td>
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<tr>
<td>Other (horse, chicken)</td>
<td>10 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Contact with soil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>233 (30.3)</td>
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<tr>
<td>Less than once a month</td>
<td>323 (42.0)</td>
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<tr>
<td>At least monthly</td>
<td>191 (24.8)</td>
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<tr>
<td>Not sure</td>
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<tr>
<td>Eat produce from home garden</td>
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<td>Yes</td>
<td>357 (46.7)</td>
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<tr>
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<td>407 (53.3)</td>
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<tr>
<td>Traveled outside the United States in past 5 y</td>
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<tr>
<td>Yes</td>
<td>14 (1.8)</td>
<td>13</td>
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<tr>
<td>No</td>
<td>750 (98.2)</td>
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<tr>
<td>Sewer connection</td>
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<tr>
<td>Septic tank</td>
<td>312 (40.7)</td>
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<tr>
<td>Cess pit</td>
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<td>Straight-pipe</td>
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<tr>
<td>Don’t know</td>
<td>125 (16.3)</td>
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<tr>
<td>Other</td>
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<td>Sewage contamination of property in the past year</td>
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<td>62 (8.4)</td>
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<td>No</td>
<td>680 (91.6)</td>
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<td>If yes, where was the contamination?</td>
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</tr>
<tr>
<td>Inside the house</td>
<td>13 (24.5)</td>
<td>9</td>
</tr>
<tr>
<td>In the yard</td>
<td>40 (75.5)</td>
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<tr>
<td>Payment of water bill</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>643 (83.3)</td>
<td>5</td>
</tr>
<tr>
<td>No</td>
<td>123 (15.8)</td>
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</tr>
<tr>
<td>Don’t know</td>
<td>6 (0.8)</td>
<td></td>
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<tr>
<td>Amount of screen time daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 h</td>
<td>128 (16.8)</td>
<td>13</td>
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<tr>
<td>2–4 h</td>
<td>336 (44.0)</td>
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<tr>
<td>&gt;4 h</td>
<td>300 (39.3)</td>
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<tr>
<td>Believe screen time prevents child from playing outdoors</td>
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<tr>
<td>Yes</td>
<td>179 (23.6)</td>
<td>20</td>
</tr>
<tr>
<td>No</td>
<td>578 (76.4)</td>
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<tr>
<td>No. stools received</td>
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<tr>
<td>0</td>
<td>73 (9.4)</td>
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<tr>
<td>3</td>
<td>676 (87.0)</td>
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</table>
*Values are no. (%) except as indicated.  
†Age was calculated on the basis of time between (self-reported) date of birth and date of form completion.
light infection as defined by the World Health Organization (i.e., 1–1,999 eggs/g) (37). We also estimated assay and combined sensitivity without considering recovery to demonstrate the theoretically ideal performance of our methods. Not accounting for recovery, we estimated 100% combined sensitivity at a concentration of 3 eggs/g (Table 3).

Discussion

Our survey findings confirmed that a substantial number of homes in our study region lack adequate sanitation, resulting in potential exposure of children to untreated sewage. However, we did not identify any cases of STH, a finding in contrast to McKenna et al. (17), who reported 19 cases of *N. americanus* infection and 4 cases of *S. stercoralis* infection among 55 persons in Lowndes County. They detected cases through qPCR at very low concentration by using a standard curve from a previous study, translating to an estimated mean burden of 1–2 eggs/g. Subsequent microscopic examination of specimens from 9 of the 19 positive persons by the Alabama Department of Public Health and the CDC did not detect any hookworm eggs. *Toxocara* seroprevalence was higher in our Alabama cohort than in the national study in comparable age ranges (3.0% in ages 6–11 years, 3.9% in ages 12–19 years) (38), indicating higher levels of exposure in the American Southeast, as is also demonstrated in a recent Mississippi surveillance study (22). Detection of *E. vermicularis* pinworms in stool samples from our cohort was rare.

There are several factors to consider why our results differ to the McKenna et al. (17) study. In the McKenna et al. study, participants were mostly adults and were tested in 2013. In endemic populations, the prevalence of hookworm and *S. stercoralis* threadworm increases with age (39, 40) because adult worms can live in the gut for several years (41); thus,

**Table 2.** Percentage of children enrolled, by age group and race per county population, in a study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Lowndes County</th>
<th>Perry County</th>
<th>Wilcox County</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>1.5</td>
<td>7.6</td>
<td>2.2</td>
</tr>
<tr>
<td>5–9</td>
<td>5.1</td>
<td>13.8</td>
<td>9.8</td>
</tr>
<tr>
<td>10–14</td>
<td>9.1</td>
<td>14.8</td>
<td>24.8</td>
</tr>
<tr>
<td>15–19</td>
<td>5.5</td>
<td>8.2</td>
<td>14.1</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black or African American</td>
<td>6.4</td>
<td>12.6</td>
<td>16.5</td>
</tr>
<tr>
<td>White</td>
<td>1.6</td>
<td>0.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Based on 2020 US Census data.
although residual infections were reported by McKenna et al., transmission may have since ceased. We only enrolled children because they are most at risk for adverse outcomes associated with STH infection, including anemia (42), cognitive deficits, potential growth faltering (43), and other outcomes (44). In addition, our studies used different methods for sample preservation before analysis; the McKenna et al. study processing stool stored initially on dry ice for up to 5 days, followed by storage until analysis at −20°C (time from collection until analysis not reported). Conclusive evidence on whether endemic human hookworm exists in rural Alabama would be the identification of a case according to standard diagnostic criteria (observation of ≥1 definitive hookworm eggs by microscopic examination of a stool specimen), without the possibility of having acquired the infection outside Alabama. To our knowledge, such evidence has not been demonstrated in the recent past. A review of Medicaid claims data from 2010–2018 indicated that STH infections continue to be clinically diagnosed in children in Alabama, but rarely (45). Without confirmatory stool diagnostic data, drawing conclusions regarding ongoing transmission is difficult because such diagnoses are frequently made empirically on the basis of parental reports of seeing worms in the stool. In 1991, microscopic examination of stool samples collected from children in Wilcox

Table 3. Estimated sensitivity to detect STH infection by assay method in a single infected child for different assumed intensity infections (for fecal testing methods used in a STH prevalence study conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022*).

<table>
<thead>
<tr>
<th>Egg/g feces from 1 child</th>
<th>Mini-FLOTAC, triplicate, %</th>
<th>qPCR, single, %</th>
<th>dPCR, single, %</th>
<th>Combined, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity accounting for recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>18</td>
<td>20</td>
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<tr>
<td>3</td>
<td>5</td>
<td>6</td>
<td>53</td>
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<td>5</td>
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<td>100</td>
<td>93</td>
<td>100</td>
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<td>100</td>
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<tr>
<td>Sensitivity not accounting for recovery</td>
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<tr>
<td>100</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*dPCR, digital PCR; qPCR, quantitative PCR.

Figure 2. Heat map demonstrating distribution of children enrolled living in homes with self-reported straight pipe sewage discharge in a study of soil-transmitted helminthiases conducted in Lowndes, Wilcox, and Perry Counties, Alabama, USA, December 2019–August 2022.
County identified 3 cases of *A. lumbricoides* infection out of 81 samples collected (16). The last published population-based survey using microscopic examination to identify STH eggs in stool samples in the United States found a single positive case of hookworm in a sample of 561 children 3–7 years of age in Kentucky in 1982 (46).

Sustained hookworm transmission requires 3 factors: infected persons shedding eggs; environmental conditions for eggs to mature into larvae, typically in sandy soil where temperature and moisture conditions are favorable (47); and exposure to susceptible new hosts through contact of the larvae with skin (47). In settings with endemic hookworm transmission, studies indicate that some persons within a population shed large numbers of eggs, sufficient to maintain transmission to others, whereas other persons may have moderate- or low-intensity infections (47,48). If hookworm were endemic to this region, we would expect to have identified some cases with microscopically detectable hookworm eggs. The negative results from microscopic examination were concordant with more sensitive qPCR and dPCR assays we performed on a subset of samples. In addition, we analyzed triplicate samples from 129 persons from Lowndes County, in contrast to McKenna et al. (17), who tested single samples from only 55 persons (48,49). Whereas our survey possibly could have missed isolated infections in this population, we do not consider that result likely given what is known about endemic hookworm transmission. We estimate high combined sensitivity for light infections (<100 eggs/g) in the subset of participants that was tested with all methods.

In conclusion, our study did not confirm endemic STH infection in the Alabama Black Belt. However, our survey data suggests a considerable number of children in this region may be exposed to raw sewage, which is itself a major public health concern.

### Acknowledgments

We thank our community partners, including BAMAKids Inc., West Central Alabama Community Health Improvement League, John Paul Jones Hospital, Sheryl Matthews, Ethel Johnson, Janice Robinson, Jasmine S. Kennedy, Perman Hardy, Sherry Bradley, and Sally McGhee. We thank Sydney Poulson for database management, Anastasia Hartzes for statistical analyses, Ariann Nassel for geocoding and map creation, and Matthew Purucker, Anastasia Ragland, and Qiana Butler for laboratory analysis.

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### About the Author

Dr. Poole is an assistant professor of pediatrics in the Division of Pediatric Infectious Diseases at the University of Alabama at Birmingham. Her primary research interests include infectious disease epidemiology of public health importance.

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Bacterial Infections

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- Emergence of Erythromycin-Resistant Invasive Group A Streptococcus, West Virginia, USA, 2020–2021
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Marine mammals are a diverse polyphyletic group of animals that are anatomically and physiologically adapted to an aquatic lifestyle and depend on marine ecosystems (1). The group includes members of the order Carnivora, such as pinnipeds, sea otters (**Enhydra lutris**), marine otters (**Lontra felina**), and polar bears (**Ursus maritimus**); order Sirenia, which includes manatees and dugongs (**Dugong dugon**); and the infraorder Cetacea, which comprises dolphins, whales, and porpoises (**1**, **2**). Those diverse species are considered ecosystem sentinels (**3**, **4**). The coast of Brazil sustains a high diversity of marine mammals, including 44 cetacean, 8 pinniped, and 1 sirenian species (**5**, **6**), at least 8 of which are classified into some level of extinction risk (**7**, **8**). Anthropogenic threats, such as pollution, climate change, and bycatch, severely effect marine mammal populations; however, infectious diseases also play a role (**9**). Information on marine mammal diseases in Brazil is limited, particularly in sirenians, and usually focuses on viral infections (**10**–**14**).

Mycoplasmas are a group of pleomorphic cell wall–deficient bacteria of the class Mollicutes that are considered the smallest self-replicant prokaryotes (**15**). Mycoplasmas have been detected in several mammal species, including humans (**16**–**19**). Mycoplasma spp. are wall-less bacteria able to infect mammals and are classified as hemotropic (hemoplasma) and nonhemotropic. In aquatic mammals, hemoplasma have been reported in California sea lions (**Zalophus californianus**) and river dolphins (**Inia spp**.). We investigated Mycoplasma spp. in blood samples of West Indian manatees (**Trichechus manatus**), pinnipeds (5 species), and marine cetaceans (18 species) that stranded or were undergoing rehabilitation in Brazil during 2002–2022. We detected Mycoplasma in blood of 18/130 (14.8%) cetaceans and 3/18 (16.6%) pinnipeds. All tested manatees were PCR-negative for Mycoplasma. Our findings indicate that >2 different hemoplasma species are circulating in cetaceans. The sequences from pinnipeds were similar to previously described sequences. We also detected a nonhemotropic Mycoplasma in 2 Franciscana dolphins (**Pontoporia blainvillei**) that might be associated with microscopic lesions. Because certain hemoplasmas can cause disease and death in immunosuppressed mammals, the bacteria could have conservation implications for already endangered aquatic mammals.
and hemotropic mycoplasmas (hemoplasmas) (15,20). Nonhemotropic mycoplasmas usually infect epithelial cells of the respiratory and urinary tracts and have been associated with persistent pneumonia, opportunistic nephritis, encephalopathies, autoimmune disease, and reproductive disorder in multiple species (21–23). Hemotropic mycoplasmas can adhere to erythrocyte surfaces and have been linked to acute and chronic anemia, starvation, and even death, especially when they occur along with other pathogens or in immunosuppressed or immature mammals (24). Regardless, most hemoplasma species cause subclinical infections (19,24).

Available studies have focused on domestic animals, but hemoplasmas have attracted the attention of wildlife researchers in the past decade (23). Hemoplasmas have high infection prevalence and genetic diversity, and hemoplasma DNA has been found in several wild mammals (25,26). In addition, occasional reports of interspecies transmission could indicate zoonotic potential (17,27). Despite the potential for zoonoses, few taxa have been described, and little is known about hemoplasma transmission routes and pathogenesis (19).

Among marine mammals, hemoplasmas have only been reported in pinnipeds, and a study of California sea lions (Zalophus californianus) described an infection rate of 12.4% (17/137) (28). We recently detected hemoplasma in 65.6% (32/50) of sampled river dolphins (Inia geoffrensis and I. boliviensis) from the Amazon Basin, indicating that the bacteria are also circulating in fully aquatic mammals (13). Nonhemotropic mycoplasmas have been detected in marine mammals, particularly in pinnipeds, but only a few cases have been reported in cetaceans (29–35). Of note, Mycoplasma phocicerebræ, one of the species isolated from harbor seals (Phoca vitulina) in 1988, is recognized as the likely cause of seal finger disease in humans, which is transmitted by direct contact with infected seals through bites or handling (35). Despite those reports, little information is available on hemotropic mycoplasma in marine cetaceans and sirensians worldwide or on epitheliotropic mycoplasma in cetaceans of the Southern Hemisphere. We surveyed and characterized Mycoplasma spp. in blood samples of marine mammals that were stranded or undergoing rehabilitation in Brazil during 2002–2022.

Materials and Methods

Samples
All collected samples described herein are stored in the Marine Mammal Blood and Tissue Bank of the Laboratory of Wildlife Comparative Pathology of the University of São Paulo, São Paulo, Brazil. Samples are periodically surveyed for other pathogens depending on necropsy and histopathological reports from sampled mammals.

Cetaceans
We analyzed samples from 130 marine cetaceans that stranded alive or dead or were bycaught (caught unintentionally) along the northeastern (62/130) or southeastern (68/130) coast of Brazil during 2011–2022. Samples were from animals of the families Delphinidae (n = 67), Pontoporiidae (n = 42), Kogiidae (n = 14), Balaenopteridae (n = 6), and Physeteridae (n = 1), comprising 18 different species (Table 1). Blood samples were collected from the ventral caudal peduncle in live animals or directly from the heart in dead stranded animals, placed in vacutainer tubes with EDTA, and maintained at −80°C until analysis.

Animals that stranded dead or that died during rehabilitation were necropsied according to standard procedures (36). Age class was established according to total body length (6). Selected tissue samples were fixed in 10% formalin at room temperature. An additional set of samples, including lung, spleen, cerebrum, and liver, was frozen at −80°C until analysis.

Pinnipeds
We analyzed blood samples from 18 pinnipeds that stranded alive during 2018–2022 along the northeastern (1/18) or southeastern (17/18) coast of Brazil, including 9 South American fur seals (Arctocephalus australis), 4 subantarctic fur seals (A. tropicalis), 2 Antarctic fur seals (A. gazella), 1 crabeater seal (Lobodon carcinophaga), and 1 southern elephant seal (Mirounga leonina). Blood samples were collected from the caudal gluteal or interdigital veins, placed in vacutainer tubes with EDTA, and maintained at −80°C until analysis.

Sirensians
We analyzed samples of 24 West Indian manatees (Trichechus manatus) undergoing rehabilitation along the northeastern coast of Brazil. Blood samples were collected from the ventral pectoral fin, placed in vacutainer tubes with EDTA, and maintained at −80°C until processing. Of note, those blood samples were previously screened for herpesvirus and adenovirus (14).

Ethics Approvals
This study was approved by the Ethical Committee in Animal of the School of Veterinary Medicine and
We extracted total DNA from 172 blood samples by using the DNeasy Blood & Tissue Kit (QIAGEN, https://www.qiagen.com), according to the manufacturer’s instructions. We screened samples for Mycoplasma spp. DNA by using a real time PCR protocol targeting a 384-bp fragment of the 16S rRNA gene that we adapted from a previous study (25). To molecularly characterize the bacteria, we further subjected confirmed positive samples to a nested PCR targeting a 384-bp fragment of the 16S rRNA gene that could be associated with the any of the following species: Mycoplasma fermentans, M. flavoparvum, M. hominis, M. orale, M. pneumoniae, M. genitalium, and M. hominis. We purified positive amplicons by using ExoSAP-IT PCR Product Cleanup (Affymetrix–Thermo Fisher Scientific, https://www.thermofisher.com) and GFX PCR DNA and Gel purification (Affymetrix–Thermo Fisher Scientific, https://www.thermofisher.com) and confirmed by direct Sanger sequencing in both directions. We assembled sequence reads in MEGA 7.0 (39) by using ClustalW (http://www.clustal.org/clustal2) alignment and compared sequences with those available in GenBank by searching BLASTn (https://blast.ncbi.nlm.nih.gov). We calculated nucleotide and amino acid genetic distances to the closest sequences on the basis of p-distance, after editing out the primers. Finally, we used MEGA 7.0 to construct nucleotide maximum-likelihood phylogenetic trees with a bootstrap value of 1,000 replicants and a general time-reversible plus invariant site model for 23S gene and Tamura 3 parameter with in-versions and gamma distribution model for the 16S gene (329 bp). We selected those evolutionary models by using jMODELTEST 2.1.10 (My Biosoftware, https://mybiosoftware.com). We omitted all bootstrap frequency values <70.

### Statistical Analysis

We performed statistical analyses in GraphPad Prism version 5 (GraphPad Software Inc., https://www.graphpad.com) to establish whether hemoplasma could be associated with the any of the following factors.

<table>
<thead>
<tr>
<th>Cetacean scientific name</th>
<th>Common name</th>
<th>Sex</th>
<th>M</th>
<th>Total</th>
<th>No. (%) Mycoplasma-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balaenopteridae</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Megaptera novaeangliae†</td>
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<tr>
<td>Delphinidae</td>
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</tr>
<tr>
<td>Delphinus delphis</td>
<td>Common dolphin</td>
<td>1</td>
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<td>2</td>
<td>1 (50)</td>
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<td><em>Porpoidea</em></td>
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<td>Megaptera novaeangliae†</td>
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<td>Common dolphin</td>
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<td>1</td>
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<td>1 (50)</td>
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<td><em>Porpoidea</em></td>
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<td>Common dolphin</td>
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<td>1</td>
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<td><em>Porpoidea</em></td>
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<tr>
<td>Megaptera novaeangliae†</td>
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</table>

†One animal’s sex was not determined.

Mammals were stranded or caught along the coast of Brazil during 2011–2022.
variables: host family, habitat distribution, sampling region, sampling year, age, or sex. We considered \( p \leq 0.05 \) statistically significant. We used \( \chi^2 \) test to analyze differences between sexes and Kruskal–Wallis test to analyze the remaining variables. None of the analyzed variables were statistically significant.

**Histopathologic Examination**

We analyzed available formalin-fixed samples from 2 positive cases of nonhemotropic *Mycoplasma*, a Franciscana dolphin (identification [ID] number 85) and its calf (ID173). We embedded samples in paraffin wax, processed according to routine procedures of the School of Veterinary Medicine and Animal Sciences of University of São Paulo, sectioned at 5 \( \mu \)m, and stained with hematoxylin and eosin for light microscopic examination.

**Results**

**Molecular Findings**

**Cetaceans**

We detected *Mycoplasma* DNA in the blood of 13.8% (18/130) of tested animals by using the 16S rRNA gene real-time PCR (Figure 1), including 14.9% (10/67) of Delphinidae, 35.7% (5/14) of Kogiidae, and 7.1% (3/42) of Pontoporidae (Table 1). None of the tested Balaenopteridae and Physeteridae were positive (Table 1). We compiled biologic and molecular data for the *Mycoplasma*-positive cases (Table 2).

After sequencing the 16S rRNA fragments, we were able to retrieve 1,100-bp sequences from 11 cases and 330-bp sequences from another 5 cases, comprising a total of 12 sequence types. In addition, we confirmed *Mycoplasma* in 2/18 sequences; however, the low sequence quality prevented species characterization. Among the good quality sequences, 14 *Mycoplasma* nucleotide sequences from marine dolphins in our study showed \( >98.0\% \) nt identity with the closest available sequences (GenBank accession nos. ON721294, ON721301, and ON721299), which were previously detected in blood of riverine cetacean species (15). *Mycoplasma* sequences in our study were from Guiana dolphin (*Sotalia guianensis*), pygmy sperm whale (*Kogia breviceps*), dwarf sperm whale (*Kogia sima*), pygmy killer whale (*Feresa attenuata*), common dolphin (*Delphinus delphis*), Franciscana dolphin, killer whale (*Orcinus Orca*), and rough-toothed dolphin (*Steno bredanensis*). All sequences retrieved from pygmy sperm whales were identical or very similar to sequences described in dwarf sperm whale and had only 2 single point mutations.

We also detected a shared 1,100-bp sequence type in samples from a killer whale (ID95), a rough-toothed dolphin (ID81), and a Franciscana dolphin (ID79) that was very similar to the 1 detected in a common dolphin (ID62).

One of the 330-bp sequences detected in Atlantic spotted dolphin (*Stenella frontalis*) had 92.3% nt identity with an uncultured *Mycoplasma* spp. (GenBank accession no. OL985926) detected in lowland tapir (*Tapirus terrestris*) from Brazil (Table 2), likely representing a novel hemoplasma species. Of note, 1 of the retrieved consensus sequences from blood of a Franciscana dolphin had the highest nucleotide identity (98.5%) with a *Mycoplasma* spp. sequence identified in a fecal sample from Yangtze finless porpoise (*Neophocaena phocaenoides asiaeorientalis*) from China that was not classified as hemoplasma. In addition, we detected that nonhemotropic mycoplasma in spleen, liver, and lung of the same dolphin (ID85) and in the cerebrum, spleen, and lung of its calf (ID173).

We were able to recover 23S rRNA *Mycoplasma* spp. genes from 4/18 16S rRNA real-time PCR-positive cases in a killer whale (1/1), a pygmy sperm whale (1/3), a dwarf sperm whale (1/2), and a pigmy killer whale (1/1) (Table 1). The retrieved 800-bp sequences confirmed 88.4%–90.9% nt identity with a sequence of *Candidatus Mycoplasma haemolomae* (GenBank accession no. CP003731) detected in alpacas (*Vicugna pacos*) from the United States (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0903-App1.pdf). All but 2 sequences clustered in the 16S rRNA gene phylogram with other sequences from river dolphins within the *Candidatus M. haemofelis* group (Figure 2, panel A). The sequence retrieved from Atlantic spotted dolphin clustered with other sequences of hemotropic mycoplasma within the *M. haemofelis* group, and the sequence detected in Franciscana dolphin clustered with the epitheliotropic mycoplasma *M. pneumoniae* (Figure 2, panel A). In the 23S rRNA phylogram, all cetacean species clustered together (Figure 2, panel B).

**Pinnipeds**

We detected *Mycoplasma* DNA in 3 of 18 blood samples (Figure 1), comprising 2 subantarctic fur seals and 1 Antarctic fur seal. We were able to recover two 1,100-bp sequences and one 410-bp sequence of the 16S rRNA gene and two 800-bp sequences of the 23S rRNA gene (Table 1). Phylogenetic analysis of the 16S rRNA gene showed that the retrieved sequences had confirmed (99.3%–100% nt) identity with *Mycoplasma* previously detected in California sea lions (GenBank accession no. GU124613). Both retrieved sequences
of the 23S rRNA gene confirmed (99.0% and 99.1% nt) identity with a hemotropic mycoplasma also detected in California sea lions (GenBank accession no. GU905003). On the phylogram, the retrieved sequences clustered with Candidatus M. haemophilus within the haemosuis group in both genes (Figure 2). We submitted representative sequences to GenBank (accession nos. OR183450, OR184994, OR185401–4, OR193689, OR193690, OR193698–700, OR193703, OR193728, OR193739–45).

Sirenians
We analyzed samples of 24 West Indian manatees. All manatees tested PCR negative for Mycoplasma DNA.

Histopathologic Findings
We summarized all epidemiologic and biologic data of the tested marine mammals (Appendix Table). We described anatomopathological findings of the Fransiscana dolphin and calf (ID85 and ID173) that tested positive for nonhemotropic mycoplasma (Table 3; Appendix Figure 2).

Discussion
We detected hemoplasma in cetaceans and pinnipeds in Brazil. We observed a 13.8% occurrence rate in tested cetaceans (18/130), which was lower than rates observed in river dolphins (64%; 32/50) but similar to the rates reported in California sea lions (12.4%; 17/137) (13,28). The spatial distribution of the marine cetaceans we studied is wider and less restricted than the previously studied river dolphin populations, which could affect hemoplasma circulation. Furthermore, the sampling method could have influenced the different occurrence rates among marine and riverine cetaceans. In this study, we sampled marine mammals involved in stranding events that were distributed over a broad geographic area and timeframe, and multiple species and groups were represented. Our previous study involved data collected in scientific captures concentrated in a few days and usually involving animals from the same family group and species (13).

Several previous hemoplasma studies targeted the 16S rRNA gene, a highly conserved genetic region, which could affect the identification of novel species because the gene can be undifferentiable between pathogen species (19). Despite targeting this gene, the nucleotide sequence we retrieved from an Atlantic spotted dolphin showed 92.3% nt identity with the closest sequence available, likely corresponding to a novel hemoplasma species. In the phylogram, that novel species did not cluster with the other hemoplasmas described in cetaceans to date, evidencing high divergency. The species also was classified within the *M. haemophilis* group (Figure 2), which usually is associated with higher pathogenicity. All the remaining hemoplasma sequences we retrieved from cetaceans had >98.0% nt identity with sequences of previously detected hemoplasma in river dolphins.
and clustered with those sequences. That high similarity among most of the obtained cetacean sequences likely indicates co-evolution or co-divergence of the pathogen and a common ancestry. Nevertheless, the novel sequence detected in an Atlantic spotted dolphin suggests that at least 2 different species of hemotropic mycoplasmas are circulating in cetaceans in the south Atlantic.

We detected the same 16S rRNA sequence type in 3 different species, Franciscana dolphin, killer whale, and rough-toothed dolphin, which belong to 2 different taxonomic families and the samples were collected in different regions of Brazil. That pattern diverged from the pattern we observed in river dolphins, where we could link genetic structure on the basis of host and collection site (13). That finding potentially indicates interspecies transmission of hemoplasma among marine cetaceans because those species are not geographically separated by natural barriers like the river dolphins we studied. We could only amplify the 23S gene in one of the mentioned animals, which precluded a multilocus analysis.

In pinnipeds, 16S and 23S sequences retrieved in subantarctic and Antarctic fur seals were very similar to sequences previously detected in California sea lions (>98.9% nt identity). Considering the geographic and taxonomic differences among those 3 host species, our findings could indicate a low mutation rate within this bacterium. Antarctic and subantarctic fur seals belong to the genus Arctocephalus and both have reproductive colonies near the Antarctic convergence in the Southern Hemisphere, and dispersal to the Northern Hemisphere has not been reported (5). By contrast, California sea lions are of the genus Zalophus, breed in coastal and offshore islands of California (USA) in the Northern Hemisphere, and disperse farther north after breeding season (40). Thus, the species are unlikely to have natural encounters.

We detected a nonhemotropic mycoplasma in blood and available frozen lung, spleen, and liver tissues from a Franciscana dolphin and frozen brain, lung, and spleen tissues from its calf, indicating systemic infection. Nonhemotropic mycoplasmas have already been detected in other marine mammals, including cetaceans, and were mainly associated with 2 species of hemoplasma.

Table 2. Biologic and molecular data from mammals in a study of molecular detection and characterization of Mycoplasma spp. in marine mammals, Brazil*  

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Species</th>
<th>Age class/sex</th>
<th>Year stranded</th>
<th>16S rRNA sequence length, bp</th>
<th>Accession no.†</th>
<th>% nt identity</th>
<th>Closest available sequence†</th>
<th>Species of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID1</td>
<td><em>Kogia breviceps</em></td>
<td>Adult/M</td>
<td>2017</td>
<td>1,100</td>
<td>OR193701</td>
<td>98.9</td>
<td>ON712194</td>
<td><em>Inia geoffrensis</em></td>
</tr>
<tr>
<td>ID13</td>
<td><em>Kogia breviceps</em></td>
<td>Adult/F</td>
<td>2019</td>
<td>1,100</td>
<td>OR193699</td>
<td>98.9</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID14</td>
<td><em>Stenella coeruleoalba</em></td>
<td>Adult/F</td>
<td>2019</td>
<td>Inadequate sequence</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ID15</td>
<td><em>Kogia breviceps</em></td>
<td>Adult/M</td>
<td>2020</td>
<td>1,100</td>
<td>OR193701</td>
<td>98.9</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID16</td>
<td><em>Kogia sima</em></td>
<td>Adult/M</td>
<td>2020</td>
<td>1,100</td>
<td>OR193703</td>
<td>98.7</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID19</td>
<td><em>Stenella frontalis</em></td>
<td>Adult/F</td>
<td>2020</td>
<td>330</td>
<td>OR184994</td>
<td>92.3</td>
<td>OL985926</td>
<td>Tapirus terrestris</td>
</tr>
<tr>
<td>ID26</td>
<td><em>Kogia breviceps</em></td>
<td>Adult/F</td>
<td>2020</td>
<td>1,100</td>
<td>OR193701</td>
<td>98.7</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID29</td>
<td><em>Stenella frontalis</em></td>
<td>Adult/F</td>
<td>2021</td>
<td>Inadequate sequence</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
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<td>ID34</td>
<td><em>Feresa attenuata</em></td>
<td>Adult/M</td>
<td>2015</td>
<td>1,100</td>
<td>OR193702</td>
<td>98.0</td>
<td>ON712194</td>
<td><em>Inia boliviensis</em></td>
</tr>
<tr>
<td>ID62</td>
<td><em>Delphinus delphis</em></td>
<td>Juvenile/M</td>
<td>2021</td>
<td>1,100</td>
<td>OR193698</td>
<td>98.4</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID63</td>
<td><em>Sotalia guianensis</em></td>
<td>Juvenile/F</td>
<td>2021</td>
<td>330</td>
<td>OR193744</td>
<td>98.6</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID79</td>
<td><em>Pontoporia blainvillii</em></td>
<td>Calf/M</td>
<td>2019</td>
<td>1,100</td>
<td>OR193700</td>
<td>98.5</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID81</td>
<td><em>Steno bredanensis</em></td>
<td>Adult/M</td>
<td>2019</td>
<td>1,100</td>
<td>OR193727</td>
<td>98.5</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID85</td>
<td><em>Pontoporia blainvillii</em></td>
<td>Adult/F</td>
<td>2018</td>
<td>1,100</td>
<td>OR183450</td>
<td>98.5</td>
<td>JN792314</td>
<td>Neophocaena phocaenoides asiaeorientalis</td>
</tr>
<tr>
<td>ID95</td>
<td><em>Orcinus orca</em></td>
<td>Juvenile/F</td>
<td>2020</td>
<td>1,100</td>
<td>OR193728</td>
<td>98.5</td>
<td>ON712194</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID119</td>
<td><em>Sotalia guianensis</em></td>
<td>Juvenile/M</td>
<td>2022</td>
<td>330</td>
<td>OR193742</td>
<td>100</td>
<td>ON712199</td>
<td><em>I. geoffrensis</em></td>
</tr>
<tr>
<td>ID122</td>
<td><em>Pontoporia blainvillii</em></td>
<td>Calf/F</td>
<td>2022</td>
<td>330</td>
<td>OR193741</td>
<td>99.7</td>
<td>ON712194</td>
<td><em>I. boliviensis</em></td>
</tr>
<tr>
<td>ID126</td>
<td><em>Sotalia guianensis</em></td>
<td>Juvenile/M</td>
<td>2022</td>
<td>330</td>
<td>OR193743</td>
<td>98.6</td>
<td>ON712194</td>
<td><em>I. boliviensis</em></td>
</tr>
<tr>
<td>ID131</td>
<td><em>Arctocephalus tropicalis</em></td>
<td>Adult/M</td>
<td>2020</td>
<td>1,100</td>
<td>OR193739</td>
<td>100</td>
<td>GU124613</td>
<td>Zalophus californianus</td>
</tr>
<tr>
<td>ID132</td>
<td><em>Arctocephalus tropicalis</em></td>
<td>Adult/F</td>
<td>2020</td>
<td>1,100</td>
<td>OR193740</td>
<td>99.3</td>
<td>GU124613</td>
<td><em>Z. californianus</em></td>
</tr>
<tr>
<td>ID144</td>
<td><em>Arctocephalus tropicalis</em></td>
<td>Juvenile/M</td>
<td>2021</td>
<td>410</td>
<td>OR193745</td>
<td>99.3</td>
<td>GU124613</td>
<td><em>Z. californianus</em></td>
</tr>
</tbody>
</table>

* Mammals were stranded or caught along the coast of Brazil during 2011–2022. bp, base pairs; ID, identification; NA, not applicable. †From GenBank.
Figure 2. Maximum-likelihood phylograms from a study of molecular detection and characterization of Mycoplasma spp. in marine mammals, Brazil. A) 16S rRNA gene (329-bp) phylogram based on Tamura 3-parameter with inversions and gamma distribution model. Mycoplasma pneumoniae was selected as outgroup. B) 23S rRNA gene (820-bp) phylogram based on a general time-reversible model with invariant sites. Bacillus subtilis was selected as outgroup. Trees show alignment of Mycoplasma spp. consensus sequences obtained in marine mammals from this study (pink dots and bold text) and other hemotropic mycoplasmas retrieved from GenBank. Reliability of the phylograms was tested by 1,000 replicate bootstrap analyses omitting values <70. Trees generated by using MEGA 7.0 (https://www.megasoftware.net).
pneumonia and septic polyarthritis (29–35). Despite those detections, little information is available about the pathologic signature of these bacteria because the descriptions are usually linked with co-infections, like avian influenza virus or parasites, that could influence the observed lesions (29,31,41,42). In our study, both dolphins had mild to moderate granulocytic pneumonia with exfoliation of macrophages and pneumocytes in alveoli (Appendix Figure 2). Extensive pulmonary lesions, including pneumonia with granulocytes in the alveoli (43,44), have already been described, suggesting that the lesions observed in our study could be associated with mycoplasma infection. Furthermore, the brain of the calf tested positive for *Mycoplasma* spp. and demonstrated lesions compatible with neuronal suffering (e.g., neuronophagia). Detection of nonhemotropic mycoplasma in brain has been described in multiple mammals, including humans, seals, cattle, and sheep, and has been recorded mainly in nurseries (i.e., human infants and calves) (23), as observed in this investigation. In sheep, evidence of transmammary transmission and development of encephalitis in the infected lambs has been described (23). Despite isolation from the brains of harbor seals in an epizootic mortality episode in the North Sea 30 years ago, the role of mycoplasmas in central nervous system infections has not been clarified (29). Previous reports of nonhemotropic mycoplasmas in cetaceans were all in species distributed in the Northern Hemisphere, and detection was from lungs, nasopharynx, liver, preputium, and atlantooccipital joints (31,33,34).

We did not find statistically significantly higher hemoplasma occurrence in adults than in juveniles or calves, which differs from what we observed in Amazon (*Inia geoffrensis*) and Bolivian (*I. boliviensis*) river dolphins (13). Nevertheless, we detected hemoplasma in blood sampled over the years. Thus, endemnicity of detected hemoplasmas in those populations cannot yet be determined. As we observed in Amazonian manatees (*Trichechus inunguis*) (13), all the West Indian manatees tested herein were PCR negative for mycoplasma. Manatees, unlike cetaceans and pinnipeds, are herbivorous and shed different endoparasites than the other 2 groups, which could explain the lack of mycoplasma-positive manatees (45). Nevertheless, most of the tested mammals had previously stranded as neonates or calves; therefore, they would have had little contact with adult manatees that could be a source of infection.

Some of the species selected for this study are threatened and experiencing decreasing population trends. The Franciscana dolphin and the West Indian manatee are currently classified as vulnerable by the International Union for Conservation of Nature’s Red List of Threatened Species (7). Certain hemoplasma species can cause disease and even death in immunosuppressed mammals, so those pathogens could

### Table 3. Main pathologic findings of 2 bycaught Franciscana dolphins (*Pontoporia blainvillei*) positive for nonhemotropic mycoplasma in a study of molecular detection and characterization of *Mycoplasma* spp. in marine mammals, Brazil

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Gross findings</th>
<th>Microscopic findings</th>
</tr>
</thead>
</table>

*ID, identification.
potential have conservation implications in aquatic mammals. That observation is especially crucial when considering that aquatic mammals are facing diverse anthropogenic and natural threats, such as aquatic pollution, climate change, and anthropization, which are capable of affecting their immune status and increasing disease susceptibility (46).

In conclusion, we detected Mycoplasma DNA in blood samples of marine cetaceans and pinnipeds in Brazil. Our findings indicate that at least 2 divergent species of hemotropic mycoplasma are circulating in cetaceans. In addition, we detected a nonhemotropic mycoplasma in Franciscana dolphins. The Mycoplasma sequences retrieved from pinnipeds were very similar to sequences previously described in California sea lions. Mycoplasmas were not detected in any of the tested sirenians. Our findings demonstrate a wider host range of hemotropic mycoplasma in cetaceans and pinnipeds and expand epitheliotropic mycoplasmas to cetaceans of the Southern Hemisphere, reinforcing the presence of those bacteria in aquatic mammals under natural conditions. The interspecies transmission, zoonotic potential and pathogenicity of all mycoplasmas detected should prompt additional serosurveys to elucidate the range and possible implications of Mycoplasma infections for marine mammals, especially endangered species.

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Marine mammals that stranded along the São Paulo coast were rescued by the Santos Basin Beach Monitoring Project (PMP-BS/ABIO 640/215). PMP-BS is a project required by the Brazilian Institute of the Environment and Renewable Natural Resources of the Brazilian Ministry of Environment for the environmental licensing process of the oil and natural gas production and transport by Petrobras at the Santos Basin. The objective of this project is to assess the possible impacts of oil production and flow activities on birds, sea turtles, and marine mammals, through monitoring of beaches and veterinary care for live animals and necropsy of animals found dead. The project is carried out from Laguna, in Santa Catarina, to Saquarema, in Rio de Janeiro, and is divided into 15 sections. Instituto de Pesquisas Cananéia monitors the Section 7, comprising Ilha do Cardoso, Ilha Comprida, and Juréia (Iguape), Biopesca institute monitors the Section 8, between Perube and Praia Grande, and Argonauta para Conservação Marinha e Costeira institute monitors the Section 10, between São Sebastião and Ubatuba.

About the Author
Dr. Duarte-Benvenuto is a veterinarian and a doctorate student at the Laboratory of Wildlife Comparative Pathology in University of São Paulo, São Paulo, Brazil. Her primary research interest is wildlife disease and conservation, especially of aquatic mammals.

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April 2023

Vectorborne Infections

• Challenges in Forecasting Antimicrobial Resistance

• Pediatric Invasive Meningococcal Disease, Auckland, New Zealand (Aotearoa), 2004–2020

• Bacterial Agents Detected in 418 Ticks Removed from Humans during 2014–2021, France

• Association of Scrub Typhus in Children with Acute Encephalitis Syndrome and Meningoencephalitis, Southern India

• Nocardia pseudobrasiliensis Co-infection in SARS-CoV-2 Patients

• Monitoring Temporal Changes in SARS-CoV-2 Spike Antibody Levels and Variant-Specific Risk for Infection, Dominican Republic, March 2021–August 2022

• Extensive Spread of SARS-CoV-2 Delta Variant among Vaccinated Persons during 7-Day River Cruise, the Netherlands

• Adeno-Associated Virus 2 and Human Adenovirus F41 in Wastewater during Outbreak of Severe Acute Hepatitis in Children, Ireland

• Outbreaks of SARS-CoV-2 Infections in Nursing Homes during Periods of Delta and Omicron Predominance, United States, July 2021–March 2022

• Effectiveness of BNT162b2 Vaccine against Omicron Variant Infection among Children 5–11 Years of Age, Israel

• Monkeypox Virus Infection in 2 Female Travelers Returning to Vietnam from Dubai, United Arab Emirates, 2022

• Experimental Infection and Transmission of SARS-CoV-2 Delta and Omicron Variants among Beagle Dogs

• Highly Pathogenic Avian Influenza A(H5N1) Virus Outbreak in New England Seals, United States

• Emergence and Persistent Dominance of SARS-CoV-2 Omicron BA.2.3.7 Variant, Taiwan

• Mapping Global Bushmeat Activities to Improve Zoonotic Spillover Surveillance by Using Geospatial Modeling

• Yezo Virus Infection in Tick-Bitten Patient and Ticks, Northeastern China

• Effects of Seasonal Conditions on Abundance of Malaria Vector Anopheles stephensi Mosquitoes, Djibouti, 2018–2021

• Tularemia in Pregnant Woman, Serbia, 2018

• Ocular Trematodiasis in Children, Sri Lanka

• Serial Intervals and Incubation Periods of SARS-CoV-2 Omicron and Delta Variants, Singapore

• Serial Interval and Incubation Period Estimates of Monkeypox Virus Infection in 12 Jurisdictions, United States, May–August 2022

• Two-Year Cohort Study of SARS-CoV-2, Verona, Italy, 2020–2022

• Chikungunya Outbreak in Country with Multiple Vectorborne Diseases, Djibouti, 2019–2020

• Blackwater Fever Treated with Steroids in Nonimmune Patient, Italy

To revisit the April 2023 issue, go to: https://wwwnc.cdc.gov/eid/articles/issue/29/4/table-of-contents
Newcastle disease is a common and highly contagious zoonotic infection affecting wild and domestic birds globally; it is caused by virulent strains of avian paramyxovirus type 1 (APMV-1) (1,2). APMV-1 is a negative-sense, single-stranded RNA virus belonging to the Orthoavulavirus genus, Paramyxoviridae family (1). Strains of this virus are categorized into 2 classes, I and II, on the basis of their fusion protein sequence (1). Substantial variation in virulence exists among strains of APMV-1; class I strains are classically avirulent, whereas class II strains can be avirulent or virulent. Class II genotype VI strains of APMV-1, known as pigeon avian paramyxovirus type 1 (PPMV-1), commonly have high pathogenicity; pigeons and doves are reservoirs (3,4).

Newcastle disease in birds can manifest with fatal central nervous system (CNS), respiratory, and digestive diseases, depending on tropism of the particular strain (2). Whereas AMPV-1 primarily affects birds, the virus can infect nonavian hosts, including primates (humans, monkeys), rabbits, and pigs (1). In humans, cases reported have typically manifested as mild conjunctivitis, although fatal cases have been reported (1). We report a fatal case of neurologic infection caused by PPMV-1 in a child in Australia.

**Case Report**

A 2-year-old child with a history of infantile pre-B cell acute lymphoblastic leukemia (ALL) was admitted to hospital with nausea and vomiting after 3 weeks of upper respiratory tract symptoms. As part of the Associazione Italiana di Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Münster Acute Lymphoblastic Leukemia (AIEOP-BFM ALL) clinical trial, of Clinical Pathology and Medical Research, Westmead (A.R. Howard-Jones, J. Kok); Kids Cancer Centre, Sydney Children’s Hospital, Randwick (A. Anazodo); University of New South Wales Faculty of Medicine and Health, School of Clinical Medicine, Sydney (B. McMullan, K. Kim); Prince of Wales Hospital and Community Health Services, Sydney (W. Rawlinson); University of New South Wales Schools of Clinical Medicine, Biotechnology and Biomolecular Sciences, Sydney (W. Rawlinson)

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1These first authors contributed equally to this article.

2These senior authors contributed equally to this article.
she had been randomized to receive blinatumomab, a bispecific T-cell–engaging antibody targeting CD19-expressing B cells; her last dose was 6 months before admission (5). The patient had completed her second cycle of reinduction chemotherapy with cytarabine, 6-mercaptopurine, and cyclophosphamide 6 weeks before she was brought for care. No recent travel, pets, or unwell contacts were reported. Her condition progressed over 4 days; she became febrile and experienced sudden-onset superrefractory status epilepticus in the setting of fever. The clinical syndrome was consistent with febrile infection-related epilepsy syndrome (FIRES) (6).

Results of initial cerebral magnetic resonance imaging (MRI) were unremarkable. Investigations for autoimmune causes of encephalitis all yielded negative results, including testing for antibodies to myelin oligodendrocyte glycoprotein, aquaporin 4, N-methyl-d-aspartate (NMDA) receptor, contactin-associated protein-like 2 (CASPR2), leucine-rich glioma-inactivated 1 (LGI-1), gamma aminobutyric acid-B (GABA-B), dipeptidyl-peptidase-like protein 6 (DPPX), and immunoglobulin-like cell adhesion molecule 5 (IgLON5). Neurotropin was elevated in cerebrospinal fluid (CSF) at 1,752 nmol/L (reference range 6–30 nmol/L), representing significant CNS inflammation. Ultra-rapid exome sequencing performed by Acute Care Genomics, a tool that enables diagnosis of genetic disorders within 5 days, yielded no genetic abnormalities (7). Results of extensive culture and PCR-based investigations for infectious causes, including bacterial, viral, fungal, and mycobacterial pathogens, were all negative.

Haematoxylin and eosin (H&E) staining of brain biopsy tissue performed 20 days after admission demonstrated almost complete cortical necrosis with some subpial sparing. The cortex was replaced by sheets of foamy macrophages with some gliosis and scattered, predominantly interstitial, CD3-positive T cells. Only rare residual small neurons were identified in the superficial cortex with NeuN immunostaining. No microglial nodules, viral inclusions, or viral cytopathic effects were apparent (Figure 1, A).

Figure 1. Histology of brain tissue and imaging from a fatal neurologic infection in an immunocompromised child in Australia that was caused by APMV-1. A) Brain biopsy showing extensive cortical necrosis with a dense infiltrate of macrophages. Hematoxylin and eosin stain; original magnification ×20. B) Immunohistochemistry of brain biopsy showing cytoplasmic APMV-1 nucleoprotein, probably in neurons, with axon-like processes (arrowheads). Original magnification ×20 (inset ×40). C) Immunohistochemistry of APMV-1 nucleoprotein, demonstrating the absence of immunolabeling in normal brain tissue. APMV, avian paramyxovirus.

Figure 2. Magnetic resonance imaging of the brain of an immunocompromised child with avian paramyxovirus type 1 infection, Australia. Image, captured 16 days after hospital admission, shows predominantly left frontal and insular T2 signal hyperintensity evolving into laminar necrosis (white arrow) and hyperintensity of deep gray-matter structures (red arrows).
Panel A). No viral pathogens were present in a range of samples, including CSF, plasma, and brain tissue; relevant negative results were for HHV6, BK virus, SARS-CoV-2, parvovirus B19, herpes simplex viruses (HSV-1, HSV-2), varicella zoster virus, cytomegalovirus, Epstein-Barr virus, parainfluenza virus, adenovirus, human metapneumovirus, enterovirus, rhinovirus, bocavirus, coronavirus, and JC virus. Culture results for bacterial and fungal pathogens on those samples, panmycobacterial PCR, and 16S ribosomal RNA PCR performed on brain tissue were all negative.

Despite therapy with broad-spectrum antimicrobial drugs, antiseizure medications, immunomodulators, and a ketogenic diet, the patient’s condition did not improve. Over the course of 2 weeks, serial cerebral MRI showed progressive and widespread inflammatory changes with increasing left frontal and insular T2 signal hyperintensity evolving into laminar necrosis as well as T2 hyperintensity of deep gray-matter structures (Figure 2). Treatment was withdrawn, and the child died 27 days after admission. A postmortem examination was not performed.

We conducted comprehensive, hypothesis-free metagenomic testing of the biopsied brain tissue using established panviral hybridization-capture (8) and unbiased metatranscriptomic (9) sequencing methods performed in parallel. After sequence quality filtering, host nucleic acid removal, and alignment to the National Center for Biotechnology Information nonredundant/nucleotide database, both approaches independently identified APMV-1 as the dominant nonhuman sequences. Hybrid capture methods identified 39,928 APMV-1 sequences using Twist Biosciences viral panel (https://www.twistbioscience.com/resources/product-sheet/twist-pan-viral-panel) and 281,601 sequences by in-house methods. The only other nonhuman sequences of note included low levels (<230 reads) of human pegivirus (HPgV) sequences identified using both methods, as previously identified in similar studies (data not shown).

We performed de novo assembly of the nonhuman sequences from the brain metatranscriptomic library, which yielded a complete APMV-1 genome with a mean read depth of 2,568×. Comparative analysis of potential virulence markers identified evidence of P-gene editing and a polybasic F gene cleavage site, suggestive of a virulent (velogenic) strain (Figure 3). The avian paramyxovirus detected in this case was classified as a type-1 APMV species using a phylogenetic approach based on the L-protein sequence, in accordance with International Committee on Taxonomy of Viruses guidelines (10). Specifically, we aligned the complete large (L) protein sequences of reference and case viruses using MAFFT version 7 (https://mafft.cbrc.jp/alignment/
Fatal Infection Caused by Avian Paramyxovirus-1

software) before phylogenetic analysis with PhyML version 3.3.20180214 (www.atgc-montpellier.fr/phyml) using the Le-Gascuel (LG) protein substitution model (Figure 4). An analysis of the F gene (Figure 4) indicated the virus belonged to a putative Australian lineage of the pigeon variant, PPMV-1, belonging to class II, genotype VI, sublineage 2.1.1.2.2 (Figure 5). The complete genome sequence was submitted to GenBank (accession no. OR636618).

APMV-1 infection of the brain tissue was confirmed using virus-specific real-time quantitative PCR assays (data not shown) and immunohistochemistry at the Australian Centre for Disease Preparedness. The APMV-1 nucleoprotein identified using immunohistochemistry was present in occasional clusters within cells in the brain tissue. At least some of those small pyramidal cells appeared to be neurons with an axon-like process (Figure 1, panel B). No staining was identified in appropriate negative controls, which were healthy juvenile brain and lymphoid (tonsil) tissue (Figure 1, panel C).

Discussion

The first documented case of APMV-1 infection in humans was reported in 1942 in Australia (1). Since then, an additional 485 human cases have been reported globally, most (288 cases) in the United Kingdom (1). Most infections have been mild and self-limiting, commonly presenting as conjunctivitis (1). There have been 4 recorded human deaths, all caused by the PPMV-1 strain, in the Netherlands, United States, China, and France (1–3,11); 1 case-patient who died had a clear exposure to pigeons (11). Case-patients in the Netherlands, United States, and China all experienced respiratory symptoms and died from respiratory failure (3,11). Two of those case-patients were immunosuppressed with an underlying hematologic malignancy.

Figure 5. Phylogeny of avian paramyxovirus type 1 strain from an immunocompromised child in Australia (bold). Tree was prepared from MAFFT-aligned fusion gene sequences of genotype VI sublineage 2.1.1.2.2 strains (classified as pigeon avian paramyxovirus 1) using PhyML with the Hasegawa-Kishino-Yano + gamma DNA substitution model and rooted with a genotype VI sublineage 2.1.1.2.1 outgroup (HM063425/Pigeon/CHN/P4). Red dot indicates virus from this case; GenBank accession numbers are provided for reference sequences. Gray box indicates the branch containing the virus we identified, expanded to show detail. Colored circles at tips indicate country of sampling. The virus from our study appears to be related to viruses circulating in Australia since at least 2011. Node support values show Shimodaira-Hasegawa—like approximate likelihood ratio test statistics; branch lengths are proportional to the scale of the number of substitutions per site. Scale bars indicate number of substitutions per site. All strains used in this analysis are listed in the Appendix (https://wwwnc.cdc.gov/EID/article/29/12/23-0250-App1.pdf).
Combined immunodeficiency (1,3). The fourth, with marked similarities to the case we describe, involved a young girl who died following the sudden onset of progressive seizures, 3 months after hematopoietic stem cell transplantation for combined immunodeficiency (2). Those cases highlight the virulence of PPMV-1 and the potential for severe disease in contrast to other APMV-1 genotypes.

Although we detected a small number of HPgV reads (<230 reads), there is no clear association between HPgV and human disease, nor evidence of neurotropism. Because HPgV has a reported seroprevalence of up to 20% in the human population, this finding most likely represents passive transport into CSF (9). In the absence of other co-infecting viral, bacterial, or fungal pathogens, or other major organ pathology such as pneumonia, the child’s death was most likely caused by encephalitis from overwhelming PPMV-1 CNS infection.

In birds, APMV-1 infects respiratory, neural, lymphoid, and other tissues, causing a range of signs that are partially dependent on the viral strain. In the human case we report, it is likely that the PPMV-1 infection began in the upper respiratory tract before the onset of FIRES and spread to the CNS, given the history of respiratory signs. Although no exposure was identified, it is likely the virus was transmitted inadvertently via direct contact with pigeon feces or infected fluids. The virus is known to remain stable in pigeon feces and can be spread by windborne dust, extending the risk beyond just localized environments (3). In the absence of seroprevalence data of PPMV-1 in humans, the relationship between exposure and development of disease remains unknown and worthy of further investigation.

The term FIRES refers to an epileptic encephalopathy characterized by intractable seizures after a febrile illness. It is diagnosed in the absence of infectious encephalitis or a defined trigger and most commonly affects otherwise healthy children and young adults (6). FIRES has a high rate of sequelae; up to two thirds of surviving patients experience some degree of cognitive impairment. Severity ranges from mild intellectual disability to vegetative states; almost all patients have refractory seizures (6). The mortality rate is not insignificant, 10% in the acute phase and 13% in chronic phase (6). In cases in which a cause is identified, autoimmune pathologies predominate, followed by cerebral viral infection. An association between avian viruses and FIRES could overcome that limitation (13). The ability to test samples for such a breadth of organisms also conserves sample volume for difficult-to-obtain sample types such as cerebral tissue. From a laboratory workflow perspective, this approach could simplify diagnostics, avoiding the need to develop, validate and verify individual assays for each novel pathogen.

Incorporating metagenomic sequencing into routine diagnostics has several barriers and challenges. Metagenomic sequencing is expensive. High-throughput sequencing requires highly skilled staff, including bioinformaticians with expertise in data analysis (15); however, the use of robust cloud-based metagenomic sequence classification software tools could overcome that limitation (16). The potential for low-cost, robust testing with reduced local bioinformatic expertise requirements may enable lower-income and middle-income countries to use metagenomics as a diagnostic method, which would provide tertiary-level diagnostic abilities in a greater range of clinical settings. Care in handling samples is of utmost importance to avoid contamination and nucleic acid degradation that can affect the interpretation of results (15). As with conventional PCR, results should be interpreted in the appropriate clinical context.
(15). Incorporating pathogen-agnostic metagenomic testing into routine laboratory workflows improves laboratory diagnostics and our response to novel emerging disease threats, including emergent zoonoses. Improved diagnosis and monitoring of conditions with unknown etiology, such as FIRES, using metagenomic analyses has the potential to uncover novel and emergent pathogens, which expands the breadth of infectious-disease diagnosis. Such advances will also increase the potential for future targeted treatments and improved outcomes for children and adults with neurologic infections.

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Japanese encephalitis (JE) is associated with an immense social and economic burden. Published cost-of-illness data are primarily from decades-old studies. To determine the cost of care for patients with acute JE and initial and long-term sequelae from the societal perspective, we recruited patients with laboratory-confirmed JE from the past 10 years of JE surveillance in Bangladesh and categorized them as acute care, initial sequelae, and long-term sequelae patients. Among 157 patients, we categorized 55 as acute, 65 as initial sequelae (53 as both categories), and 90 as long-term sequelae. The average (median) societal cost of an acute JE episode was US $929 ($909), of initial sequelae US $75 ($33), and of long-term sequelae US $47 ($14). Most families perceived the effect of JE on their well-being to be extreme and had sustained debt for JE expenses. Our data about the high cost of JE can be used by decision makers in Bangladesh.

Japanese encephalitis (JE) is among the most common viral causes of encephalitis in the world; an estimated 67,900 new cases of JE occur annually in JE-endemic countries, and case-fatality rates are 20%–30% (1,2). Among JE survivors, 30%–50% experience long-term neuropsychological sequelae (1,3,4). Clinical signs/symptoms develop in only ≈1 of 250 JE patients (5,6). JE transmission is a risk in 24 countries, totaling 3 billion persons at risk (7). JE is associated with immense social and financial burden because of the severe neuropsychiatric sequelae and the need for physical and cognitive therapy throughout the patient’s lifetime (4). Previous economic evaluations of JE have examined the cost of illness for acute JE and sequelae in Cambodia, China, India, Indonesia, and Nepal. However, few of those studies assessed how the cost of sequelae care changes with level of sequelae severity and over time (8–11). Only the study in Nepal explored how the economic burden of JE sequelae care differed by level of disability; however, the cost data reflected only sequelae care provided within 12 months after hospital discharge (11). To estimate the annual cost of sequelae care across a longer period, another study in China surveyed caregivers of JE patients with sequelae the first year after hospital discharge, but that study did not examine how those costs varied with the sequelae severity (10). In addition, published cost-of-illness data for acute JE and sequelae come primarily from studies conducted >1 decade ago and, given improvements during this time may not reflect the current standard of care in hospitals, rehabilitation facilities, and other settings.

The first JE outbreak in Bangladesh was reported from the central part of the country in 1977 (12). Not until 2003–2005 was a systematic hospital-based study conducted in 4 hospitals to assess the JE...
Materials and Methods

IRB) approved the study protocol. Institutional Review Board and Copernicus group (WCG-Review Committee of icddr,b and the Western Institute (for children) included in the study. The Ethical formed consent from all participants or their guard-

15 mating a population mean (\( \bar{\mu} \)) to calculate the target sample size formula for esti-

\[ n = \frac{Z^2 \sigma^2}{\delta^2} \]

We used the standard sample size formula for estimating a population mean \((\bar{\mu})\) to calculate the target sample size (Appendix, https://wwwnc.cdc.gov/EID/article/20/12/23-0594-App1.pdf). By setting precision at ±10% of the mean cost, setting coefficient of variation of 0.5, and considering the estimated 195 annual JE patients/year since 2011 and the Bangla-
desh case-fatality rate of 10% (13,16), we estimated a total sample size of 229: 64 patients for the acute group, 75 patients for the initial sequelae group, and 90 patients for the long-term sequelae group. Because of the small number of JE patients identified during the COVID-19 pandemic (November 2019–December 2021), to achieve the target sample size we adminis-
tered acute and initial sequelae questionnaires to enrolled participants.

Sequelae Assessment

The team used the Liverpool Outcome Score (LOS) (17), a tool to measure outcome after encephalitis, to screen for JE sequelae. The LOS had a total of 15 questions divided into 2 sections. During the first section, administered by telephone, we asked the parent/caregiver 10 questions, after which we recruited pa-
tients experiencing some level of sequelae to partici-
pate in the study, as indicated by an outcome score of <5 in the first LOS section. During the second sec-
tion, to obtain total LOS outcome score we visited the households to observe the patient performing simple motor tasks. For acute patients, we assessed severity of illness by their level of consciousness. We categor-
ized patient condition as conscious (mild illness), altered consciousness (moderate illness), or uncon-
scious (severe illness).

Data Collection Procedure

We collected primary and secondary data. During primary data collection, we interviewed consenting caregivers or patients with regard to socioeconomic status, costs of care, strategies for responding to JE-related expenses, and effect on the family. During secondary data collection, from consenting partici-
pants and with hospital approval, we extracted direct medical costs from the patient files, main order book, and register books of the hospitals where the patients received care.

Cost Estimates

We measured direct medical, nonmedical, and indirect costs. Direct medical costs included medicines (prescription and over-the-counter drugs, homeopathics, traditional medicines), diagnostics, procedures, and interventions (in hospital and from other provid-
ers such as acupuncturists and physical therapists), facility/provider fees (registration, consultation,
hospital bed), and other informal payment. For direct medical costs, we included informal payments because most informal payments were made for various direct medical services (e.g., payments to nurses and cleaning and ward support staff for help with availing a wheelchair or a bed at the hospital). Direct nonmedical costs included travel, lodging, meals, and other miscellaneous costs. Indirect costs were measured by household income lost while providing care for patients and accompanying them to healthcare facilities. We collected information for each day of income lost by patients and their household members while seeking healthcare facilities and providers and while caring for the patients. We estimated the household income lost by multiplying the number of total working days missed by the patient and the caregivers because of the disease and the income that they had to forgo each day. We also collected information about missed school days by patients and other household members; we did not monetize that cost but rather reported it as effect on the family’s overall well-being. For acute patients, we collected the cost of prehospitalization, hospitalization, discharge, and follow-up visits up to 90 days after discharge. For
sequelae patients, we collected costs for JE-related care in the previous 90 days.

We considered the healthcare system perspective and the household perspective together representing a societal perspective. Healthcare system costs were direct medical costs covered by the healthcare system. Costs from the household perspective included direct medical costs and direct nonmedical costs that were paid out-of-pocket by the patients and their families. Indirect costs were also borne by the households. The societal costs included all the aforementioned costs and were paid by the household along with the government healthcare system.

Data Analyses
We used descriptive statistics to analyze costs by patient age group, patient sex, patient wealth quintile, and illness severity. We calculated the costs by multiplying the quantity of the items by unit costs, which were charges from providers or payment made by the families. Costs for patients in the acute group were the average total costs for the acute episode, and costs for those in the initial sequelae and long-term sequelae groups were average monthly costs. We calculated the proportion of expenditure for JE sequelae to household income by dividing monthly costs for sequelae by household monthly income, multiplied by 100 for a patient. All costs were collected in Bangladesh Taka (BDT) and converted to US dollars. The conversion rate was adjusted to reference year 2021, and the exchange rate was 85.08 BDT to 1 US dollar (18). To assess the group differences with a 5% level of significance (i.e., p<0.05), we performed the Mann-Whitney U-test for binary group variables and the Kruskal-Wallis test for polytomous group variables.

Results
We collected data for 3 study groups from a total of 157 patients. The acute care group consisted of 55 patients, initial sequelae 65 patients, and long-term sequelae 90 patients; 53 patients were considered for acute and initial care costs and received both the acute and initial sequelae questionnaires. The average duration of initial sequelae care was 9.24 (median 6.41) months and for long-term sequelae care was 62.29 (median 64.17) months.

Most patients were <18 years of age (Table 2). More patients were male than female. Average monthly income was US $171–$193 and for more than half the families in all groups was US $118–$353 (Table 2).

Societal Costs
The average societal cost of an acute JE episode was US $929 (median [SE] US $909 [$68]) (Table 3). The percentage of total costs for patients with acute JE was 40% (US $370) for direct medical costs and 38% (US $351) for indirect costs. For initial sequelae patients, the average monthly total cost was US $75 (median [SE] US $33 [$13]), and for long-term sequelae patients, US $47 (median [SE] US $14 [$8]). For initial sequelae and long-term sequelae patients, indirect cost was the highest and was incurred for 60% (39/65) of the initial sequelae patients and 53% (48/90) of the long-term sequelae patients (Table 3). Drug and diagnostic costs were the most common—and the highest direct medical cost—for acute patients. However, drug and consultation/registration fees were low but most common for initial and long-term sequelae care. Costs for procedures/intervention (physical therapy) were highest for the initial sequelae group but were incurred for only 6% (4/65) of the initial sequelae patients. Medical equipment (braces, wheelchairs, sticks) was most costly for long-term sequelae patients, and costs were incurred for 11% (10/90) of those patients (Table 3). A total of 5% of the initial sequelae patients and 7% of the long-term sequelae patients reported that they stopped seeking care for issues associated with JE illness and consequences.

When study participants were analyzed by age, sex, illness severity, and wealth, the cost of JE was significantly higher (p = 0.011) among male than female patients (Table 4). For acute JE care, the cost increased with severity levels, although not significantly (p = 0.064). For initial and long-term JE sequelae care, the cost for patients with mild illness was significantly less than for those with moderate and severe sequelae (p = 0.038 for initial group and p = 0.035 for long-term group). The costs did not...
significantly differ among age groups and wealth categories, except for the long-term sequelae among those 41–50 years of age, which may be affected by the small number of cases (n = 5).

**Household Costs**
The average household cost of an acute JE episode was US $825 (median [SE] US $797 [64]), which accounts for 89% (825/929) of the total cost of ill-

### Table 2. Sociodemographic characteristics of patients with acute onset, initial sequelae, and long-term sequelae of Japanese encephalitis, Bangladesh, November 2011–December 2021

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Acute, n = 55</th>
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<th>Long-term sequelae, n = 90</th>
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<tr>
<td>F</td>
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<td><strong>Sex</strong></td>
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<tr>
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<td>37 (1)</td>
<td>40 (1)</td>
</tr>
<tr>
<td>Age group, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–30</td>
<td>16 (29)</td>
<td>20 (31)</td>
<td>18 (20)</td>
</tr>
<tr>
<td>31–40</td>
<td>21 (38)</td>
<td>25 (38)</td>
<td>34 (38)</td>
</tr>
<tr>
<td>41–50</td>
<td>12 (22)</td>
<td>12 (18)</td>
<td>24 (27)</td>
</tr>
<tr>
<td>51–60</td>
<td>4 (7)</td>
<td>7 (11)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>2 (4)</td>
<td>1 (2)</td>
<td>6 (7)</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>12 (22)</td>
<td>15 (23)</td>
<td>23 (26)</td>
</tr>
<tr>
<td>Primary school</td>
<td>9 (16)</td>
<td>14 (22)</td>
<td>23 (26)</td>
</tr>
<tr>
<td>Secondary school</td>
<td>24 (44)</td>
<td>18 (28)</td>
<td>23 (26)</td>
</tr>
<tr>
<td>Higher secondary school</td>
<td>8 (15)</td>
<td>15 (23)</td>
<td>11 (12)</td>
</tr>
<tr>
<td>Some college or technical school training</td>
<td>0</td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Bachelor’s degree or higher</td>
<td>2 (4)</td>
<td>3 (5)</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

*Values are no. (%) patients except as indicated. All monetary amounts are in US dollars. NA, not applicable (other groups located in urban and rural areas only.)

†Odd US$ are used in income categories because they were based on Bangladesh currency.
The average health system cost of an acute JE episode was US $106 (median [SE] $66 [$14]), which accounts for 11% (106/929) of the total societal cost (Table 3). The average healthcare system costs of sequelae care were US $6 per month (median [SE] US $3 [$3]) for the initial sequelae group and US $5 per month (median [SE] US $3 [$3]) for the long-term sequelae group (Table 3).

**Coping Strategies**
Most families reported that they coped with the expenses of the illness by using savings and then borrowing money (Table 5). For expenses during the acute phase, families that used savings spent an average (SE) of US $403 ($75). Those who sought a loan required an average (SE) of US $734 ($112), not accounting for interest. Those who sold household assets used an average (SE) of US $683 ($146) to cov-
er expenses. The economic burden was experienced not only during the acute stage but also during the sequelae stages because most families used savings and one third took a loan for monthly JE-related expenses. To cover expenses, the families also received donations from relatives, other household members, friends, charity, and local community or government leaders.

Most (82%, 53/65) respondents reported that they had sustained debt from borrowing during the acute phase of the illness (Table 5). The average monthly payment for sustained debt was US $96 for the initial sequelae group and US $74 for the long-term group. Borrowing from relatives was most common during the acute phase, and a bank was the common source during the sequelae phase (Table 3). Families most commonly identified their plan to repay debt as working extra hours.

### Effects on the Family’s Overall Well-Being

Most families of acute and sequelae patients reported that the effects were extreme, particularly for expenses as well as stress and fear associated with the disease (Table 6). An extreme effect on missed school was reported by 49% of the acute, 43% of the initial, and 42% of the long-term sequelae patients (Table 6). Altogether, the patients and caregivers of acute patients missed 92 school days during the entire episode. On the other hand, 26% of the initial patients (19 days/mo) and 19% of the long-term patients (21 days/mo) missed school days (Table 6).

### Discussion

We provide an estimate of the economic burden of JE in Bangladesh. The average societal cost of an acute JE episode estimated from 55 patients was US $929 (median US $909), and the average household cost
The average monthly household cost of long-term sequelae care, estimated from 90 patients, was US $47 (median US $14). Although during the acute phase, approximately one tenth of the cost was borne by the healthcare system, almost all costs during the sequelae phase were borne by the households. The monthly cost for sequelae care was measured over 3 months and then assumed to remain constant over the rest of the patient’s life. The monthly costs were then aggregated over the long term and created substantial economic burden for the families.

Similar to findings of a study conducted in India (19), our study also noted that cost per case for sequelae patients decreases over time. In addition, similar to findings of another study (20), we found that among acute patients, direct costs were higher than indirect costs, which might be explained by direct medical costs consisting of medication, diagnoses, and other procedures. On the other hand, similar to findings of another study in China (10), we found that among sequelae patients, indirect costs were higher than direct medical costs, which might result from more wage loss because the patient and the caregiver were unable to be involved in income-generating activities.

More than half of the JE patients in Bangladesh were children (2,14). The cost of a child disabled for life by JE is a heavy burden for the families. For a patient who recovers completely, the treatment is expensive in terms of financial as well as productivity loss of the child’s caregiver. Similar to findings of other studies (20), our study found a higher cost for male than female patients. Those findings were consistent within the context of Bangladesh because discrimination against women exists for health-associated behavior (21). In Bangladesh, male household members are usually prioritized for healthcare-seeking practices (21,22). In low-income countries, a household usually spends 2%-5% of its income on healthcare (23). In our study, most of the cost was borne by the households for acute (89%), initial (99%), and long-term (100%) sequelae care, which is similar to findings from a separate study (24). Furthermore, for patients with JE sequelae, an average of 44%-56% of the household monthly income was spent on healthcare, indicating a catastrophic expenditure for the families. In 2017, the illness cost borne by households (out-of-pocket expenditure) was 74% of the total health expenditure in Bangladesh (25). During the same year, 3.3% of the population of Bangladesh was pushed into poverty because of out-of-pocket health expenditures (26).

Values are no. (%) patients except as indicated. Missed school days were reported as total for acute care patients and monthly for sequelae patients.

### Table 6. Effects of on acute care, initial sequelae, and long-term sequelae of Japanese encephalitis patients on family’s overall well-being, Bangladesh, November 2011–December 2021

<table>
<thead>
<tr>
<th>Level of effect</th>
<th>Acute, no. (%)</th>
<th>Initial sequelae, no. (%)</th>
<th>Long-term sequelae, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expenses associated with the disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Little</td>
<td>3 (5)</td>
<td>4 (6)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Moderate</td>
<td>7 (13)</td>
<td>6 (9)</td>
<td>10 (11)</td>
</tr>
<tr>
<td>Extreme</td>
<td>44 (80)</td>
<td>54 (83)</td>
<td>78 (87)</td>
</tr>
<tr>
<td><strong>Income of members in the household</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (5)</td>
<td>5 (8)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Little</td>
<td>4 (7)</td>
<td>4 (6)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Moderate</td>
<td>8 (15)</td>
<td>13 (20)</td>
<td>12 (13)</td>
</tr>
<tr>
<td>Extreme</td>
<td>40 (73)</td>
<td>43 (66)</td>
<td>70 (78)</td>
</tr>
<tr>
<td><strong>Missed school because of illness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient’s missed school (d missed)</td>
<td>20 (80)</td>
<td>16 (20)</td>
<td>15 (22)</td>
</tr>
<tr>
<td>caregivers’ missed school (d missed)</td>
<td>6 (116)</td>
<td>1 (1)</td>
<td>4 (10)</td>
</tr>
<tr>
<td>Total missed school, no. (d missed)</td>
<td>25 (92)</td>
<td>17 (19)</td>
<td>17 (21)</td>
</tr>
<tr>
<td>None</td>
<td>25 (45)</td>
<td>32 (49)</td>
<td>38 (42)</td>
</tr>
<tr>
<td>Little</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>6 (7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>2 (4)</td>
<td>4 (6)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Extreme</td>
<td>27 (49)</td>
<td>28 (43)</td>
<td>38 (42)</td>
</tr>
<tr>
<td><strong>Stress and fear associated with the disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1 (2)</td>
<td>0</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Little</td>
<td>1 (2)</td>
<td>2 (3)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>5 (9)</td>
<td>8 (12)</td>
<td>11 (12)</td>
</tr>
<tr>
<td>Extreme</td>
<td>48 (87)</td>
<td>55 (85)</td>
<td>71 (79)</td>
</tr>
</tbody>
</table>

*Values are no. (%) patients except as indicated. Missed school days were reported as total for acute care patients and monthly for sequelae patients.
sequelae patients stopped seeking treatment for JE. The level of expenditure of JE, from acute phase to prolonged treatment, often forces households to use savings, sell assets (e.g., livestock), work extra hours, borrow money, and continue to pay high debts, thus leading to impoverishment (29). That downward spiral of assets and income loss may lead some families into abject poverty and subsequently perpetuate the cycles of generational poverty (30). Moreover, the extreme effects on psychosocial health may also cause different illnesses among family members and may affect the health and well-being of the overall family.

Among our study limitations, recall bias was a concern for collecting cost data from participants by using a micro-costing approach (most precise assessment). We tried to limit recall bias in several ways (e.g., extracting direct medical costs from hospital records rather than asking participants, reporting cost/visit rather than cost/episode). In addition, to reduce recall bias, we used a 90-day retrospective reference period for the initial and long-term sequelae costs, as recommended in the literature (31). Out of respect for the families, we did not include cases that ended in death; thus, cost of death is out of the scope of our study. Although not being able to find the required number of patients for acute- and initial-phase cost data collection limited our generalizability, we enrolled all the available JE patients for those 2 phases.

Our findings are useful for providing information about the magnitude of economic burden and the effects of JE among the affected families in Bangladesh. In addition, our findings can be used with regard to JE prevention, which plays a crucial role in alleviating the burden of the disease. The most effective and sustainable way to prevent JE in humans is vaccination. Updated evidence on the cost of JE illness, and on initial and long-term rehabilitation and sequelae care by severity, will further underscore the value of JE vaccination and can be used for national decision-making for JE vaccine introduction and sustainability. Our findings that the average societal cost of an acute JE episode was US $929, average monthly cost of initial sequelae was US $75, and average monthly cost of long-term sequelae was US $47 provide an economic estimation that can be used for policy decisions with regard to vaccine introduction in Bangladesh.

Acknowledgment

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About the Author

Dr. Sultana is a medical anthropologist and health economist. She specializes in infectious disease research, implementation research, and outbreak investigation. Her particular interests include epidemiology and emerging and re-emerging infectious diseases, including zoonotic emerging diseases in Bangladesh.

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Detection of Anopheles stephensi Mosquitoes by Molecular Surveillance, Kenya

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The Anopheles stephensi mosquito is a major vector of malaria in south Asia, the Middle East, and southern China, where it is endemic and is known to transmit both Plasmodium falciparum and P. vivax. This mosquito differs from other malaria vectors because of its ability to grow and reproduce in human-made containers in clean or contaminated water. Those traits have enabled An. stephensi mosquitoes to colonize urban settings, in addition to their native rural foci, where they can potentially sustain malaria transmission (1).

The Anopheles stephensi mosquito was first reported in Djibouti in the Horn of Africa in 2012 (2). Since then, it has been reported in multiple urban and rural settings in Ethiopia, Sudan, Somalia, Nigeria, and Ghana (3–6) and could be responsible for sustaining malaria transmission in Ethiopia. The species has the potential to increase P. falciparum incidence by 50% according to recent mathematical modeling (7,8), as has been observed in Djibouti (9).

An. stephensi mosquitoes could spread south and west from their original foci of detection in the Horn of Africa, as has been observed in Nigeria (4) and Ghana (6). This vector has the potential to establish or increase transmission in urban settings where the malaria burden is generally lower than in rural settings, particularly in areas where poorly planned drainage and waste disposal systems create conducive larval habitats (10). The behavior of adult mosquitoes in their invasive range in Africa is not well understood.

The Anopheles stephensi mosquito is an invasive malaria vector recently reported in Djibouti, Ethiopia, Sudan, Somalia, Nigeria, and Ghana. The World Health Organization has called on countries in Africa to increase surveillance efforts to detect and report this vector and institute appropriate and effective control mechanisms. In Kenya, the Division of National Malaria Program conducted entomological surveillance in counties at risk for An. stephensi mosquito invasion. In addition, the Kenya Medical Research Institute conducted molecular surveillance of all sampled Anopheles mosquitoes from other studies to identify An. stephensi mosquitoes. We report the detection and confirmation of An. stephensi mosquitoes in Marsabit and Turkana Counties by using endpoint PCR and morphological and sequence identification. We demonstrate the urgent need for intensified entomological surveillance in all areas at risk for An. stephensi mosquito invasion, to clarify its occurrence and distribution and develop tailored approaches to prevent further spread.

Author affiliations: Kenya Medical Research Institute, Nairobi, Kenya (E.O. Ochomo, S. Milanoi, B. Abong’o, B. Onyango, M. Muchoki, D. Omoke, D. Matoke-Muhia, L. Kamau, J. Mwangangi, M. Maia, M.K. Rono, C. Mbogo); PMI Kinga Malaria Project, Abt Associates Inc., Kisumu, Kenya (E. Olanga); National Museums of Kenya, Nairobi (L. Njoroge); Pan-African Mosquito Control Association, Nairobi (E.O. Juma, D. Matoke-Muhia, C. Mbogo); World Health Organization, Kenya Country Office, Nairobi (J.D. Otieno); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C. Rafferty, J.E. Gimnig); US President’s Malaria Initiative, Washington, DC, USA (C. Rafferty, J.E. Gimnig, M. Shieshia, D. Wacira); University of Oxford, Oxford, UK (M. Maia); KEMRI-Wellcome Trust, Kilifi, Kenya (M. Maia, M.K. Rono); Division of National Malaria Program, Ministry of Health, Nairobi (C. Chege, A. Omar); Moi University, Eldoret (W.P. O’Meara, A. Obala); AMPATH, Eldoret, Kenya (L. Abel); Duke University, Durham, North Carolina, USA (W.P. O’Meara, L. Kariuki)

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especially as they continue to colonize new areas in the continent, but their spread has been predicted using modeling (10).

The World Health Organization recently called for heightened surveillance and development of response strategies to limit the spread of this vector in Africa (4). The initiative highlights 5 key focus areas: increased collaboration across sectors and borders, strengthening surveillance, improving information exchange, developing national guidelines, and prioritizing research to evaluate tools against this vector.

In Kenya, the Division of National Malaria Program (DNMP) at the Ministry of Health and its partners have been on high alert and instituted surveillance efforts after the World Health Organization initiative (4). Surveillance efforts have been focused along the Kenya coast and the northern counties bordering Sudan and Ethiopia. Current surveillance efforts are aimed at the collection of both larval and adult mosquito samples. Samples collected are identified using morphological keys and PCR at the reference laboratories located at the Kenya Medical Research Institute (KEMRI). Here we detail the process that led to detecting and identifying An. stephensi mosquitoes in Kenya.

Methods

Surveillance Sites

The DNMP and its partners collected mosquitoes in 14 counties in December 2022 as part of routine surveillance. Counties where DNMP supported vector surveillance in December 2022 were categorized as malaria endemic (Kilifi, Taita, and Taveta), highland epidemic prone (Elgeyo Marakwet, West Pokot, Kisii, and Nandi), low risk (Garissa, Makueni, Kajiado, Kirinyaga, and Laikipia), or seasonal (Marsabit, Baringo and Turkana) (Figure 1). For the purpose of this work, we present results for Marsabit and Turkana Counties, where samples were collected, identified, and confirmed to be An. stephensi mosquitoes. Marsabit and Turkana are neighboring counties in northern Kenya, located on either side of Lake Turkana. Marsabit County borders Ethiopia to the north, Turkana County to the west, Samburu County to the south, and Wajir and Isiolo Counties to the east. Turkana County borders Uganda to the west, South Sudan to the north, and Ethiopia to the northeast. Directly east lies Lake Turkana, and Marsabit lies just beyond. The counties lie 300–900 meters above sea level.

Mosquito sampling in Marsabit was conducted in the subcounties of Moyale, Laisamis, and Saku and focused on urban and rural settings along the northern transport corridor connecting Kenya and Ethiopia (Table 1). Sampling in Turkana focused on Lodwar, the capital of the county and a major town on the land transport corridor into Kenya. The main economic activities of the rural population are nomadic pastoralism because of the semiarid terrain; urban trade centers are set up along the northern transport corridor. Urban trade centers were the focus of the sampling efforts.

Sampling

We conducted mosquito sampling in Marsabit for adult and larval samples. We collected adult mosquitoes using US Centers for Disease Control and Prevention (CDC) light traps and collected larvae by dipping. We set CDC light traps overnight indoors, next to a person sleeping under a bednet, or outside, 10 m from the structure without regard for the presence of animals, between 6 PM and 7 PM and collected them the next morning between 7 AM and 8 AM. In addition, we dipped for larvae in animal watering pens, containers, tires, and other standing water in the area (Figure 2). We collected Anopheles larvae and placed them in whirlpacks for transportation to the entomology laboratory at KEMRI for additional assays. The mosquitoes were reared in the infection room; the room was equipped with a triple door and curtains at the entrance and sealed windows to prevent escapes. Surviving larvae were reared to adults for morphologic identification using standard conditions (25 ± 2°C; 80% ± 4% relative humidity; 12 h/12 h light/dark cycle). We fed larvae on Tetramin baby fish food and brewer’s yeast daily and maintained adults on 10% sugar solution.

In Turkana, larval sampling focused on water pans near the seasonal river and cement water cisterns. We visited 11 suspected larval sites throughout the town every 2 weeks and dipped 5 times at each site to quantify larval density. We separated Anopheles larvae and placed them in tubes with 95% ethanol for shipment to the PEARL laboratory in Webuye. Collections occurred during November–December 2022.

Molecular Characterization

We isolated DNA from 55 mosquito carcasses (either whole or legs and wings) consisting of field-collected larvae or laboratory-reared F1 adults from larvae collected in Marsabit using the ethanol precipitation method (11) in the KEMRI Kisumu laboratory. We conducted amplification using an endpoint PCR assay that included 3 primers: St-F (5'-CGTATCTTTCTCGCATCCA-3'), an An. stephensi-specific forward primer targeting the internal transcribed spacer
(ITS2) region; U5.8S-F (5′-ATCACTCGGCTCATG-GATCG-3′), a universal forward primer flanking the conserved 5.8S rDNA region; and UD2-R (5′-GCAC-TATCAAGCAACACGACT-3′), a universal reverse primer flanking the conserved D2 domain of 28S rDNA (12). We performed the reactions using 0.15 µL of the DNA template alongside a positive control with the following set of cycling conditions: 95°C for 5 min, followed by 95°C for 30 s, 55°C for 30 s and 68°C for 45 s for 30 cycles, and a final extension at 68°C for 7 min. Thereafter, we ran 15 µL of each of the PCR products on 2% agarose gel alongside 3 µL of a 100-bp DNA ladder for size comparison. We visualized the products in the gel documentation system for an expected amplicon size of ≈438bp. This visualization was the primary method of identification given the relative inexperience of the laboratory teams in morphological identification of An. stephensi mosquitoes.

Samples collected in Turkana were processed at the AMPATH Laboratories in Eldoret, Kenya. We rinsed field-collected larval samples preserved in ethanol with nuclease-free water and pooled in groups of 3 from the same breeding site. We extracted triads in a single well of a 96-well plate using the Hotshot protocol, performed amplification using a previously published protocol (13) with corrected primer sequences, and visualized reactions on 2% agarose gels. If a band of the expected size was observed, we separated the larvae in the pool and extracted them individually using the DNeasy Blood and Tissue Kit (QIAGEN, https://www.qiagen.com), after which we repeated amplification and electrophoresis as
Detection of Anopheles stephensi Mosquitoes, Kenya

We subsequently sequenced positive samples as described in the following section.

Morphologic Identification and Sequencing

We taxonomically identified emerging adults that were a subset of the larvae collected in Marsabit using the keys described by Coetzee et al. (14) to detect the distinct banding on the maxillary palps, pale scales on the scutum, and the 3 dark spots on wing vein 1A (Figure 3). We randomly selected 4 adult specimens that were a subset of the samples from Marsabit identified as An. stephensi mosquitoes by morphology and shipped them to CDC (Atlanta, GA, USA), where DNA from a single mosquito leg was extracted using the Extracta DNA Prep for PCR kit (Quantabio, https://www.quantabio.com).

We performed amplification targeting the ITS2 (as previously described) and the cytochrome c oxidase subunit 1 gene (CO1) locus. For CO1 amplification, we used specific LCO1490F (5'-GTTCAACAAATCATAAAGATATTGG-3′) and HCO2198R (5′-TAACTTCAGGTGACCAAAAAATCA-3′) primers (15). The PCR cycling conditions included an initial step at 95°C for 1 min, then 30 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min. We ran Amplitcons for both ITS2 and CO1 on a 2% agarose for confirmation, then used the positive PCR products for Sanger sequencing. We performed BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) homology searches of both ITS2 and CO1 sequences using the default parameters to confirm the matching species.

![Figure 2. Habitats where Anopheles stephensi mosquitoes were collected, Kenya. A) Animal watering pan in Marsabit County; B) disposed containers containing standing water in Marsabit County; C) old tire in Marsabit County; D) seasonal river pan in Turkana County. Persons pictured gave consent for their photographs to be published in this article.](image-url)
We sequenced 4 larval samples from Lodwar after the ITS2 band was puriﬁed from the agarose gel at the KEMRI Wellcome Trust (Kilifi, Kenya). We constructed sequencing libraries using Oxford Nanopore Technologies Ligation Sequencing Kit and multiplexed samples using the Native Barcoding Expansion Kit (https://nanoporetech.com). We performed adaptor ligation on the barcoded amplicon pool and the ﬁnal library loaded on a SpotON R9.4.1 ﬂow cell and sequenced on the GridION (Oxford Nanopore Technologies).

Using SPADES assembler (16), we performed de novo assembly on the ﬁltered reads. We performed species identiﬁcation through a BLAST search using ITS2 sequences from GenBank as the subject database and the assembled contigs as the query dataset.

Phylogenetic Analyses
We constructed phylogenetic trees for both CO1 and ITS2 sequences by incorporating sequences from diverse isolates retrieved from GenBank along with isolates from Kenya. We used MAFFT software version 7.520 (17,18) for all sequence alignments and reconstructed maximum-likelihood phylogenies using the Bayesian Information Criterion with general time-reversible (GTR) as the best substitution model as inferred by jModelTest in IQ-TREE version 2.0.7 (19,20). We performed tree visualization using MEGA version 11 (21) and took the bootstrap consensus tree inferred from 1,000 replicates to reliably show the evolutionary history.

Results

Molecular Surveillance Results
We collected Anopheles larvae from 11 locations in 3 subcounties in the 2 counties (Table 1). In Marsabit, a total of 59 larvae were collected. Of those, 11 died in transit and were immediately prepared for PCR identiﬁcation using the An. stephensi protocol; 7 were conﬁrmed as An. stephensi mosquitoes (Table 2). We pooled the 48 remaining larvae by subcounty to rear adult samples. Of the 12 samples that emerged, we identiﬁed 9 adults by morphology (Figure 3). We correctly identiﬁed 7 of the 9 samples as An. stephensi mosquitoes, which were later conﬁrmed by PCR through ITS2 ampliﬁcation (Table 2). The other 2 were identiﬁed as An. gambiae mosquitoes by morphology but were conﬁrmed to be An. stephensi mosquitoes by PCR. We shipped 4 of those samples to the CDC for sequencing as described previously; 36 samples did not amplify using An. stephensi, An. gambiae, or An. funestus PCR protocols and are the subject of further investigation. We did not conduct morphologic identiﬁcation on those samples before DNA extraction; the samples will be sequenced to determine species at a later date. No adult mosquitoes were collected in light traps. In summary, of the 59 mosquito samples tested by PCR from collections in Marsabit, 23 were conﬁrmed to be An. stephensi mosquitoes.

Of the 9 sites monitored in Lodwar town during November 8–December 22, 2022, two had only culicine larvae and 7 had Anopheles larvae. A total of 1,415 larvae were collected and screened by PCR; 1,218 were collected from river pans, 50 from cisterns, 147 from drainage ditches, and the remaining from other sources. Two pooled extracts from river pans on the Turkwel River screened positive for An. stephensi. We separated, extracted, and retested 5 larvae; 5 were conﬁrmed to be An. stephensi mosquitoes.

Sequencing
The sequences for 3 of the 4 adult samples matched CO1 isolates from GenBank and were conﬁrmed as An. stephensi mosquitoes (Figure 4). One sample failed

Table 2. Numbers of mosquito larvae or adults used for PCR to identify Anopheles stephensi mosquitoes, Kenya*

<table>
<thead>
<tr>
<th>Subcounty</th>
<th>Total Anopheles larvae collected</th>
<th>Larvae used for PCR</th>
<th>Adults used for PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample size</td>
<td>An. stephensi</td>
<td>Unamplified</td>
</tr>
<tr>
<td>Saku</td>
<td>42</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Laisamis</td>
<td>17</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Moyale</td>
<td>51</td>
<td>51</td>
<td>2</td>
</tr>
<tr>
<td>Turkana Central†</td>
<td>193</td>
<td>193</td>
<td>5</td>
</tr>
</tbody>
</table>

*Unampliﬁed samples are those that failed to amplify with the An. stephensi, An. gambiae, and An. funestus PCR protocols. NA, not available.
†Including only a single site that was positive for An. stephensi. Five larvae were shipped to KEMRI-Wellcome Trust for sequencing.
to amplify, possibly because of DNA degradation (Table 3). BLAST searches using default parameters for isolates 2 and 3 matched to *An. stephensi* sequences with 100% identity to the hap 10 5.8S ribosomal RNA gene, ITS2; isolate 1 had 99.4% identity to the same gene but 100% identity to the *An. stephensi* isolate 141 steph 5.8S ribosomal RNA gene, ITS2. However, when we focused on the CO1 genes in BLAST searches, we found that isolates 1 and 2 exhibited a striking similarity of 100% (isolate 1) and 99.7% (isolate 2) to *An. stephensi* isolate SM147. Conversely, isolate 3 displayed a substantial 99% identity to *An. stephensi* isolate ANST15 (Table 3).

In-depth phylogenetic analyses of the CO1 sequences from Kenya isolates 1 and 2 demonstrated a close relationship with sequences from Ethiopia isolates; isolate 3 exhibited a close association with sequences from India (Figure 5). On the other hand, phylogenetic analysis of sequenced isolates with other isolates of ITS2 for *An. stephensi* available in GenBank demonstrated that the isolates from Marsabit and Turkana matched quite closely; however, because ITS2 is a nuclear marker, it was not used to infer relatedness. The isolates closely matched the Iraq, India, Yemen, and Nigeria isolates (Figure 6). The *An. stephensi* sequences from

---

**Table 3.** Results for sequencing analysis of 4 mosquito samples from Marsabit County, Kenya, sent to CDC for identification in study of *Anopheles stephensi* mosquito, Kenya

<table>
<thead>
<tr>
<th>Origin sample ID</th>
<th>CDC sample ID</th>
<th>Morphologic ID</th>
<th>Confirmed species</th>
<th>ITS2 GenBank accession no.</th>
<th>% Identity match</th>
<th>CO1 GenBank accession no.</th>
<th>% Identity match</th>
</tr>
</thead>
<tbody>
<tr>
<td>KE83GY</td>
<td>2023KEN00001</td>
<td>Suspected <em>An. stephensi</em></td>
<td><em>An. stephensi</em></td>
<td>OQ275144 FJ526599.1</td>
<td>99.40</td>
<td>OR607949</td>
<td>100</td>
</tr>
<tr>
<td>KE83QF</td>
<td>2023KEN00002</td>
<td>Suspected <em>An. stephensi</em></td>
<td><em>An. stephensi</em></td>
<td>OQ275145 MW732931.1</td>
<td>100</td>
<td>OR607950</td>
<td>99.70</td>
</tr>
<tr>
<td>KE83FZ</td>
<td>2023KEN00003</td>
<td>Suspected <em>An. stephensi</em></td>
<td><em>An. stephensi</em></td>
<td>OQ275146 MW732931.1</td>
<td>100</td>
<td>OR607951</td>
<td>99.10</td>
</tr>
<tr>
<td>KE83I1</td>
<td>2023KEN00004</td>
<td>Suspected <em>An. stephensi</em></td>
<td>Did not amplify</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
this study have been uploaded to GenBank (ac-
cession nos. OQ275144, OQ275145, and OQ275146
[ITS2 sequences from Marsabit]; OQ878216,
OQ878217, and OQ878218 [sequences from Turka-
na]; and OR607949, OR607950, and OR607951 [CO1
sequences from Marsabit]).

**Discussion**

We report collection and detection of *An. stephensi*
mosquitoes from Marsabit and Turkana Counties
in northern Kenya. From the samples collected, we
used multiple methods for identification, including
morphologic keys, standard ITS2 and CO1 PCR,
and Sanger and next-generation sequencing. Molecular
methods were instrumental in confirming the pres-
ence of *An. stephensi* mosquitoes. The mosquitoes
were collected as larvae. The lack of adult mosquitoes
found in the light traps indicates the need for studies
to characterize adult vector bionomics and behavior
to elucidate how they contribute to transmitting ma-
laria and to design appropriate tools for surveillance
of adult *An. stephensi* mosquitoes.

Reports from other sites have documented the
difficulty of trapping adult mosquitoes (7). The bio-
nomics and behavior of this vector in its recent inva-
sive geographic foci are poorly understood; the only
available detailed descriptions are from Asia (4,13,22).
However, reports from Ethiopia on this vector have
indicated that crepuscular biting behaviors and rest-
ing outside houses could translate to reduced efficacy
of core vector-control interventions, insecticide-treat-
ed bed nets, and indoor residual spraying, indicating
the importance of accurate parameters (8,13,23). In
addition, the effectiveness of any insecticide-based
control method will depend on the insecticide resis-
tance of the *An. stephensi* mosquito; insecticide-resis-
tance surveys are needed (8,23).

On the basis of the phylogenetic analysis of ITS2,
the Kenya *An. stephensi* isolates from Turkana and
Marsabit matched one another closely, but because
we only conducted CO1 analysis of mosquitoes col-
lected from Marsabit, data were insufficient to infer
relatedness. The isolates also matched closely with
isolates from India, Iraq, Yemen, Iran, and Nigeria
but were more distant from the isolates from Ethio-
pia. However, phylogenetic analysis of CO1 demon-
strated 2 of the Marsabit samples matched closely
with isolates from Ethiopia, meaning they are likely
related and suggesting a southward invasion of *An.
stephensi* mosquitoes from Ethiopia. This finding as-
serts the importance of sequencing CO1 amplicons
to infer common phylogenetic origins of *An. stephensi*
species. Additional population genetics studies using
whole-genome sequencing approaches to describe
these clades are needed, along with intensive surveil-
lance to describe their bionomics and behavior.

Our findings also suggest potential introduc-
tion routes; *An. stephensi* mosquitoes were found
along highways connecting Kenya to Ethiopia and
South Sudan, highlighting the need for increased
surveillance along major transportation routes, ideally targeting such areas as truck stops and resting sites, weighbridges, and borders. Future work should include phylogenetic analysis of CO1 isolates of *An. stephensi* mosquitoes to understand their origin and spread. Further, tracking parasites that cause malaria cases around the areas where *An. stephensi* mosquitoes have been introduced will be key given that the species is an efficient vector of both *P. falciparum* and *P. vivax*.

Because of rapid, often unplanned, urbanizing in Africa, many urban centers have poor refuse disposal and drainage systems that are potential larval habitats of *An. stephensi* mosquitoes. In addition, because of inadequate social amenities in informal urban settlements, most inhabitants rely on water storage containers for domestic use. Such containers can thus become major breeding habitats for *An. stephensi* mosquitoes, further compounding the problem of malaria transmission in urbanized areas (2,10). A recent report described the role of construction in urban areas in Ethiopia in propagating *An. stephensi* larval breeding through uncovered cisterns, plastic containers, and pits dug out for brick manufacturing (S. Yared et al., unpub. data, https://www.biorxiv.org/content/10.1101/2023.05.23.354190v1). In this study, *An. stephensi* larvae were collected in riverbeds, which is notable because *An. stephensi* mosquitoes are thought to confine themselves to habitats similar to those of *Aedes* spp. mosquitoes. That level of plasticity in colonizing larval habitats demonstrates the potential for this species to invade rural and urban areas alike. In addition, climate change, which creates suitable climatic conditions for mosquito breeding, also means the potential for the spread and establishment of *An. stephensi* mosquitoes in cities in Africa is great.

When *An. stephensi* mosquitoes were introduced into Djibouti (2), the country was at the preelimination stage for malaria but then spiked to nearly 3,000 reported malaria cases in 2013, just 1 year after the mosquito was first reported. In 2019, just 6 years later, Djibouti reported 49,402 malaria cases (24). Modeling of the potential effects of *An. stephensi* mosquito establishment in Ethiopia predicts a surge in *P. falciparum* cases by 50% overall if no additional interventions are put in place; areas of lowest transmission (∼0.1%) are forecast to be affected the most (8). Similar models need to be conducted in all areas that are newly invaded to predict the spread and effects of the vector and to learn more about the potential effects of additional interventions.

The breeding habitats of *An. stephensi* mosquitoes are similar to those of *Ae. aegypti* mosquitoes, but the resting and biting behavior of adult *An. stephensi* mosquitoes in their invasive range in Africa is less well understood (1,25). Evidence of outdoor, crepuscular feeding by this species suggests it might be less affected by insecticide-treated bed nets or indoor residual spraying as a vector-control intervention. Furthermore, *An. stephensi* mosquitoes in Ethiopia were reported to be highly resistant to pyrethroids, carbamates, and organophosphates (13). Those traits indicate that alternative vector-control measures and non–vector-control measures might be needed to address the threat of this invasive mosquito. As the national malaria control program develops a vector-control strategy, integrated vector management...
approaches offer advantages because of the potential benefit of targeting additional vectors on the basis of World Health Organization guidance (26,27), particularly because of the poor understanding of this vector’s behavior when it colonizes new areas. Deploying an integrated approach provides opportunities to target *Ae. aegypti* and *An. stephensi* vectors for surveillance and control using similar interventions, which could optimize resource allocation and use in the resource-limited settings where *An. stephensi* mosquitoes are currently being reported. Managing larval sources, including by applying larvicides, reducing larval sources, and modifying the environment to make it less conducive to productive mosquito aquatic stages, has been pointed out as a potential strategy for targeting *An. stephensi* mosquitoes, given their tendency to breed in human-made containers in urban areas (3,13,23,28; S Yared et al., unpub. data). Other potential vector control tools, including those currently under evaluation, include spatial repellents (29), attractive targeted sugar baits (30), endectocides (31), insecticide-treated clothing (32), and genetically modified mosquitoes (33). Given the mosquito’s outdoor, early-evening biting behaviors, its resistance to multiple insecticides, and the threat it poses to malaria control efforts, these alternative vector-control approaches might be necessary to sustain gains made against malaria over the past 2 decades.

The first limitation of our study is that samples were collected over a short time frame in a limited number of sites; in Turkana County, we conducted 4 collections in 2 months at 9 sites, and in Marsabit County, collections were performed at 6 sites over 2 months. Therefore, the temporal and spatial extent of the *An. stephensi* mosquito is still largely unknown and is likely more widespread than this initial report would suggest. Furthermore, only larval samples of *An. stephensi* mosquitoes could be collected, pointing to gaps in our understanding of adult behavior and optimal adult sampling tools and methods. Collection of other *Anopheles* species was likely lacking because collections occurred in the dry season, which also demonstrates the potential for *An. stephensi* mosquitoes to sustain transmission in dry seasons, as has been predicted elsewhere (34). Last, 75% of samples collected in Marsabit could not be amplified by any of the species identification PCR protocols available in the KEMRI laboratory and will be sequenced once the budget is available. Amplifying those samples is a critical first step in combating this emerging threat. Expanding surveillance activities to mitigate the spread of *An. stephensi* mosquitoes will be key, as will learning more about how this invasive vector is related to recent malaria outbreaks in both counties.

In conclusion, we confirm the presence of *An. stephensi* mosquitoes in northern Kenya, which points to the urgent need to reexamine and expand vector surveillance and control efforts to include this vector. This mosquito vector is likely to sustain and possibly increase malaria transmission in northern Kenya and spread further southward to highly populated urban areas and existing malaria-endemic counties, further compounding the problem of malaria control in the country. Our findings emphasize the need for heightened and tailored surveillance to elucidate the scope of this invasive vector’s spread, to initiate research on the bionomics of the vector, and to advise on targeted control using existing interventions, including those currently under trial.

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Anopheles stephensi Mosquitoes, Kenya

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March 2023 World TB Day

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- Multicenter Retrospective Study of Vascular Infections and Endocarditis Caused by Campylobacter spp., France
- Yellow Fever Vaccine–Associated Viscerotropic Disease among Siblings, São Paulo State, Brazil
- Bartonella spp. Infections Identified by Molecular Methods, United States
- COVID-19 Test Allocation Strategy to Mitigate SARS-CoV-2 Infections across School Districts
- Using Discarded Facial Tissues to Monitor and Diagnose Viral Respiratory Infections
- Postacute Sequelae of SARS-CoV-2 in University Setting
- Associations of Anaplasma phagocytophilum Bacteria Variants inIxodes scapularis Ticks and Humans, New York, USA
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Neurotropic Highly Pathogenic Avian Influenza A(H5N1) Virus in Red Foxes, Northern Germany

Christine Baechlein, Sven Kleinschmidt, Dorothee Hartmann, Patricia Kammeyer, Anne Wöhlke, Tobias Warmann, Louise Herms, Bianca Kühl, Andreas Beineke, Peter Wohlsein, Timm Harder, Martin Runge

In a 1-year survey of wild terrestrial predators in northern Germany, we found that 5 of 110 foxes were infected with contemporary avian influenza A(H5N1) viruses, forming a temporal cluster during January–March 2023. Encephalitis and strong cerebral virus replication but only sporadic mammalian-adaptive viral polymerase basic 2 protein E627K mutations were seen.

Since emergence of the highly pathogenic avian influenza virus (HPAIV) H5 A/Goose/Guangdong/1/1996 (gs/GD) lineage in 1996, successors continue to circulate in waves around the world, leading to massive losses in wild bird and domestic poultry populations (1). Until 2020–2021, gs/GD HPAIV infections in poultry holdings characteristically paralleled waterfowl migration patterns. Since then, this seasonality has virtually disappeared and gs/GD HPAIV, currently of subtype H5N1 assigned to clade 2.3.4.4b, are detected year-round in wild birds and poultry in Europe (2,3). The virus has been found at increasing frequency in domestic and wild living mammals, mostly affecting carnivorous species (4) and massive die-off events raised concern about potential mammal-to-mammal transmission in dense populations (5,6). HPAIV infections were regularly characterized by high viral loads in the brain and associated clinical signs of the central nervous system with corresponding morphologic changes (7–11). Although Germany has had high HPAIV infection rates in avian species, prevalence studies on HPAIV infections in terrestrial predators, which feed on (infected) waterfowl, are not available. We performed a 1 year-survey to detect HPAIV in wild terrestrial predators in northern Germany.

The Study
We studied HPAIV infections in 170 wildlife predators of several animal species: red foxes (Vulpes vulpes, n = 110), racoons (Procyon lotor, n = 28), badgers (Meles meles, n = 15), martens (Martes foina or Martes martes, n = 9), and racoon dogs (Nyctereutes procyonoides, n = 8) by using PCR and in situ methods. We performed avian influenza virus real-time PCR on individual brain samples of those 170 terrestrial wildlife predators and an H5-specific assay. The carcasses originated from different geographic locations in the German federal state of Lower Saxony and were delivered to the Lower Saxony State Office for Consumer Protection and Food Safety during February 2022–April 2023 (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0938-App1.pdf).

We detected viral RNA with cycle threshold values of 13.75–36.35 in the brains of 5 red foxes (4.5%), which were submitted with differing preliminary reports partly involving signs of disease specific for the central nervous system. Virus-positive animals were found in the first 3 months of 2023 (Table). In 4 of 5 cases, full-length influenza virus sequences could be recovered by using Illumina (https://www.illumina.com) high-throughput sequencing and were deposited in GISAID (https://gisaid.org) (Appendix Table 2). The hemagglutinin sequences of the 4 red foxes had nucleotide identities of 98.53%–99.06% and had a cleavage site typical for a highly pathogenic phenotype (REKRRKRG).

For the phylogenetic analyses, we included avian influenza virus nucleotide sequences representing the first 3 BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search hits, as well as sequences detected in mammals during 2021–2023 in Europe and...
Table. Overview of case history, qRT-PCR results, nucleotide frequency at amino acid position 627 of the PB2 segment, and virus isolation experiments of HPAIV H5N1-positive foxes, Germany*

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Case history, date of submission</th>
<th>PB2 mutation E627K</th>
<th>Ct values, M segment/H5 segment</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>51023-60</td>
<td>Adult, clinical signs, nonspecific, 2023 Jan</td>
<td>99.54/0.46</td>
<td>13.75/19.49; 20.29/25.05; 18.07/21.78</td>
<td>Positive</td>
</tr>
<tr>
<td>51023-61</td>
<td>Found dead, 2023 Jan</td>
<td>ND/ND/ND</td>
<td>28.98/34.60; 29.95/36.35; 30.45/34.36</td>
<td>ND/ND/ND</td>
</tr>
<tr>
<td>51023-113</td>
<td>Found dead, 2023 Jan</td>
<td>ND/ND/ND</td>
<td>29.16/32.32; 31.02/35.55; 29.05/32.51</td>
<td>ND/ND/ND</td>
</tr>
<tr>
<td>51023-124</td>
<td>Adult, clinical signs, CNS, 2023 Feb</td>
<td>99.93/0.07</td>
<td>23.16/27.61; 25.91/30.04; 25.40/29.77</td>
<td>Positive</td>
</tr>
<tr>
<td>51023-261</td>
<td>Pup, clinical signs, CNS, 2023 Mar</td>
<td>99.76/0.23</td>
<td>16.90/19.17; 21.80/23.72; 21.01/23.38</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*CNS, central nervous system; H, hemagglutinin; HPAIV, highly pathogenic avian influenza virus; M, matrix; ND, not determined; nt, nucleotide; PB2, polymerase basic 2; qRT-PCR, quantitative reverse transcription PCR.

Figure 1. Phylogenetic analyses of highly pathogenic avian influenza virus H5 sequences of mammals and wild birds, Germany. The maximum-likelihood tree was built with 500 bootstrap iterations. H5 variants included 4 red foxes from Lower Saxony, Germany, their 3 closest relatives according to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analyses, and distinct H5 sequences detected in wild birds from northern Germany and in mammalian species from Europe during 2021–2023. Bold text indicates virus variants found in this study. Numbers along branches indicate percentage bootstrap values. Scale bar indicates nucleotide substitutions per site. H, hemagglutinin.
Neurotropic Influenza A(H5N1) Virus in Red Foxes

relevant HPAIV H5N1 sequences recovered from wild birds from northern Germany. The phylogenetic tree showed that the H5 sequences detected in red foxes in this study belonged to clade 2.3.4.4b. They were closely related to sequences previously found in avian and mammal hosts in Europe but did not form a separate cluster (Figure 1). Analyses of all 8 genome segments assigned the genomes to genotype Ger-10–21-N1.5, Ger-11-21-N1.4, or Ger-02-23-N1.1 (Appendix Figure) (12). We further analyzed viral RNA segments for the presence of mutations, which have been described in mammalian H5Nx infections, involving amino acid residues in the polymerase basic (PB) 2 segment (7,9,11). The PB2 E627K substitution was found in the consensus sequence in only 1 of 4 cases (animal no. 51023–113) (Table).

HPAIV H5N1 isolates were obtained from 3 of 4 central nervous system tissue homogenates during the first passage in MDCK II cells. Hemagglutinating activity and high viral loads (cycle threshold values <20) were evident in the supernatant of inoculated cell cultures. An influenza-like cytopathic effect proceeded to affect the whole cell monolayer within less than 72 hours postinoculation (Table).

None of the 5 AIV-positive foxes showed macroscopic brain lesions. Microscopically, we observed mild-to-moderate multifocal lymphohistiocytic encephalitis with predominant perivascular infiltrations in the midbrain of 4 foxes and in the brain stem of 3 foxes. One animal showed vasculitis. Minor lesions were seen in the rhinencephalon and the cerebellum in 2 foxes. Neuronal necrosis was present in the midbrain and rhinencephalon in 1 animal, whereas another animal showed multifocal gliosis in the brain stem and midbrain. Immunohistologic analysis showed influenza A nucleoprotein in morphologically affected and unaffected areas of the brain. This protein was located in the nucleus and perikaryon and cell processes of neurons, as well as in glial cells (Figure 2).

Conclusions

Recent H5N1 virus infections with dramatic losses in sea bird breeding colonies in Europe have proven the deleterious implications of a year-round presence of HPAIV in northern Europe (13). The sustained occurrence of HPAIV outbreaks in wild birds might enhance spillover risks to wild carnivores that prey on infected birds or scavenge on their carcasses. An alimentary route of infection has been proven experimentally (14).

Our phylogenetic analyses confirm that virus strains similar to those circulating in the wild bird population have the potential to be transferred to terrestrial carnivores. Wild birds are suspected to be the most likely animal reservoirs sustaining HPAIV replication in Europe. The role of other animal species, in particular mammalian predators, is still equivocal. Clinically conspicuous cases have been reported throughout Europe in a sporadic fashion, but the true prevalence remains unknown. Our data of a 1-year survey from the federal state Lower Saxony in Germany showed a temporal clustering of cases in red foxes found within the first 3 months of 2023. This period coincided with a peak in HPAIV detections in wild birds in northern Europe (4). Studies from the Netherlands also described positive cases in the winter period of 2021–2022 (10,11). Conditions in the cold season could favor virus transmission to carnivorous mammals.

Virus variants from foxes in Germany did not form a separate phylogenetic cluster confirming independent infection events. According to several reports, gs/GD-like HPAIV shows a strong neurotropism in mammal species. This finding is true for H5N8 infections in harbor seals (9), as well as for disease outbreaks in terrestrial predators (7,8,10,11). Also, in this study, HPAIV infection of the brain was shown by high viral loads and immunohistochemical analysis. In previous studies,
point mutations suspected to increase viral replication in mammals have been frequently described, especially the E627K mutation in the PB2 segment. Ten of 14 HPAIV H5N1-positive wild carnivores detected in the Netherlands carried the mammal-adaptive variant (10,11), and those viruses replicated to higher titers in mammalian cells than in an avian cell line (10). In our study, only 1 of 4 analyzed cases had the E627K substitution. Those results support previous observations that PB2 627K is not a prerequisite for virus replication in mammalian cells (10).

Little is known about the pathogenesis of HPAIV infection in wild mammal predators. Further virologic and serologic studies are planned and are needed to monitor those potential hosts as indicators for enhanced zoonotic spillover. Recent serologic findings of a clinically silent HPAIV H5N1 infection in a pig herd in Italy suggest that the neurologic cases seen in carnivores in Europe and elsewhere might represent just the tip of an iceberg (15). Widespread HPAIV H5N1 infection in those hosts would provide ample opportunities to further adapt to mammals, which could be associated with increased infection risks for humans.

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We thank the local authorities in Lower Saxony for submitting wild animals and Sabine Baumann, Hannah Habbeck, Sina Korn, Bozena Ohlhäuser, Sara Walter, Diana Wessler, and Kristin Trippler for excellent technical assistance with diagnostic samples and virus isolation.

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References

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Lobomycosis, also known as lacaziosis, is an exceedingly uncommon chronic fungal infection primarily affecting the skin and subcutaneous tissues (1). It was identified in the mid-1930s (2). This tropical mycosis is caused by the dimorphic fungus *Paracoccidioides* (*Lacazia*) *loboi* (hereafter *P. loboii*; see Conclusions) (3), and it affects primarily persons residing in regions of Central and South America, particularly the Amazon rainforest (1). Clinically, lobomycosis manifests as slow-growing, nodular lesions that can lead to disfigurement and functional impairment if left untreated (1). Because of its rarity, diagnostic challenges, and limited therapeutic options, each case of lobomycosis offers valuable insights into its epidermiologic and ecologic aspects, clinical manifestations, treatment strategies, and overall management.

We report a detailed description of a recent patient from Panama who had lobomycosis. We used combined clinical-epidemiologic and phylogenetic data, including a new gene sequence dataset on this fungus in Panama, for analysis. Findings contribute useful insights to limited knowledge of this fungal infection in the Mesoamerican Biologic Corridor.

**The Study**

An 87-year-old woman from Capira in western Panama was referred to a hospital in Panama in September 2022 to rule out leishmaniasis versus deep fungal infection. Her condition began 40 years earlier with a hyperpigmented nodular lesion on the left leg that progressed to multiple, nonfluctuant, indurated, and exophytic nodular lesions throughout the affected limb, which later involved the scalp. Initially painless, her lesions did not interfere with daily farming activities, but after the first year, occasional pain, partial ulceration, seropurulent/hemorrhagic discharge, and edema in her left leg occurred. No history of penetrating trauma was reported, but she admitted to walking barefoot during agricultural activities. A biopsy performed 3 years earlier reported sporotrichosis, treated with itraconazole and cryotherapy, yielding poor response and persistent lesions. Sizes of lesions had been reduced. Previous clinical and histopathologic reports were not available.

On physical examination, the patient appeared alert and oriented to person and space. Multiple nodular (keloid-like), confluent, indurated, hyperpigmented, and painless lesions were evident throughout the anterior, medial, lateral, and posterior regions of the left leg; a lesion showing the same characteristics was located in the mid-distal third of the posterior aspect of her left thigh (Figure 1). The patient was tested for leprosy and other cutaneous mycobacterial infections, sporotrichosis, atypical cutaneous leishmaniasis, and neoplasia. The result of a leishmania intradermal reaction test was negative. Direct examination of slit-skin smears for leprosy and leishmania did not show microorganisms. Routine cultures did not grow mycobacteria or fungi. Results of tissue PCR for mycobacteria were negative. However, a biopsy sample...
submitted for histologic examination showed an extensive chronic granulomatous sclerosing inflammatory reaction, with abundant large and refractile yeast-like structures arranged in chains, consistent with lobomycosis (Figure 1).

Electron microscopy showed multiple tissue-bound yeast forms exhibiting well-preserved cell walls and classic tubular-filamentous radiations between cells (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/29/12/23-1092-App1.pdf). We extracted DNA from formalin-fixed, paraffin-embedded tissue and subjected the DNA to PCR for fungal detection. We performed species identification by using the MinION Sequencing System (Oxford Nanopore, https://nanoporetech.com) for P. loboi ADP-ribosylation factor (ADP-rf), Gp43 protein (Gp43), and internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) genes, as described (3,4) (Appendix). We evaluated phylogenomic relationships of the isolate sequenced in this study (5,6) by comparison against other Paracoccidioides species, confirming the organism to be P. loboi (Figure 2).

In August 2022, the patient was given itraconazole (200 mg orally 1×/d). However, in November 2022, the patient had pyrosis, dyspepsia, and increased liver enzyme levels, leading to the decision to taper her dose of itraconazole to 100 mg/day, resulting in slight improvement of lesions. In June 2023, it was decided to increase the dose of itraconazole to 200 mg/day because of slow improvement of lesions while waiting for adjuvant cryotherapy. Despite showing a slow and minor regression of her nodules, the patient reported a noticeable improvement of dysesthesias associated with the lesions.

Conclusions
Lobomycosis is a puzzling disease caused by the dimorphic Onygenale fungus P. loboi (8). One of the intriguing aspects of this organism is its inability to grow in laboratory cultures and its resistance to specific antifungal treatments (1). Lobomycosis was documented in the 1930s when Brazilian dermatologist Jorge Lobo described a patient with skin lesions resembling keloids (2). Although the fungus is believed to be primarily in soil and vegetation, its presence in marine mammals has been increasingly observed (1). Furthermore, human infections have been linked to water proximity, leading to the hypothesis that P. loboi might be a hydrophilic microorganism that enters the skin through traumatic means (9).

Because of challenges of culturing the fungus in vitro, nomenclature has been a highly debated aspect among mycologists, resulting in diverse classifications of the organism. Proposed taxonomic designations have included Glenospora loboi, Glenosporopsis amazonica, Blastomyces loboi, Loboa loboi, and the family Lobomyces (1). In 1999, Taborda et al. proposed a new genus, Lacazia, and renamed the agent as Lacazia loboi (10). Subsequent studies by Mendoza et al. contributed to resolving the taxonomic puzzle surrounding this agent. By conducting amplification of the 18S small subunit rDNA and 600 bp of the chitin synthetase gene, the organism was placed within the group of Onygenales dimorphic fungi (11). Moreover, those studies suggested a close phylogenetic relationship between L. loboi and P. brasiliensis, which
Figure 2. Phylogenetic relationship of ADP-rf, Gp43, and internal transcribed spacer (ITS) 1 and 2 genes from patient in Panama who had lobomycosis with other Paracoccidioides species. Trees indicate phylogenetic analysis inferred by maximum-likelihood method of the ADP-rf (A), Gp43 (B), and ITS1–ITS2 (C) genes. Analyses included sequences obtained from research conducted by Vilela et al. (7) and the Panama case analyzed in this study (red). Each color in the trees represent different clusters identified: yellow, Paracoccidioides cetti; white, P. restrepiensis/venezuelensis; gray, P. brasiliensis; green, P. americana; light blue, P. lutzii; and light purple, P. loboi. Gray triangles indicate genomes of the same clade belonging to P. restrepiensis, P. venezuelensis, or P. brasiliensis (to the ADP-rf gene, 49 genomes; to the Gp43 gene, 32 genomes; and to ITS1-ITS2 gene, 52 genomes). Histoplasma capsulatum and Ajellomyces capsulatus DNA sequences were used as outgroups for ADP-rf and ITS genes, respectively. Scale bars indicate nucleotide substitutions per site.
was previously inferred on the basis of their morphologic and antigenic similarities (1).

More recently, DNA sequence analyses of dolphins samples have shown that the samples align with those of *Paracoccidioides* species (12). This discovery has resulted in grouping of this pathogen within a monophyletic cluster, positioned as a close relative to *P. americana* and other *Paracoccidioides* species (3,7). Consequently, the taxonomy of the dolphin pathogen has been updated, now referred to as *P. cetii*, and the designation of the human-infecting pathogens remains classified as *P. loboi* (3,7,12,13). This recent amendment in the taxonomy reflects newfound understanding of the relationships between these pathogens.

Lesions of lobomycosis show various morphologic patterns; 5 well-known subtypes have been identified: infiltrated, keloidal, gummatus, ulcerative, and verrucoid (1). Among these subtypes, the keloid subtype is the most common, characterized by well-defined, infiltrative papules and nodules with a shiny, pink surface with overriding telangiectasias (I). Nodules are firm to the touch and can merge together. Lobomycosis lesions can appear hyperchromic, hypochromatic, or achromic and are typically painless or accompanied by dysesthesia (I,14). Distinguishing lobomycosis from other infectious causes, such as leishmaniasis, leprosy, sporotrichosis, paracoccidioidomycosis, cryptococcosis, and blastomycosis, can be challenging (I). Verrucoid lesions, observed in advanced stages, are commonly found on the legs and show a gray-white coloration mimicking chromoblastomycosis (I). Ulcerated lesions resemble those seen in leishmaniasis and mycetomas, further complicating differential diagnosis (I).

No antifungal treatment has been consistently effective against *P. loboi*. Some drugs have shown partial effectiveness, including amphoterin C, imidazoles, triazoles, expanded spectrum azoles, and clofazimine, but complete remission is rare. Surgical excision remains the most effective option, although multiple or disseminated lesions often recur after surgery (I).

Little is known about the ecoepidemiologic and biogeographic aspects of lobomycosis in Panama. This disease was reported in Panama in 1972, followed by 2 additional cases in 1978 (15) (Appendix Figure 2). An addendum to the study from 1978 reports 11 additional cases diagnosed and reported by a pathologist in the Chiriqui region (in northern Panama close to the border with Costa Rica) (15). Our case-patient came from Capira, which is ≈386 km from Chiriqui in southwestern Panama, suggesting that lobomycosis is endemic to Panama, might be more widespread than previously suspected, and might be underreported (Appendix Figure 2). Increased awareness and further research are needed to better understand the endemicity of this fungal disease in Panama.

DNA sequences have been deposited and are available in the European Nucleotide Archive under bio project no. PRJEB64904 (ERA26631335).

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Lobomycosis is the name given to the cutaneous mycosis for which *Lacazia loboi* is the etiologic agent. *L. loboi* lives primarily in dense tropical rain forests and the oceans of the Central and South America Coast. Humans and dolphins are the only known hosts for this fungus (1–4). *L. loboi* cannot be cultured and is identified by histologic analysis of excised lesions. This uncultivable characteristic played a role in the convoluted path the fungus has traversed in arriving at the current binomial designation.

The etymologic journey of *L. loboi* began in 1931 when Brazilian dermatologist Jorge O. Lobo reported a case in a 52-year-old man who had keloid-like lesions over his sacral region (5). Lobo called this novel disease *Blastomicose keloidiana*, the first misstep in the nomenclatural misadventures. Lobo believed that the fungus was similar to *Paracoccidioides brasiliensis*. *Paracoccidioides loboi* was proposed by Fonseca and Lacaz in 1971 (6), honoring Lobo with the species name, but inadequate Latin description resulted in rejection. This resemblance with *Paracoccidioides* caused nearly endless taxonomy problems (7).

Sufficient Latin validation gathered, Lacaz re-submitted an updated proposal in 1996 (8). Lacaz was unable to locate Lobo’s original sample. Taborda and colleagues (9) studied specimens stored in the US National Fungus Collections (10), concluding “no existing genus can accommodate this taxon” (9). In 1999, they advanced *Lacazia loboi* for validation, heralding Lacaz, an esteemed physician and director of the Tropical Medicine Institute of São Paulo, with the genus designation.

At least 6 genera (*Glenosporella, Blastomyces, Glenosporosis, Paracoccidioides, Lobomyces, and Loboa*) and 2 species (*brasiliensis* and *amazonica*) preceded *Lacazia loboi*. The repetitive *Loba loboi* was proposed in 1956 (5), but deemed “nomem nudum and illegitimate” and incorrectly identified as *P. brasiliensis*. Herr and colleagues showed that *L. loboi* is in the sister taxon of *P. brasiliensis* and confirmed their confusing similarity (11). Vilela and colleagues, using updated phylogenetic DNA data analysis, identified the uncultivable *P. ceta*, isolated from dolphins, and *L. lobo* as species that belonged in the genus *Paracoccidioides* (12).

Because *P. loboi* had been discarded, *Paracoccidioides lobogeorgii* (georgii represents an Anglicization of the Spanish Jorge) was submitted as the replacement. For the disease itself, Francesconi and colleagues catalogued 8 monikers and mercifully declared, “Lobomycosis is the correct name for this disease” (13).

Figure 1. A) Grocott methamine silver–stained section from a skin biopsy specimen of a bottlenose dolphin (*Tursiops truncatus*) showing abundant *Lacazia loboi* yeast cells individually and in chains connected by thin tubular bridges. Source: Emerging Infectious Diseases 15 (4) April 2009. B) *L. loboi* yeast cells in chains connected by thin tubular bridges. Source: Centers for Disease Control and Prevention.
Figure 2. A, B) Extensive lobomycosis-like disease on the beak and dorsal fin of a bottlenose dolphin (Tursiops truncatus) stranded on Margarita Island, Venezuela. Source: Emerging Infectious Diseases 15 (8), August 2009. C) Lobomycosis in a 41-year-old soldier from Colombia. Erythematous, lobulated plaque (4 cm × 2.5 cm) on the sternal notch with hematic crust and black areas on the surface. Source: Emerging Infectious Diseases 25 (4), April 2019.

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Tecovirimat is an antiviral drug approved in 2018 by the United States Food and Drug Administration to treat orthopoxvirus infection. Tecovirimat inhibits orthopoxvirus viral protein (VP) 37, which is involved in membrane formation required for egress-competent virions (1–3). Outside of a clinical trial, the Centers for Disease Control and Prevention recommends tecovirimat only for persons who have (or are at risk for) severe manifestations of mpox (4,5).

A low barrier to tecovirimat resistance by mutations in the VP37 protein encoded by the F13L gene of monkeypox virus (MPXV) has been demonstrated through in vitro and animal studies (6). Cases of suspected tecovirimat resistance during the ongoing mpox outbreak have occurred in immunocompromised patients receiving prolonged or repeated courses of tecovirimat (7,8).

Reports of tecovirimat-resistant mpox have emerged after widespread use of antiviral therapy during the 2022 mpox outbreak. Optimal management of patients with persistent infection with or without suspected resistance is yet to be established. We report a successfully treated case of severe mpox in California, USA, that had suspected tecovirimat resistance.

Treatment of Mpox with Suspected Tecovirimat Resistance in Immunocompromised Patient, United States, 2022

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The Study

A 35-year-old man with no notable history sought care at an urgent care clinic in California, USA, because of painful diffuse skin lesions (Figure 1). He was given a diagnosis of mpox by quantitative PCR from a lesion sample and completed 2 weeks of oral tecovirimat (600 mg 2×/d). No testing for sexually transmitted infections was ordered during his initial examination. He was unable to take the medication with a high-fat meal as recommended. Five weeks after his mpox diagnosis, he sought care for persistent ulceration and swelling of his finger and underwent surgical debridement. Tissue cultures grew methicillin-sensitive Staphylococcus aureus, and pathologic analysis showed mixed exuberant inflammation, fibrinopurulent exudates, and pseudoepitheliomatous hyperplasia. Mpox was not suspected and MPXV testing was not obtained. He was prescribed cephalaxin for presumed paronychia.

The patient’s lesions continued to worsen, and he came to Santa Clara Valley Medical Center (San Jose, California, USA) ≈8 weeks after his initial mpox diagnosis. He had painful ulcerated lesions on his nose, finger, soles of his feet, anogenital region, and abdomen (Figures 1, 2). The patient had no previous

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More data are needed to inform the management of severe mpox, including cases of possible resistance. We report a case of a patient who had HIV/AIDS and prolonged severe mpox with F13L gene mutations concerning for possible tecovirimat resistance who was successfully treated with cidofovir, brincidofovir, and vaccinia immune globulin (VIGIV). Written consent was obtained from the patient to share all protected health information and images included in this case report.
HIV testing; HIV screening obtained at admission was positive with a viral load of 687,772 copies/mL and a CD4+ T-lymphocyte count of 73 cells/mm³ (CD4% of 4). His skin lesions were positive for MPXV DNA by quantitative PCR with low cycle threshold (Ct) values, suggesting active infection (nonvariola orthopoxvirus Ct 15.9, MPXV Ct 15.1). Biopsy of an abdominal lesion showed interface dermatitis and marked inflammatory dermal infiltrate.

Oral tecovirimat was again given, and bictegravir, emtricitabine, and tenofovir alafenamide were given within 72 hours of HIV diagnosis. After 1 month of treatment...

Figure 1. Initial lesions on trunk (day 0, when the patient first sought care at an urgent care clinic) and visual timeline of right index finger lesion of immunocompromised patient with mpox, California, USA, 2022. Treatment received, CD4+ T-cell count, and viral load are indicated below images. IV, intravenous.

Figure 2. Visual timeline of facial lesions (top) and left plantar lesions (bottom) of immunocompromised patient with mpox, California, USA, 2022. Treatment received is indicated between images. IV, intravenous.
antiretroviral therapy (ART), his viral load decreased to 177 copies/mL, and his CD4+ T-lymphocyte count increased to 147 cells/mm³ (CD4% of 8.5). However, despite adherence to both ART and tecovirimat treatment, including taking tecovirimat with high-fat meals, the lesions became progressively larger, more painful, and more deeply ulcerated, and new lesions continued to emerge. (Figures 1, 2). The lesion progression raised concern for possible tecovirimat resistance. Repeat testing of a new chin lesion 4 weeks after restarting tecovirimat confirmed persistence of MPXV with high viral burden (nonvariola orthopoxvirus Ct 20.7, MPXV Ct 17.4). Punch biopsy of the lesion was obtained, and no other underlying etiology was identified.

Given lack of clinical improvement, tecovirimat was continued, and intravenous cidofovir (5 mg/kg/wk for 2 doses), topical 1% cidofovir, and VIGIV (6,000 units/kg) were given (Table). (Topical cidofovir was administered despite the absence of data about the role of topical use because there are no known adverse events associated with topical use and there was a chance that it would benefit the patient.) His lesions became smaller and less painful within days of the first doses of cidofovir and VIGIV. Tecovirimat was continued on the basis of expert guidance from the Centers for Disease Control and Prevention because the drug is well-tolerated and might have synergy with other antiviral drugs (9,10). The patient was discharged and was receiving ART, oral tecovirimat, and brincidofovir (200 mg orally/wk for 6 doses). Follow-up examinations through day 164 showed continued slow resolution of his lesions (Figures 1, 2).

For public health surveillance, MPXV whole-genome sequencing (WGS) was performed on lesion swab samples taken during his initial hospitalization (≈8 weeks after initial diagnosis) and subsequent re-admission (≈12 weeks after initial diagnosis); lesion specimens from his initial diagnosis were not available. WGS of the week 8 lesion showed an A290V mutation in the VP37 protein, which has been associated with high-level phenotypic resistance to tecovirimat in orthopoxviruses (3,11). WGS of 2 lesions sampled at week 12 showed that the mutation VP37 A290V residue had reverted to A290A and a new mutation, D283G, was present. VP37 D283G has been associated with high-level phenotypic tecovirimat resistance in poxviruses (3,11). The data suggest that resistance to tecovirimat evolved in this case over time.

**Conclusions**

During the ongoing multicountry mpox outbreak, mutations associated with tecovirimat resistance have been identified in immunocompromised patients who had severe or persistent mpox and a high mortality rate (7,8). It is unknown whether this patient was initially infected with a tecovirimat-resistant strain. However, resistance could have emerged during prolonged infection in this immunocompromised patient, who was receiving extended tecovirimat treatment, because 2 resistance mutations were detected in lesions sampled at different sites and timepoints. Different VP37 resistance mutations have been documented in separate lesions from persons who had severe mpox and underwent prolonged tecovirimat treatment (12). In addition, the patient might have had subtherapeutic tecovirimat serum concentrations and subsequent selection for resistance when he took his initial course of tecovirimat without high-fat meals (ideally 600 calories or 25 g of fat) (13). Challenges for patients living with advanced HIV infection often include lack of housing (14) and access to regular food sources, which can pose additional burdens to the specific dietary requirements for tecovirimat absorption (4,15).

Both the failure to recognize the lesions as progressive mpox and delayed HIV diagnosis probably contributed to the severity of disease in this patient. Current guidelines advise that screening for sexually transmitted infections and HIV be offered to all sexually active patients who have mpox (https://www.cdc.gov/poxvirus/mpox/clinicians/people-with-HIV.html). Earlier ART initiation would probably have benefited the patient. Clinicians caring for patients who have persistent mpox should evaluate for underlying immunodeficiency, particularly HIV, and consider the possibility of antiviral resistance in cases that fail to respond to standard of care. WGS is helping to illuminate the potential resistance mutations that might occur in mpox cases, as well as describe the genetic differences between strains. However, MPXV WGS data are not approved for clinical use, and there is a paucity

**Table. Summary of antiviral therapy received by immunocompromised patient with mpox, California, USA, 2022**

<table>
<thead>
<tr>
<th>Antiviral therapy</th>
<th>Dose</th>
<th>Days administered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tecovirimat</td>
<td>200 mg orally twice daily</td>
<td>0–14, 56–125</td>
</tr>
<tr>
<td>Cidofovir</td>
<td>5 mg/kg intravenously weekly</td>
<td>89, 96</td>
</tr>
<tr>
<td>Brincidofovir</td>
<td>200 mg orally weekly</td>
<td>103, 111, 117, 124, 132, 140</td>
</tr>
<tr>
<td>Vaccinia immune globulin</td>
<td>576,000 units intravenously</td>
<td>92</td>
</tr>
</tbody>
</table>

*Day 0 was the date the patient first sought care at an urgent care clinic.
of data guiding the clinical application of MPXV sequencing. With the likelihood for continued human-to-human transmission of MPXV, expert consultation should be sought for patients who have nonresolving mpox, and lesion samples should be obtained for genotypic and phenotypic resistance testing to inform future case management.

Acknowledgments

We thank the patient for sharing his story so that others may learn from his disease and treatment; Monica Haw, Chantha Kath, and Alex Espinosa for performing whole-genome sequencing; and Timothy Tran for providing excellent patient care.

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References


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Hepatitis A virus (HAV) is a common human pathogen found exclusively in primates. In a molecular and serologic study of 64 alpacas in Bolivia, we detected RNA of distinct HAV in ≈9% of animals and HAV antibodies in ≈64%. Complete-genome analysis suggests a long association of HAV with alpacas.

HAV infection and vaccination normally induce lifelong immunity (2). HAV infection and vaccination normally induce lifelong immunity (2). The genus Hepatovirus (family Picornaviridae) consists of 9 species, designated A–I (3). Species B–I have been detected in small wild mammals, in seals, and in a domestic goat (4–8). HAV strains of Hepatovirus A have been exclusively associated with primates. All human hepatoviruses pertain to species A. Within species A, genotypes I–III are found in humans, and genotypes IV–VI are found in monkeys (9). No wildlife or livestock reservoir has been described for human HAV.

We screened 64 alpacas and 6 llamas in Bolivia for viruses and detected a divergent nonprimate genotype of Hepatovirus species A. We provide serologic evidence for a high frequency of HAV infection in New World camelids.

The Study
We collected serum and feces samples from 64 alpacas and 6 llamas in Bolivia within the Apolobamba national protected area near the Bolivia-Peru border in 2019 (Figure 1). We tested RNA from 70 serum samples and 69 fecal samples stored in RNAlater (ThermoFisher Scientific, https://www.thermo Fisher.com).

Figure 1. Alpaca and llama sample collection sites in study of hepatitis A virus in alpacas and llamas, Bolivia, 2019. The number of animals sampled per site is shown, 70 animals in total. Colored icons indicate alpaca HAV-positive animals by quantitative reverse transcription PCR. Inset shows locations of Bolivia and study site in South America.
in pools of 8–10 using Illumina (https://www.illumina.com) high-throughput sequencing. In 3 of 16 pools, we detected matches with HAV and investigated this finding in detail.

We tested all individual samples for alpaca HAV RNA with a novel specific quantitative reverse transcription PCR (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-1123-App1.pdf). Testing did not

**Table 1. Nucleotide distance matrix of complete HAV genomes in alpacas, Bolivia, 2019***

<table>
<thead>
<tr>
<th>Genome</th>
<th>Chu-alp-14</th>
<th>Chu-alp-21</th>
<th>IA</th>
<th>IB</th>
<th>IIA</th>
<th>IIB</th>
<th>IIA</th>
<th>IIIB</th>
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<td>14.1</td>
<td>14.1</td>
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<td>19.1</td>
</tr>
<tr>
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<td>20.1</td>
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<td>14.1</td>
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<td>19.5</td>
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<td>9.2</td>
<td></td>
<td></td>
<td>17.3</td>
<td>19.5</td>
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<td>IIIB</td>
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*Alpaca HAV and existing Hepatitis A genotypes were included where complete genomes were available. Bold indicates intra-genotype distances. Genomes were aligned using Clustal Omega 1.2 (http://www.clustal.org/omega) in Geneious Prime 2023.1.2 (https://www.geneious.com). Nucleotide distances, given in percentages, were calculated in Geneious Prime 2023.1.2. Accession numbers used: IA, AB020564; IB, M14707; IIA, AY644670; IIB, AY644676; IIIA, AB279732; IIIB, AB279735; V, EU140838; VI, OQ559662. HAV, hepatitis A virus.
detect alpaca HAV in any of the 6 llamas. In contrast, alpaca HAV RNA was detected in 6/64 (9.4%) alpacas: in 5 of 64 (7.8%) serum samples and 5 of 63 (7.9%) feces samples. In 4 of those 6 alpacas, serum and feces samples were both positive. Concentrations of alpaca HAV RNA were up to $3.2 \times 10^5$ RNA copies/mL in serum and $3.6 \times 10^5$ RNA copies/mL in feces (Appendix Figure 1).

We further processed all alpaca HAV–positive samples for complete genome sequencing using undirected Illumina NextSeq sequencing (https://www.illumina.com), HAV-specific in-solution sequence capturing, and GridION reverse transcription PCR amplicon sequencing (Oxford Nanopore Technologies, https://nanoporetech.com) (Appendix Table). We generated 2 complete and 4 partial alpaca HAV genomes for further analyses (GenBank accession nos. OR452339–44). In a phylogenetic tree, alpaca HAVs form a distinct monophyletic clade within other Hepatovirus A sequences (Figure 2, panel A). Implied membership in the species Hepatovirus A is confirmed by sequence composition. The alpaca HAV polyprotein amino acid sequence is 9%–11% distant from other Hepatovirus A, well within the species demarcation criterion of 30% set by the International Committee on Taxonomy of Viruses (3). In addition, alpaca HAV’s distance from established HAV genotypes (18%–22%) is similar to HAV genotypes’ distances from each other (14%–21%; Table 1). Alpaca HAV regions are also similarly distant to all 3 human HAV genotypes across the genome; highest divergence is in the N terminus of 2C and in 3A (Appendix Figure 3). Therefore, alpaca HAV likely represents a distinct genotype, tentatively named genotype VII (gtVII), within Hepatovirus A.

We detected alpaca HAV at 2 of 3 locations: Ucha-Ucha and Chullumpina, which are ≈20 km apart (Figure 1). The separation is reflected in phylogenetic analysis, in which 2 monophyletic clades of alpaca HAV sequences correspond to these geographic sites (Figure 2, panel B). Representative complete genome sequences from each site (Ucha-alp-14 and Chu-alp-21) are 7.6% distant in nucleotide sequence, similar to the distance between subgenotypes A and B of human genotypes I–III (9%–11%). Thus, alpaca HAVs from both sites could be classified into 2 subgenotypes, gtVIIA and gtvIIIB (Table 1). Between both alpaca HAVs, 94.8% of nucleotide changes in the coding region are synonymous, and the dN/dS ratio, calculated with the Python dnds module (https://pypi.org/project/dnds), is 0.009, suggesting purifying selection.

Currently described HAV genotypes commonly belong to a single serotype (11,12). Neutralization epitopes are located in the capsid proteins VP1–3 (13), and most amino acids of those epitopes are conserved between HAV genotypes I–V and alpaca HAV (Appendix Figure 2). Thus, we were able to conduct a serologic analysis of alpaca and llama serum samples using an HAV IgG ELISA employing human HAV antigens (Mediagnost, https://mediagnost.de). Of 64 alpaca serum samples, 41 (64.1%) were reactive for HAV IgG, including the serum of 13 (81.3%) of 16 alpacas from Cañuhuma, where no alpaca tested positive for alpaca HAV RNA (Table 2; Appendix Figure 4). Of the 6 llama serum samples, 4 (66.7%) were reactive for HAV IgG (Table 2; Appendix Figure 4). In line with lifetime buildup of immunity, the proportion of seroreactive alpacas increased with age. Alpacas >2 years of age were more likely to have HAV antibodies than were younger alpacas; antibodies were present in 76.3% of older alpacas, compared with 46.2% of younger alpacas ($\chi^2 = 6.8$, d.f. = 2, n = 64; p = 0.034).

No sampled animal showed obvious clinical signs of a systemic or hepatic infection. However, we were not able to collect more data on the pathogenicity of HAV infection in alpacas. Other limitations of our study are the small number of samples and the limited geographic sampling range.

### Conclusions

We describe a nonprimate host association of a divergent HAV genotype in alpacas. We detected alpaca HAV RNA in both serum and feces samples, as is typically seen in acute human HAV infections. Signs of seroconversion were common, and seroreactivity increased with age. The relatively high seropositivity rate suggests that infection with alpaca HAV is common. Sequences of alpaca HAV are diversified at the nucleotide level but conserved at the amino acid level. The nucleotide diversity is consistent with a long evolutionary association of HAV with alpacas. Hepatoviruses have been observed to undergo host-switching (14). A spillover event might also have been involved in HAV emergence in alpacas.
Divergent Genotype of Hepatitis A Virus in Alpacas

our data are inconclusive regarding the origin of alpaca HAV and whether alpaca HAV spilled over to or from humans. More camelid and nonhuman primate HAV sequences are needed to resolve this question.

Detecting antibodies using a HAV ELISA kit with human HAV antigens suggests that alpaca HAV might belong to the same serotype as genotypes I–VI. HAV vaccination might thus provide protection from a potential alpaca HAV spillover from alpacas into humans and vice versa. Bolivia is currently considered an area of high-intermediate endemicity of HAV (15); increased local outbreaks and a higher burden of HAV-associated disease are expected. With that in mind, HAV vaccinations, especially for camelid handlers, should be considered to reduce spillover risk.

Acknowledgments
We thank the local communities (Cañuhuma, Chullumpina, Ucha-Ucha) and the Apolobamba national protected area for the permits and cooperation provided. We thank Vanessa Ramos and Jacrya Murillo for their support in the field. The fieldwork was carried out by the Greater Madidi-Tambopata Landscape Conservation Program of the Wildlife Conservation Society. For excellent technical assistance, we thank Philip El-Duah and Jörn Beheim-Schwarzbach from Charité-Universitätsmedizin Berlin—corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany.

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Ms. Veith is a virologist at the Institute of Virology, Charité-Universitätsmedizin Berlin, Berlin, Germany. Her research interests include disease ecology and virus diversity studies.

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References

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In May 2022, we observed a substantial die-off of wild migratory waterbirds on Maliy Zhemchuzhniy Island in the Caspian Sea, Russia. The deaths were caused by highly pathogenic avian influenza A(H5N1) clade 2.3.4.4.b virus. Continued surveillance of influenza viruses in wild bird populations is needed to predict virus spread over long distances.

The coastal and estuarine wetlands of the northern Caspian Sea, which borders southeast Russia, provide support for millions of waterfowl and shorebirds during nesting, molting, migration, and wintering periods ([1,2]); >300 species of birds are found in this region. The area is crossed by several migration flyways (Figure 1), of which the Black Sea/Mediterranean Flyway is the main migratory route ([3]). The region plays a critical role in the reproduction of colonial nesting birds, such as pelicans, cormorants, herons, gulls, and terns.

Maliy Zhemchuzhniy Island is located in the northern part of the Caspian Sea (Figure 1). Monitoring data on waterbirds has shown the high ecologic importance of this area, not only during the nesting period but also during bird migration. The island has had >150 species of birds registered since 2016. A breeding colony of Caspian gulls is located on the island, along with colonies of Great black-headed gulls and Caspian terns, which are all listed in the Red Data Book of Russia. We investigated mass deaths of wild migratory waterbirds on Maliy Zhemchuzhniy Island that occurred in May 2022. The study was approved by the Committee on Biomedical Ethics at the Federal Research Center of Fundamental and Translational Medicine in Novosibirsk, Russia (protocol nos. 2013-23, 2019-3, and 2021-10).

Highly Pathogenic Avian Influenza A(H5N1) Virus–Induced Mass Death of Wild Birds, Caspian Sea, Russia, 2022

Ivan Sobolev, Alimurad Gadzhiev, Kirill Sharshov, Olesia Ohlopkova, Kristina Stolbunova, Artem Fadeev, Nikita Dubovitskiy, Alexandra Glushchenko, Victor Irza, Maxim Perkovsky, Kirill Litvinov, Natalia Meshcheriakova, Guy Petherbridge, Alexander Shestopalov

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The Study

On April 28, 2022, near the end of the egg incubation period, we had counted a total of 26,769 Great black-headed gull nests, 7,340 Caspian gull nests, and 5,267 Caspian tern nests on Maliy Zhemchuzhniy Island. In May, 1 week later, we detected mass deaths of waterbirds on the island comprising 25,157 Great black-headed gulls, 3,507 Caspian gulls, 5,641 Caspian terns, and 14 Dalmatian pelicans (Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0330-App1.pdf). Nearly all gull and tern chicks died during the nesting period. The mass death event began during hatching of Great black-headed gulls. We only found the corpses of chicks (with down but without feathers) that were similar in age. We assume that not all of the chicks actually died from disease; death of adult birds likely led to the deaths of chicks in their nests. The Caspian terns were still incubating eggs at that time; consequently, the death of adult terns led to the death of egg clutches in their nests. We
Influenza A(H5N1)–Induced Death of Birds, Russia

In May 2022, we collected 10 samples from deceased Caspian terns on the island. All samples tested positive for H5Nx avian influenza virus (AIV) by real-time PCR. We characterized 5 isolates by using complete genome sequencing, phylogenetic analysis, and intravenous pathogenicity index testing (Table). We identified all 5 isolates as highly pathogenic avian influenza (HPAI) viruses, according to the amino acid sequence of the hemagglutinin (HA) polybasic proteolytic cleavage site (PLREKRRKR/G) (Appendix 1 Figure 2) and according to intravenous pathogenicity index values of 2.92–2.93 (reference values were for chickens) (Appendix). We determined the HPAI neuraminidase (NA) subtype was N1 by using sequence analysis.

The A/goose/Guangdong/1/96 (Gs/GD) strain, which was isolated in 1996 from a domestic goose, is considered the ancestor of AIV carrying the highly pathogenic H5 HA subtype (4,5). The Gs/GD lineage of HPAI H5N1 viruses evolved into several sublineages that subsequently reassorted with low pathogenicity avian influenza (LPAI) viruses, leading to the formation of H5Nx reassortant variants (6,7). Viruses of the Gs/GD lineage initially circulated in southern China. However, during 2004–2005, they began spreading throughout Asia, Europe, the Middle East, and Africa (8). An increased number of available sequences led to the creation of the H5 virus classification and identification of 10 clades (4,8–13). Subsequently, clade 2 was split into 5 subclades, each containing numerous genetic subgroups. Clade 2.3.4.4 is dominant and has been divided into several genetic subgroups, including 2.3.4.4.b, which, in turn, diverged into 2 sublineage branches, B1 and B2 (14). According to phylogenetic analysis of HA segments, we found the Caspian Sea strains (the AIV strains isolated from dead birds in the Caspian Sea region) belonged to HPAI H5N1 clade 2.3.4.4.b (Figure 2). Furthermore, we found the HPAI H5N1 virus isolates from Maliy Zhemchuzhniy Island belonged to the B2 sublineage because isoleucine was present at position 548 in HA (14).

The polymerase basic (PB) 1, polymerase acidic (PA), HA, nucleoprotein (NP), NA, and matrix (M) protein gene segments of the Caspian Sea strains were phylogenetically related to H5N1 viruses previously identified in wild birds in Israel in January 2022. However, all 8 gene segments were similar to those of strains from Romania isolated in February 2022.

Table. Sequenced viruses isolated from Caspian terns on Maliy Zhemchuzhniy Island in study of highly pathogenic avian influenza A(H5N1) virus–induced mass death of wild birds, Caspian Sea, Russia, 2022

<table>
<thead>
<tr>
<th>Isolate</th>
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<th>Sample type</th>
<th>Collection date</th>
<th>IVPI</th>
<th>GISAID no.</th>
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<tr>
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<td>45°02′ N, 48°19′ E</td>
<td>Intestine</td>
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<td>2.92</td>
<td>EPI_ISL_16020401</td>
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<td>Intestine</td>
<td>2022 May 15</td>
<td>2.92</td>
<td>EPI_ISL_16020402</td>
</tr>
<tr>
<td>A/Caspian_tern/Astrakhan/34/2022</td>
<td>45°02′ N, 48°19′ E</td>
<td>Intestine</td>
<td>2022 May 15</td>
<td>2.93</td>
<td>EPI_ISL_16020403</td>
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<tr>
<td>A/Caspian_tern/Astrakhan/36/2022</td>
<td>45°02′ N, 48°19′ E</td>
<td>Liver</td>
<td>2022 May 15</td>
<td>2.93</td>
<td>EPI_ISL_16020404</td>
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<tr>
<td>A/Caspian_tern/Astrakhan/38/2022</td>
<td>45°02′ N, 48°19′ E</td>
<td>Liver</td>
<td>2022 May 15</td>
<td>2.92</td>
<td>EPI_ISL_16020405</td>
</tr>
</tbody>
</table>

*Nucleotide sequences were deposited in the GISAID database (https://www.gisaid.org); IVPI, intravenous pathogenicity index.
2022 (Appendix 1 Figures 3–9). The phylogenetic relationships of PB2 and nonstructural (NS) gene segments between AIV strains from Israel and Romania remains unclear, because no sequences were available for the segments from Israel in the GISAID EpiFlu database (https://www.gisaid.org).

Conclusions

On the basis of virus phylogeny and chronology of virus detection, we hypothesize that the HPAI virus found in the Caspian Sea region was present in birds migrating to their wintering sites during autumn 2021 and was detected in Israel during the winter months of 2022. During spring migration in 2022, the virus strain spread from the Middle East to nesting areas, leading to wild bird deaths on Mal’y Zhemchuzhniy Island. Because of the lack of some genetic data on closely related viruses, it remains unclear whether the Caspian Sea strains were transmitted through Europe (Romania) from Israel or directly from Israel.

The HPAI H5N1 viruses detected during the mass death of birds on Mal’y Zhemchuzhniy Island evolved from sequential reassortment of multiple genetic variants of LPAI and HPAI viruses (Appendix 1 Figures 3–9). The new variants probably acquired M and HA gene segments from viruses (Egyptian-like) detected in Siberia and Kazakhstan in 2020 (15). PB2, PB1, PA, NP, and NA gene segments from HPAI viruses likely emerged as a result of reassortment with

Figure 2. Phylogenetic analysis of viruses isolated from 5 dead Caspian terns in study of highly pathogenic avian influenza A(H5N1) virus–induced mass death of wild birds, Caspian Sea, Russia, 2022. Maximum-likelihood phylogenetic tree was constructed for hemagglutinin gene segments. Black circles indicate highly pathogenic avian influenza (HPAI) A H5N1 virus strains isolated from the Caspian Sea region; black triangles indicate Egyptian-like HPAI virus strains from Russia isolated in 2020; red box indicates HPAI strains from Israel and Romania that were closely related to viruses from the Caspian Sea. Viruses belonging to clade 2.3.4.4b and B1 or B2 sublineages and those with hemagglutinin genes found in Europe are indicated. Sequences were obtained from the GISAID EpiFlu database (https://www.gisaid.org); identification numbers are provided. Scale bar indicates nucleotide substitutions per site.
Alyamzhimbetova et al. found that gene segments of HPAI viruses from the Caspian Sea were related to variants identified in the Far East, indicating widespread distribution and exchange of influenza virus genes well beyond the major flyways. Therefore, continued surveillance and monitoring of AIVs (primarily HPAI viruses) in wild bird populations will be needed worldwide to track and predict the spread of these viruses over long distances.

Acknowledgments
We thank those persons who provided sequence information to the GISAID’s EpiFlu database (Appendix 2 Table, https://wwwnc.cdc.gov/EID/article/29/12/23-0330-App2.xlsx).

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About the Author
Dr. Sobolev is a researcher at the Research Institute of Virology, Federal Research Center of Fundamental and Translational Medicine, Siberian Branch, Russian Academy of Sciences, Russia. His primary research interest is the molecular diagnosis and epidemiology of avian influenza viruses.

Influenza A(H5N1)–Induced Death of Birds, Russia

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Surveillance for Soil-Transmitted Helminths in High-Risk County, Mississippi, USA

Richard S. Bradbury, Lora Martin, Lacy Malloch, Maygan Martin, John M. Williams, Kayla Patterson, Cameron Sanders, Gurbaksh Singh, Irene Arguello, Eduardo Rodriguez, Paul Byers, Lisa Haynie, Yvonne Qvarnstrom, Charlotte V. Hobbs

Soil-transmitted helminths (STHs) are endemic in resource-limited settings worldwide. Because of its subtropical climate and socioeconomic factors, the southeastern United States historically has been at elevated risk for STH diseases. Because of improved sanitation and economic development, hookworm and other STHs were assumed to be eliminated from the US South (1). Mississippi has many areas with poor sanitation, but STHs have not been reportable since 1984 (2). Locally, expertise in clinical microscopic methods for STH diagnosis is lacking. At the University of Mississippi Medical Center (Jackson, MS, USA), stool samples are sent to the Mayo Clinic (Rochester, MN, USA), for routine ova and parasite examinations.

A recent report of suspected locally acquired cases of strongyloidiasis and hookworm in Alabama (3) initiated a parallel STH surveillance program in historically high-prevalence counties of Mississippi. After review of regions with high-risk criteria, including those with the soil type most conducive to human hookworm transmission (i.e., loamy soil) (4), review of Rockefeller Sanitary Commission data of areas with historically high prevalence, and review of sanitation data with the Mississippi Department of Health, we identified Rankin County as high risk. During 1910–1914, the Rockefeller Sanitary Commission found a very high prevalence of hookworm infection (42.1%) in Rankin County (5). The last formal surveillance study in Mississippi (1932–1933) found the prevalence in this county had decreased to 3.8% (5).

The Study
We conducted a cross-sectional study in which we recruited parents and guardians of children in Rankin County at health fair and community events (February 2020–September 2021) and asked participants to submit 3 stool specimens per participating child (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0709-App1.pdf). Participants submitted specimens directly to designated clinics or drop sites, where study personnel collected the specimens. Because of the connection between STH and poverty (3), we targeted areas within the county that had higher deprivation indices (https://www.neighborhoodatlas.medicine.wisc.edu).

We preserved ≈250-mg aliquots of fresh stool specimens in 70% ethanol at room temperature. For DNA extraction, we washed the specimens once in phosphate-buffered saline, followed by freezing at −80°C for 30 min, snap-thawing at 100°C for 10 min, then bead beating for 1 min with zirconium beads (Benchmark, https://www.benchmarkscientific.com) instead of the kit-supplied beads. Otherwise, we performed DNA extraction according to the manufacturer’s...
instructions. We processed the first 128 specimens by using the SurePrep Soil DNA Isolation Kit (Fisher Scientific, https://www.fishersci.com). This kit was discontinued by the manufacturer during the study, so we validated and processed the remaining 660 specimens by using the PowerFecal ProDNA kit (QIAGEN, https://www.qiagen.com) (6). We immediately stored DNA extracts at −80°C and sent them to the Division of Parasitic Diseases and Malaria, Center for Global Health, at the Centers for Disease Control and Prevention for quantitative PCR (qPCR) analysis.

Where specimen volume allowed, we performed microscopic analysis by using saturated sodium nitrate (NaNO₃) centrifugal flotation as previously described but with a 500 × g (instead of 3,000 × g) centrifugation step (7). We performed Kato–Katz microscopy as previously described (8). We prepared Koga agar plate cultures (APCs) and inoculated as previously described (9). We sealed the plates with parafilm, incubated them at 28°C, and checked for larval tracks on days 3 and 5.

We quality-control tested DNA extracts for PCR inhibitors by using a human cytochrome B qPCR (10). We tested DNA samples without inhibition by using multiparallel qPCR specific for Nector americanus, Ancylostoma duodenale, Trichuris trichiura, Strongyloides stercoralis (11), and Ascaris lumbricoides (12). We considered a cycle threshold value <40 to be positive. We incorporated positive (genomic DNA from worms) and negative (water and DNA extracted from STH-free stool specimens) controls into each qPCR run.

We collected data regarding risk factors for STH by using case report forms from parents or guardians representing 354 children 2–18 years of age at the time of enrollment. The median age of children enrolled was 8 years (interquartile range 5.0–11.5 years); 55.4% were boys and 44.6% girls; 78.2% were White and 16.9% Black (US Census data for Rankin County [https://www.census.gov/quickfacts/rankincountymississippi] indicate the population is 74.5% White and 22.5% Black) (Table 1). Most (94.6%) reported non-Hispanic ethnicity, although US Census data indicate 72.2% are non-Hispanic in this county (Table 1). According to survey responses, 12.5% had traveled outside the United States in the previous 5 years, 7% had prior treatment for an intestinal parasite (80% had treatment for Enterobius vermicularis pinworm), most (89.7%) children had some contact with soil, and all had a flushable toilet (Table 2; Appendix Table [data for children for whom stool specimens were received]).

We received 784 stool specimens from 277 of the 354 survey respondents, representing 129 households (Figure). Three specimens taken over 3 days were submitted by 245 participants, 15 submitted only 2 specimens, and 17 submitted a single specimen. Laboratory processing was performed within 72 hours of specimen collection for 98% of specimens received. Sufficient specimen was present to perform

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>196 (55.4)</td>
</tr>
<tr>
<td>F</td>
<td>158 (44.6)</td>
</tr>
<tr>
<td>Total</td>
<td>354 (100)</td>
</tr>
<tr>
<td><strong>Ethnicity†</strong></td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>15 (4.3)</td>
</tr>
<tr>
<td>Not Hispanic</td>
<td>332 (94.6)</td>
</tr>
<tr>
<td>Prefer not to answer</td>
<td>3 (0.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Total</td>
<td>351 (100)</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td>277 (78.2)</td>
</tr>
<tr>
<td>White</td>
<td></td>
</tr>
<tr>
<td>Black or African American</td>
<td>60 (16.9)</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>AI/AN</td>
<td>0</td>
</tr>
<tr>
<td>NHOPI</td>
<td>0</td>
</tr>
<tr>
<td>White and Black or African American</td>
<td>10 (2.8)</td>
</tr>
<tr>
<td>White, Asian, and AI/AN</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>White and AI/AN</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>White, AI/AN, and NHOPI</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>Prefer not to answer</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>354 (100)</td>
</tr>
</tbody>
</table>

*Median age of children enrolled was 8 years (interquartile range 5.0–11.5 years). AI/AN, American Indian or Alaska Native; NHOPI, Native Hawaiian or Other Pacific Islander.
†Records missing for 3 children.
We performed NaNO₃ flotation microscopy on 731 specimens. We performed Kato–Katz microscopic analysis on 730 specimens and conducted APC on 728 specimens. All NaNO₃ flotations, Kato–Katz microscopy, and APC tests showed no STH. Two participants (0.6%) were positive in 1 of 3 specimens each for *Enterobius vermicularis* eggs by NaNO₃ flotation only.

We extracted DNA from all specimens and subjected them to qPCR analysis. Negative results in a DNA quality-control assay excluded 9 specimens (1.15%) from further qPCR analysis. We subjected the remaining 775 DNA extracts to multiparallel STH qPCR, all of which were negative. Of note, the 9 excluded specimens were all negative by NaNO₃, Kato–Katz microscopic analyses, and APC.

**Conclusions**

Our findings suggest the absence of STH infections among the children surveyed in Rankin County, Mississippi. We suspect that our survey results may not have captured sanitation and hygiene data: a limitation of our survey design was that it did not enquire about the endpoint of flush toilets, so any household effluent released nearby through straight pipes was possibly not detected by our survey tool. Furthermore, a stigma associated with having substandard sanitation or fear of ramifications for the need to install appropriate sanitation may have limited the veracity of some responses. However, our laboratory data are consistent with our prior surveillance in the Mississippi Delta (10) and work with postdiagnostic specimens in Mississippi (2), which demonstrated no human hookworm, *Ascaris* or *Trichuris* spp. infections by microscopic analysis or qPCR. Rare *S. stercoralis* infections were detected previously in Mississippi residents by serologic analysis, but whether those infections were autochthonously acquired, travel-acquired, or chronic or persistent infections many decades after exposure is unclear (2). We have observed sporadic cases of *Ascaris* hookworm infections in Mississippi (C. Hobbs, unpub. data), usually in association with pig farming, and those cases may therefore represent zoonotic acquisition of *A. lumbricoides* pig genotype. Both hookworm and *S. stercoralis* worms are common in children in STH-endemic areas globally but increase in prevalence with age (13,14). Further surveillance of adults might identify rare STH infections, particularly persistent cases of strongyloidiasis.

**Table 2.** Selected risk factors for soil-transmitted helminth infection among 354 school-age children enrolled in study of soil-transmitted helminth infection, Rankin County, Mississippi, USA, February 2020–September 2021

<table>
<thead>
<tr>
<th>Question and answer</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has your child travelled outside the United States in the past 5 years?</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>44 (12.5)</td>
</tr>
<tr>
<td>No</td>
<td>308 (87.5)</td>
</tr>
<tr>
<td>Total</td>
<td>352* (100)</td>
</tr>
<tr>
<td>Has your child been treated for intestinal parasites?</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24 (7)</td>
</tr>
<tr>
<td>No</td>
<td>322 (91)</td>
</tr>
<tr>
<td>Not sure</td>
<td>7 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>353* (100)</td>
</tr>
<tr>
<td>If yes to treatment for an intestinal parasite above, which one?</td>
<td></td>
</tr>
<tr>
<td>Hookworm</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Roundworm</td>
<td>0</td>
</tr>
<tr>
<td>Whipworm</td>
<td>0</td>
</tr>
<tr>
<td>Pinworm</td>
<td>20 (80)</td>
</tr>
<tr>
<td>Not sure</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Total</td>
<td>25 (100)</td>
</tr>
<tr>
<td>Has your child played outside with bare hands or bare feet (has there been contact with soil) in the past 3 years?</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>34 (9.7)</td>
</tr>
<tr>
<td>Sometimes/less than once a month</td>
<td>61 (17.3)</td>
</tr>
<tr>
<td>Often/at least monthly</td>
<td>104 (29.5)</td>
</tr>
<tr>
<td>All the time</td>
<td>151 (42.9)</td>
</tr>
<tr>
<td>Not sure</td>
<td>2 (0.6)</td>
</tr>
<tr>
<td>Total</td>
<td>352* (100)</td>
</tr>
<tr>
<td>If yes to contact with soil as above, how often?</td>
<td></td>
</tr>
<tr>
<td>Daily</td>
<td>31 (12.1)</td>
</tr>
<tr>
<td>Weekly</td>
<td>81 (31.8)</td>
</tr>
<tr>
<td>Monthly</td>
<td>104 (40.8)</td>
</tr>
<tr>
<td>Yearly</td>
<td>39 (15.3)</td>
</tr>
<tr>
<td>Total</td>
<td>255 (100)</td>
</tr>
<tr>
<td>What type of toilet is in the home where your child lives?</td>
<td></td>
</tr>
<tr>
<td>Flushable toilet†</td>
<td>350* (100)</td>
</tr>
</tbody>
</table>

*Data were missing for some of the 354 children.
†Questionnaire response options were flushable toilet, outdoor toilet, or prefer not to answer.
Even though 12.5% of respondents in our investigation had traveled outside of the United States, none harbored travel-acquired helminthic infections.

The prevalence of enterobiasis was much lower in our study compared with the most recent surveillance from the United States, which reported prevalence of 11.6%-38.9% in southern California elementary schools in the early 1980s (15). Our results may represent actual lowered prevalence or may be attributable to the poor sensitivity of NaNO₃ flotation for *E. vermicularis* detection.

Further study is needed in several other counties that have had historically high levels of hookworm infection. However, considered in the context of other recent surveillance studies (2,10), it is becoming increasingly likely that continued transmission of STH is not occurring in this high-risk county in Mississippi.

Acknowledgments

We thank the patients, their parents, and guardians for their participation in this study. We also thank Susan P. Montgomery, Emily Dodd, and Anne Straily.

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Author contributions: R.S.B. cowrote the draft paper, assisted in study design, and provided technical support to University of Mississippi Medical Center. C.V.H. and J.M.W. performed data analysis. G.S., J.M.W., K.P., and I.A. performed microscopic analysis, agar plate culture, and all DNA extractions. L.M., L.M., M.M., C.S., and L.H. conducted specimen collection. P.B. provided input into manuscript writing. Y.Q. analyzed PCR data and reviewed the manuscript. E.R. performed PCR. C.V.H. obtained funding and specimens, designed the study, and supervised and managed ethical approvals at University of Mississippi Medical Center, supervised and aided in specimen collection, salt flotation microscopic analyses, and DNA extraction and cowrote the paper. All authors reviewed and approved the final draft of the paper.

About the Author

Dr. Bradbury is a parasitologist and microbiologist with expertise in laboratory diagnostics and epidemiology. He maintains a research program in parasitic diseases, zoonoses, and parasite diagnostics. Dr. Hobbs is a professor of pediatric infectious disease and attending physician at Children’s of Mississippi and professor of cell and molecular biology at University of Mississippi Medical Center, Jackson, Mississippi, USA. Her research interests include parasitic diseases in children in resource-limited settings.

References


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**EID Podcast**

**Comprehensive Review of Emergence and Virology of Tickborne Bourbon Virus in the United States**

In 2014, the first case of tickborne Bourbon virus (BRBV) was identified in a man in Bourbon County, Kansas. Since its initial identification, at least 5 human cases of BRBV-associated disease have been confirmed in the Midwest region of the United States. Because little is known about BRBV biology and no specific treatments or vaccines are available, further studies are needed.

In this EID podcast, Dr. Christopher Stobart, a microbiologist and associate professor at Butler University in Indianapolis, Indiana, discusses the emergence and virology of tickborne Bourbon virus in the United States.

Visit our website to listen: [https://bit.ly/3w0vefK](https://bit.ly/3w0vefK)
In Europe, dengue primarily is imported from endemic countries. However, an increasing number of autochthonous cases and limited outbreaks have been described since 2010. In 2023, a total of 105 autochthonous cases were reported: 66 in Italy, 36 in France; and 3 in Spain (1). We describe an autochthonous outbreak of dengue in the Paris Region of France during September–October 2023.

The Study

On September 13, 2023, a 36-year-old woman from Limeil-Brévannes, a city 15 km south of Paris, who had no consistent medical history started having symptoms of fever (>38°C), malaise, and headache. Her 7-year-old son experienced the same symptoms during September 11–13 and had a maculopapular rash that disappeared 5 days after the onset. Also, the woman’s partner had symptoms begin on September 14 and had a fever (>38°C), chills, frontal headache, myalgia, a papular rash, and itching on the trunk and upper limbs. The case-patients reported no travel abroad or to other regions of France.

On September 19, the woman continued to be febrile daily and began to have nausea and vomiting. She consulted the emergency department of Hôpital Henri Mondor, Créteil, south of Paris, where blood tests were performed. Tests revealed thrombocytopenia (105,000 cells/μL; reference range 150,000–450,000 cells/μL), leukopenia (700/μL; reference range 1,000–4,000 U/L), and increased alanine aminotransferase (213 U/L; reference range <35 U/L), aspartate aminotransferase (266 U/L; reference range <35 U/L), and gamma-glutamyl transferase (135 U/L; reference range <40 U/L). C-reactive protein was 2.4 mg/L (reference range <5 mg/L). Major and common causes of viral hepatitis were excluded, and serologic tests for hepatitis A–C, HIV, and cytomegalovirus were negatives. Epstein-Barr virus serology was compatible with a previous infection.

Because a diagnosis was not made during clinical examination, we performed arbovirus serologic testing, including for dengue virus (DENV), on September 22. DENV serologic results were IgM positive; thus, we performed a retrospective analysis of blood samples collected on September 19. In that analysis, results for DENV nonstructural protein 1 (NS1) antigen in plasma were positive, as was the real-time reverse transcription PCR (RT-PCR), which could identify DENV type 2 (cycle threshold 35), confirming the diagnosis of acute DENV infection.

We performed a second serologic test 25 days after symptom onset, which showed seroconversion for DENV, IgM persistence, and apparition of IgG (Table). The patient’s son and partner also had DENV serologic testing on October 13, and results were IgM and IgG positive. All 3 patients fully recovered, and no case was classified as severe dengue.

According to national guidelines, we made a notification to the national health authority of France on October 16, which prompted a door-to-door survey in the family’s neighborhood during October 19–20. Pesticide spraying for mosquito control was also performed in the neighborhood.

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DOI: https://doi.org/10.3201/eid2912.231472

These authors contributed equally to this article.
In Europe, since 2010, autochthonous dengue cases have been reported in several countries (1–3). In France, a case was reported in Nice in 2010, after which the number of case reports has been increasing, and 9 events of autochthonous DENV transmission were identified in 2022, resulting in 65 autochthonous cases (3,4). To date, all autochthonous dengue cases occurred in the south of France, and none had been described in the Paris Region.

In the reported family cluster, the virus was probably introduced into mainland France through viremic travelers returning from an endemic area. Indeed, among countries that reported a considerable increase in dengue cases in recent years, some are overseas territories of France (5,6). In an ongoing 2023 outbreak in Martinique and Guadeloupe, DENV-2 serotype has been identified in most cases (7). The cluster we report was caused by DENV-2, but past autochthonous DENV transmission events in France were caused by DENV-1 and DENV-3 (4). Clinicians should consider the serotype of DENV because infection with any DENV serotype will cause an adaptive immune response to develop, which provides short-term immunity against heterologous DENV serotypes (8,9). In addition, priming with 1 DENV serotype can increase the risk for severe dengue upon future infection with a heterologous virus (8,9).

Dengue is transmitted by the bite of an infected female mosquito. The *Aedes aegypti* mosquito, the primary vector, has not been established in continental Europe (10,11). *Ae. albopictus*, a less competent vector, might act as an epidemic driver in areas where *Ae. aegypti* is absent (10,11). Since the 1990s, *Ae. albopictus* has increasingly been detected in Europe and France. In 2022, *Ae. albopictus* was found in 71 of 95 French departments, and since 2015, it has been found in the area where the described family lives (12).

Two main elements might contribute to an increasing risk for autochthonous arbovirus transmission: intensification in international travel observed in recent decades and more stable vector mosquito populations outside previously known endemic areas. International travel raises arbovirus importation in nonendemic countries, and global warming could contribute to establishment of a more stable vector population, such as *Ae. albopictus*, in France. Those factors underline several points from this case report.

Like most viral infections, dengue has a large spectrum of clinical manifestations, as seen in the cases we report. Most persons remain asymptomatic or develop minor symptoms, but ≈25% experience a self-limited febrile illness, accompanied by mild-to-moderate hematologic and biochemical abnormalities (13). Faced with compatible clinical cases without other confirmed diagnoses, clinicians in nonendemic areas should also test for an arbovirus infection during summer and autumn. Case detection is crucial for implementing the necessary public health measures to prevent further virus transmission.

The choice of laboratory test depends on the number of days after illness onset. Before day 7, dengue can be diagnosed by detection of viral RNA or antigens (13). DENV IgM starts to rise from day 4, peak around days 10–14, and then decline and...
disappear after ≈3 months. In primary infections, dengue IgG can be detected at low concentrations by the end of the second week; the concentration increases slowly thereafter and is thought to persist for life (13). Often, paired acute and convalescent samples are required if direct detection of the virus is not available. Diagnosis can be accelerated when clinicians make every effort to obtain RT-PCR or antigenic testing, including reanalyzing samples taken in the viremic phase, when possible, which enables diagnosis without waiting for seroconversion.

Conclusions
In this report, we described a cluster of autochthonous dengue in the Paris Region of France. We assume that this cluster might be only the tip of the iceberg of a wider latent DENV reservoir in the Paris area due to the stable presence of the vector coexisting with viremic persons returning from endemic countries. We believe that paying close attention to the autochthonous transmission of arboviruses is crucial because recent increasing trends in cases, global warming, and the growth of international travel very likely mean that such infections will increase during warm seasons in France. Because of the short stay, implications for tourists visiting Paris are negligible and public health authorities do not recommend specific prophylactic strategies (14). However, for the 2024 Summer Olympic Games, when millions of visitors are expected in Paris, increased attention to tracking arboviruses, including dengue virus, is advisable.

Acknowledgments
The case-patients or guardian provided informed consent for their details to be described in published in articles.

Author contributions: M.Z. and W.V. managed the patients. S.B. performed the second-level laboratory exam. M.Z. and S.G. conceived and wrote the article. G.M. and S.B. reviewed the article. All authors have seen and approved the manuscript.

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Rabies is a viral zoonosis with high mortality rates caused by Lyssavirus rabies lineages (rabies virus, RABV) (1). Opossums of the genus Didelphis are marsupials widely distributed in the Americas, synanthropic in urban scenarios, and considered resistant to RABV (2). The main urban reservoirs of RABV in Brazil are nonhematophagous bats with distinct lineages and epidemiologic aspects (3). In 2021, passive surveillance programs detected an unusual case of rabies in a white-eared opossum (D. albiventris) by a RABV lineage of frugivorous bats of genus Artibeus spp. in Campinas, São Paulo state, Brazil, the 10th most urbanized city in the country (4). To elucidate the dynamics of this spillover, we describe the results of passive surveillance for rabies in bats and opossums in Campinas in 2021.

The Study
In 2021, we tested samples of frozen brain tissue from 930 bats and 22 opossums for rabies by direct fluorescent antibody test and confirmed infection by virus isolation in cell culture (5) in Campinas. Fixed formalin brain tissue fragments in 15 of these 22 opossums were analyzed by histopathology. In addition, for the opossum that tested positive for rabies, we performed reverse transcription PCR and subsequent phylogenetic analysis of the glycoprotein gene of RABV in the frozen brain tissue and conducted immunohistochemical analysis for rabies in fixed formalin tissues (cerebrum, cerebellum, heart, lungs, liver, spleen, kidney, and adrenal glands) (Appendix 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0373-App1.pdf; Appendix 2, https://wwwnc.cdc.gov/EID/article/29/12/23-0373-App2.xlsx). Ethics approval was granted by the Ethics Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science, University of São Paulo (approval no. 8227140222), according to the Ethical Principles in Animal Research.

Of the 22 opossums tested for rabies, 1 (4.5%) adult female white-eared opossum (D. albiventris) had a positive result. Death was caused by traumatic lesions in 10 (45.4%) opossums; 4 (18.2%) of those deaths were caused by interspecies interactions with dogs. Of the 15 opossums analyzed by histopathology, 14 (93.3%) were found in the urban zone, inside households in densely urbanized areas, or in residences on the

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DOI: https://doi.org/10.3201/eid2912.230373

1These first authors contributed equally to this article.
2These senior authors contributed equally to this article.
3These authors were co–principal investigators.
outskirts of the city; death was caused by traumatic lesions in 10 (66.7%) opossums, 4 of those deaths were caused by interspecies interactions with dogs. On histopathologic examination, we observed no lesions in 8 opossums, hemodynamic lesions in 4, autolysis in 2, and mononuclear meningoencephalitis in the rabies-positive opossum (Appendix 1 Figure 1). In the rabies-positive opossum, RABV antigen was detected by immunohistochemistry in the cerebrum (Appendix 1 Figure 2), cerebellum, adrenal gland, liver, and heart. The RABV-positive opossum was found in a zoo located within a park in the urban center of Campinas and demonstrated signs of the paralytic form of RABV infection. Phyllogenetic reconstruction demonstrated that the RABV clustered within the frugivorous fruit-eating bats (Artibeus spp.) lineage circulating in Brazil (GenBank accession no. ON604858) (Figure 1).

During 2021, the frequency of rabies detected in bats was 3.2% (30/930). Among the rabies-positive bats, 17 (56.7%) were frugivorous species of fruit-eating bats (Artibeus spp.); the other 13 (43.4%) were insectivorous bats of Eptesicus spp., Myotis spp., and Tadarida spp. (Appendix 2 Table 3). In total, bats from those 4 genera represented 153 (16.4%) of the total bats investigated. Bats were tested periodically, and different seasonality peaks were noted in frugivorous and insectivorous bats (Appendix 1 Figures 5, 6). Rabies-positive bats were found in the urban perimeter of the

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Figure 1. Rabies virus G gene phylogenetic tree showing specific clusters for different genera of bats in Brazil and dog-related samples in study of naturally acquired rabies in a white-eared opossum, Brazil (red text). The phylogeny was reconstructed by maximum-likelihood estimation from nucleotide sequences. Bootstrap values of >50% are depicted (1,000 bootstrap replicates). CVS corresponds to a fixed strain of the rabies virus. European bat lyssavirus-1 was used as an outgroup. The tree was visualized using iTOL version 6 (6). GenBank accession numbers are provided for reference sequences.
municipality of Campinas; 73.3% were found in areas of sparse vegetation and 26.7% in areas of remnants of vegetation (Appendix 1 Table). We identified bats in a regular spatial distribution throughout the city; we observed a small area of concentration in the north and a slight concentration of rabies-positive bats in the center of the city (Appendix 1 Figure 3). According to genus classification, *Artibeus* spp. bats were found in medium and high concentrations and overlapped spatially with a high concentration of insectivorous bats. Of note, opossums were found near areas of medium to high bat concentrations, and the rabies-positive opossum was captured in a vegetated area with a high concentration of *Artibeus* spp. bats (Figure 2). We also found a spatial diffusion of *Artibeus* spp. bats that overlapped with the rabies-positive opossum (Appendix 1 Figure 4), demonstrating a time overlap in August 2021.

**Conclusions**

Experimental virus inoculation in the 1960s led to initial suggestions of resistance to infection by RABV in *Didelphis* spp. opossums. (7). Reports of RABV in opossums are scarce; their low body temperature (34.4–36.1°C [94–97°F]) and the minimal possibility of surviving an attack by a rabid animal have been suggested as probable causes of the low prevalence of this disease in opossums in North America, where wild carnivorous mammals are natural reservoirs (2). Despite the low reports of rabies in opossums, a seroprevalence study conducted in São Paulo state observed a prevalence of RABV of 1.6% (5/312) in *Didelphis* spp., indicating contact between this animal population and RABV (8). Neurologic signs demonstrated by the rabies-positive opossum in this study are associated with paralytic form rabies, a common form transmitted by bats (9), and detection of viral particles in other organs indicates a phase of systemic spread. Interspecies interactions with bats in urban centers could be hypothesized as a route of RABV to the opossum, as has been observed in recent episodes of RABV in cats in Campinas (10,11). Unlike the scenario described in North America (2), opossums might survive interactions with...
bats. Opossum deaths detected in this study occurred in anthropic areas of the city; they were more prevalent in homes and were caused by traumatic events, such as attacks by dogs, warning of the possible risk for infection with RABV in domestic animals.

Frigivorous and insectivorous bats are reservoirs of RABV in urban centers of South America; bat lineages are replacing RABV canid lineage after successful vaccination efforts were adopted in Brazil in the dog population (11,12). The spatial distribution of captured bats and opossums revealed an overlap in habitats between rabies-positive bats and opossums in urban areas. The rabies-positive opossum was found in a vegetated area within a very urbanized area densely occupied by Artibeus spp. bats; those areas of dense bat population might create conditions in which rabies transmission, and development of new hosts and strains, is more tied to ecologic factors than to the phylogenetic characteristics of the hosts (13).

In São Paulo state, vaccination campaigns for dogs and cats were discontinued after dog RABV lineages had not been detected for >20 years. Spillover cases such as those described in this study indicate the importance of wildlife mammal surveillance to detect RABV, particularly in urban areas, where those animals can assume the role of host and act as a source of infection for humans. Spatial analysis can be a powerful tool to assist in rabies surveillance. Although some studies have conducted mapping of bat populations in cities in Brazil (11,14), such studies are scarce and need structured surveillance programs with trained teams. In addition, we noted seasonality in the RABV bat genus and in rabies-positive bats; insectivorous bats were commonly positive in summer and spring and the frugivorous genus Artibeus bats were more commonly positive in fall and winter, as described by Dias et al. (11).

This case shows that opossums are susceptible to rabies and can potentially acquire RABV from bats, as was suggested by the ecospatial analysis. Elucidating this possibility—through the detection of the dead opossum—occurred through integrated surveillance involving motivated field and laboratory teams. Our findings highlight the need for continuous surveillance of wildlife to clarify the dynamics of zoonotic diseases and to prevent their occurrence in humans and domestic animals, in agreement with a One Health approach.

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Serosurvey results for Crimean-Congo hemorrhagic fever virus antibodies in dromedary camels in Algeria indicate that the pathogen is circulating endemically in desertic areas, despite the hostile environment. Thus, dromedaries are suitable sentinels for detecting human risk for Crimean-Congo hemorrhagic fever in desertic areas.

Crimean Congo hemorrhagic fever virus (CCHFV) is a tickborne Orthobunyaviruse that causes a potentially fatal hemorrhagic systemic disease in humans, Crimean-Congo hemorrhagic fever (CCHF). The virus is sustained in the ecosystem through wild and domestic animals, which act as tick amplification hosts and are asymptomatic (1). CCHF has recently increased in Africa and is emerging in new regions (2). However, knowledge of CCHFV epidemiology in North Africa is limited.

In Algeria, CCHFV has been detected in ticks (3), but no human cases have been reported, probably because of inadequate surveillance (2). In recent years, breeding of dromedary camels (Camelus dromedarius) has increased (4); dromedaries could be ideal indicators of CCHFV circulation because they are widely distributed across Algeria and are commonly reared in open environments with exposure to ticks. To evaluate the distribution of the virus and the potential risk factors associated with CCHFV exposure, we conducted a serosurvey of CCHFV in dromedaries from the northeastern Saharan region of Algeria.

During 2020–2021, we collected 294 serum samples from dromedaries, of which 215 were from 23 different herds, and 79 samples from an abattoir, all from a region that included 4 provinces (wilayas): Biskra, El Oued, Touggourt, and Ouaregla. We tested samples for CCHFV antibodies by using a commercial kit (ID Screen CCHF Double Antigen Multi-species ELISA; IDvet, https://www.id-vet.com). We obtained data on risk factors associated with the individual animals (e.g., age) and management (e.g., breeding system) and evaluated their effect on CCHFV seropositivity with a mixed-effect logistic regression model with herd as a random effect, using R software (The R Project for Statistical Computing, https://www.r-project.org). We also collected data with regard to patterns of movements (i.e., duration and seasonality) for grazing. To evaluate the characteristics of the environment in which the dromedaries were reared, we created a buffer area around each herd (with a 100-km radius, considering the pattern of movements), and obtained the features of the land cover (5).

Animal-level CCHFV seroprevalence was 75.5% (95% CI 69.9–79.8; 222/294), and herd-level seroprevalence was 95.7% (95% CI 93.3–98.0; 22/23) (Figure). Odds of being seropositive for CCHFV were higher among dromedaries bred in the traditional system (i.e., grazing outdoors all year) (7.2, 95% CI 1.1–48.7) and the semitraditional system (i.e., grazing outdoors all year except for winter) (4.5, 95% CI 1.04–19.1) than among animals kept in permanent confinement. Odds of being seropositive were also higher among animals 4–10 years of age (6.5, 95% CI 2.2–19.5) and animals >10 years of age (14.9, 95% CI 3.2–69.4) than among animals <4 years of age (Table). The environment in which the dromedaries grazed was composed essentially of sandy desert (48.0%), bare areas (26.1%), and consolidated bare areas (i.e., bare rocks or stones) (11.5%).

Our results show that exposure of dromedaries to CCHFV is widespread; seroprevalence was high at both the herd and individual animal levels. Individual seroprevalence (74.8%) was similar to that reported in other countries of North Africa (6,7), suggesting that dromedaries may play a role in the epidemiology of CCHFV. Increased age was associated with higher CCHFV seroprevalence, which probably indicates that the virus has been endemic for some years. However, our finding that 22/56 (39.3%) dromedaries ≤1 year of age were seropositive indicates intense recent CCHFV circulation in the area. Traditional and semitraditional breeding systems increased the likelihood of CCHFV seropositivity because of the increased probability of exposure to CCHFV-infected ticks. Traditionally, dromedaries have been reared in constant movement.
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across large pastoral areas in the Sahara desert, but nomadism is being replaced by transhumance (i.e., shorter and seasonal movements), especially in the northeastern Saharan region of Algeria (8). None of the herds in our study was nomadic; most movements were <4 days, implying that seropositive animals were exposed within the study area. Therefore, CCHFV circulation in dromedaries from this region most likely maintains itself without the need for repeated introductions from neighboring areas.

Attempts to map the distribution of CCHF risk have indicated that the areas at risk in Africa were basically restricted to the sub-Saharan region, where CCHF was associated with the presence of shrub or grassland (9). Because we found that bare or sandy desert areas are also favorable for CCHFV transmission, more studies are needed to evaluate animal hosts and tick vectors involved in CCHFV spread in those areas. Moreover, in the northeastern Saharan region of Algeria, the practice of breeding dromedaries in peri-urban areas has recently increased (10), which could increase the risk for human exposure to CCHFV. Developing a robust surveillance system for detecting human cases and monitoring CCHFV infection in peri-urban dromedaries is essential for early detection of the risk and implementation of preventive measures.

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Microfilaremic Dirofilaria repens Infection in Patient from Serbia

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We report a case of *Dirofilaria repens* infection causing microfilaremia in a patient from Serbia. Serum samples tested positive for *D. repens* IgG by ELISA. Our findings and those of others suggest the parasite’s progressive adaptation to humans. Clinicians should be aware that microfilaremia can develop during *Dirofilaria* spp. infections.

*Dirofilaria repens* is a vectorborne filarial helminth of carnivores, mainly domesticated dogs (1). Humans are considered accidental hosts, in which the parasite induces local inflammation causing granulomatous reactions primarily detected in subcutaneous and ocular tissues. Because humans are not natural hosts, microfilariae are typically absent from peripheral blood; thus, diagnostic procedures require morphologic and molecular analyses of removed worms (2). Immunodiagnostic tests are being designed as potential alternatives to invasive diagnostic procedures (3). This parasite rarely evades the human host’s immune system to reach sexual maturity. The literature reports 22 cases of human *D. repens* microfilaremia, of which several have been confirmed through molecular examination (3,4). We describe a case of human dirofilariosis with circulating microfilariae in a patient from Serbia.

A 43-year-old professional soldier in the army of Serbia was first seen for a walnut-sized swelling accompanied by itching on the inner side of his thigh, which we promptly treated with ciprofloxacin (1 g/d) for 14 days. Two months after the initial swelling, the patient noted another similar protuberance on his inner thigh that migrated toward the back of the thigh every 2–3 days. An ultrasound detected a 13.5 × 8 mm subcutaneous nodule. Biochemical analyses of the patient’s blood and blood cell counts were within reference ranges,
including eosinophil levels; however, surgical intervention was required 1 month after the ultrasound to excise the nodule and investigate its origin. Examination of the removed nodule revealed a *Dirofilaria repens*–like specimen. Twenty days after nodule removal, the percentage of eosinophils in the patient’s peripheral blood increased to 14%. We performed a modified Knotts test on EDTA blood, which revealed the presence of 2 microfilariae/mL. The patient had an eosinophil count within reference ranges. No further microfilariae were detected in the peripheral blood smears during monitoring. Two months after the *D. repens* diagnosis, the patient had no symptoms or complications.

In conclusion, we report a case of *D. repens* microfilaremia in a professional soldier frequently exposed to vector biting because of prolonged outdoor activities during military training in northern Serbia, a dirofilariasis-endemic area (10). The presence of circulating microfilariae highlighted by this and previous studies shows the parasite’s progressive adaptation to humans and suggests a potential role for humans as a definitive *D. repens* host. Although a role for humans as an infection reservoir remains to be clarified, clinicians should be aware that microfilaremia can develop during *Dirofilaria* spp. infections.

**Figure.** Microscopic image of *Dirofilaria repens* microfilaria in case study of microfilaremic *D. repens* infection in patient from Serbia. A blood sample from the patient was processed and stained with methylene blue. Scale bar indicates 200 μm.
Anthropogenic Transmission of SARS-CoV-2 from Humans to Lions, Singapore, 2021

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In Singapore, 10 captive lions tested positive for SARS-CoV-2 by real-time PCR. Genomic analyses of nanopore sequencing confirmed human-to-animal transmission of the SARS-CoV-2 Delta variant. Viral genomes from the lions and zookeeper shared a unique spike protein substitution, S:A1016V. Widespread SARS-CoV-2 transmission among humans can increase the likelihood of anthropogenesis.

We investigated natural SARS-CoV-2 infection in captive African (Panthera leo) and Asiatic (Panthera leo persica) lions at a zoo in Singapore during increased Delta variant community infections. Understanding virus dynamics in different hosts is crucial for preventing interspecies transmission and protecting endangered species (1,2).


Eighteen zookeepers cared for and had close (within ≈1 m) but not direct contact with the lions. Six zookeepers tested COVID-19-positive beginning November 1, 2021, and 4 experienced mild respiratory symptoms starting on November 2.

To minimize stress on the animals, only 2 lions that had more severe signs, AS-M1 and AS-F1, were anesthetized for nasal and oropharyngeal sample collection on November 8. On November 9, we confirmed SARS-CoV-2 infection in the lions by real-time reverse transcription PCR (rRT-PCR); cycle quantitation (Cq) values were <40. Nasal swab samples from AS-M1 and AS-F1 had the highest viral loads (Cq 23.05 for AS-M1, 24.47 AS-F1). We conducted noninvasive infection monitoring for 3 weeks by collecting...
and testing individual and pooled fecal samples from both lion enclosures; AF-M3 had the highest fecal sample viral load, Cq 36.02.

Within 5 days of the index case, 10 lions (all 9 Asiatic and 1 African) were infected. Most (8/10) clinically recovered from respiratory signs within 2 weeks; 2 lions took longer to recover, but all animals had recovered by December 3, 2021. Full recovery in the lions was determined by low viral RNA loads (Cq >40), absence of clinical signs, and resumption of normal behavior.

We sequenced RNA from nasal swab samples of AS-M1 and AS-F1 and 1 fecal sample from AF-M3 on the MinION R9.4.1 (Oxford Nanopore Technology, https://nanoporetech.com) platform using ARCTIC-CoV V1/V3 protocols (J.R. Tyson et al., unpub. data, https://doi.org/10.1101/2020.09.04.283077). The 3GS analysis pipeline from Genome Detective (3) generated preliminary contigs, which we stitched together by using sequence alignment information from a zookeeper’s publicly available SARS-CoV-2 sequence (GISAID accession no. EPI_ISL_6600690; https://www.gisaid.org). We assessed the assembled sequences by using NextClade (4) to identify mutations and frameshifts compared with a wild-type reference sequence (GenBank accession no. NC_045512.2) and subsequently corrected alignment artifacts in the bam file (Appendix 2, https://wwwnc.cdc.gov/EID/article/29/12/22-1916-App2.xlsx).

We assembled 2 complete SARS-CoV-2 genomes (GenBank accession nos. OP393893.1 and OL677176.2) from nasal swab samples collected from AS-M1 and AS-F1. We obtained a partial genome assembly from a fecal sample from AF-M3 but did not analyze it further.

We conducted a phylogenomic analysis on 39 complete viral genomes, comprising 2 genomes from lions in this study and 37 sequences from GISAID, including the zookeeper’s sequence. We built a maximum-likelihood tree by using RAxML-ng version 1.1.0 (https://github.com/stamatak/standard-RAXML) with 2,000 bootstrap replicates and used the wild-type reference sequence as the outgroup. The tree revealed that sequences from the zookeeper and Asiatic lions nested within the same subclade (Figure). Those findings and the high (99.98%) viral genetic similarity between the lions and zookeeper strongly suggest that SARS-CoV-2 infection in the

Figure. Maximum-likelihood phylogenomic tree from a case of anthropogenic transmission of SARS-CoV-2 from humans to lions, Singapore, 2021. Tree reconstructed from sequences of 2 lions and 1 zookeeper (red bold text), along with 36 other publicly available sequences representing 4 variants of concern from Singapore, cases of infected lions from the Bronx Zoo, and the wild-type reference genome (GenBank accession no. NC_045512.2) as the outgroup. Scale bar indicates nucleotide substitutions per site. EPI, GISAID (https://www.gisaid.org) EpiFlu database.
lions occurred through a human-to-animal (anthropogenic) transmission route.

We used Pangolin (https://github.com/cov-lineages/pangolin) to identify the subclade as Delta variant (AY.23.1), consistent with the predominant circulating strain in Singapore at that time. Both lions’ sequences had 10 key Delta variant spike protein mutations and 2 open reading frame 8 amino acid deletions at positions D119- and F120-, compared with sequences from cases in Singapore (Appendix 1) (5). The lions, zookeeper, and 1 community case shared a unique spike protein mutation (S:A1016V), suggesting a potential founder’s effect from this anthropogenic transmission event. Our investigation determined that the zookeepers were likely infected 6 days before the lion index case. The lions were not vaccinated against SARS-CoV-2, but 94% of the population of Singapore was fully vaccinated by November 2021.

This study highlights the vulnerability of captive and endangered animal populations to SARS-CoV-2 transmission from humans (5–8). Close contact between zookeepers and the lions likely led to the transmission, emphasizing the crucial need for strict infection control measures in captive animal facilities, especially during periods of increased community transmission of viruses (7).

The implications of SARS-CoV-2 infection in captive lions extend beyond animal health and welfare and can have consequences for the conservation of protected species. Insights from studies on minks and hamsters shed light on the potential for animal-to-human transmission (6,8). However, mass culling, as noted in those studies of small mammals, is an impractical approach for large or endangered animal species.

Lions already face numerous threats, including habitat loss, poaching, and disease; introduction of a novel virus like SARS-CoV-2 could have devastating consequences for their populations (7). Therefore, strengthening biosecurity measures in wildlife conservation centers and promoting vaccination of susceptible animal species whenever feasible and safe are crucial for mitigating viral transmission and protecting vulnerable wildlife populations (1,9).

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All data are available in the main text or the Appendix materials and can be found on the Zenodo repository at https://doi.org/10.5281/zenodo.8362203. Raw sequence reads generated in this study have been uploaded to the National Center for Biotechnology Information Sequence Read Archive under BioProject no. PRJNA1021696.

The ethical handling of animal subjects during the course of this research has been supervised and approved by National Parks Board, Singapore.

Author contributions: C.L., J.O., and W.K.W. conducted the molecular diagnostic tests; Y.C.A.I. performed the nanopore sequencing for viral genome assembly. Y.C.A.I and A.T. performed data analyses. The figures and the manuscript draft were prepared by Y.C.A.I. with input from C.J.F. Study initiation and guidance was by K.B.H.E, and supervised by C.J.F., S.F.C., Y.H.H., and K.B.H.E. Y.C.A.I. wrote the manuscript and all authors contributed to the manuscript editing process, approved the final version for publication, and declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References
We report a massive mortality of 5,224 sea lions (Otaria flavescens) in Peru that seemed to be associated with highly pathogenic avian influenza A(H5N1) virus infection. The transmission pathway may have been through the close contact of sea lions with infected wild birds. We recommend evaluating potential virus transmission among sea lions.

The panzootic (2020–2023) caused by the highly pathogenic avian influenza (HPAI) A(H5N1) caused numerous global outbreaks in 2022 (1). At the end of the year, the H5N1 virus reached South America, causing alarming bird mortalities in Peru (2). Comprehensive surveys suggest the virus killed >100,000 wild birds by the end of March 2023 only in protected areas (and >200,000 birds including other areas); particularly affected were Peruvian boobies (Sula variegata), guanay cormorants (Leucocarbo bougainvilliorum), and Peruvian pelicans (Pelecanus thagus) (3). The large biomass of infected wild birds may have led to a spillover event affecting marine mammals cohabiting with them, as reported in other parts of the world (4). Here, we report the death of several thousand sea lions (Otaria flavescens) on the coast of Peru within a few months; the sea lions manifested neurologic and respiratory signs. Clinical signs we observed suggest they were affected by HPAI H5N1, which was later confirmed by government and scientific reports (5,6).

During January–April 2023, we performed detailed surveillance of dead and agonal sea lions in protected marine areas of Peru (Figure). We found 5,224 animals dead or dying on beaches (Table). The synchronized high mortality rate we observed was concerning: up to 100 dead animals were found floating together in the sea, and 1,112 animals died on 1 island that has one of highest populations of sea lions in Peru (San Gallan, Ica, Reserva Nacional Paracas; Table). Those unprecedented massive mortalities for this region and even the entire world killed ≈5% of Peru’s population of this species in a few months (Figure, panels A, B; Appendix Figure, https://wwwnc.cdc.gov/EID/article/29/12/23-0192-App1.pdf) (7).

Mass Mortality of Sea Lions Caused by Highly Pathogenic Avian Influenza A(H5N1) Virus

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1These two authors contributed equally to the study.
The animals also showed respiratory signs such as dyspnea, tachypnea, and nasal and buccal secretions (Figure, panel C). The body condition of the necropsied sea lion ranged from good to very good. We observed substantial quantities of whitish secretions filling the upper respiratory tract (trachea and pharynx) (Figure, panel C). Lungs were congestive, with hemorrhagic focus compatible with interstitial pneumonia. Brain was also congestive, with hemorrhagic focus compatible with encephalitis (Figure, panel D).

Given the epidemiologic situation produced by HPAI H5N1 in wild birds that cohabit with the sea lions (2,3), the most plausible diagnosis causing this mass mortality event was acute disease caused by the virus. Clinical signs observed were similar to those reported in marine mammals infected with HPAI H5N1 in the United States (4). Official information from the Peru government and associated scientific research confirmed that not only birds but also sea lions tested positive for H5N1 virus (3,5,6). As of April 2023, sea lion deaths have surpassed 5,000 in Peru; thousands of sea lions with similar clinical signs died in Chile (8). This massive mortality event associated with HPAI H5N1 could be attributed to the large aggregations of sea lions that occur during the December–March breeding season (9).

In conclusion, sea lions in Peru experienced a deadly outbreak of disease that has caused mass deaths in several regions of the coastline (Figure). The sea lion mass mortality we described is compatible with systemic HPAI H5N1 that resulted in acute encephalitis and pneumonia. The source of the H5N1 virus affecting these sea lions was most probably the large number of infected live birds or their carcasses on the Peru coastline (2,3). Sea lions may be infected by close contact with those carcasses and through consuming them (Figure, panel E). The potential for direct transmission among sea lions from their colonial breeding behavior, in which they congregate by hundreds in the same area, should be evaluated, as should the large number of animals affected and the findings that many animals died simultaneously in groups in both Peru and Chile. Recent research described potential mammal-to-mammal infection in minks (Neovison vison) (10). In fact, unique mutations that merit further surveillance were found through viral sequencing of some of the deceased sea lions we surveyed (5).

Further research is required to confirm the HPAI H5N1 virus as the main factor affecting the sea lions and to address the transmission pathway in this social species. We call for more attention to human–infected animal interaction in this geographic region (Figure, panel F) to identify any rise in infections and prevent a new pandemic.
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Influenza Resurgence after Relaxation of Public Health and Social Measures, Hong Kong, 2023

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Soon after a mask mandate was relaxed (March 1, 2023), the first post–COVID-19 influenza season in Hong Kong lasted 12 weeks. After other preventive measures were accounted for, mask wearing was associated with an estimated 25% reduction in influenza transmission. Influenza resurgence probably resulted from relaxation of mask mandates and other measures.

To control COVID-19, Hong Kong, China, put in place several public health and social measures (PHSMSs), including mandatory mask wearing, school closures, hand hygiene, and avoidance of gatherings. In early 2020, those measures also reduced influenza transmission (1), and according to laboratory surveillance records, influenza virus did not circulate in the community for 3 years (2). From mid-2022 through 2023, PHSMSs were progressively relaxed, and on March 1, 2023, the local mask mandate was lifted. We investigated the effects of PHSMSs on influenza transmission in Hong Kong.

We collected weekly influenza-like illness consultation rates reported by private general practitioners and the weekly proportion of sentinel respiratory specimens that tested positive for influenza virus in Hong Kong during October 2010–May 2023. We established a proxy for influenza virus activity by multiplying rates of influenza-like illness by the proportion of influenza-positive samples following previous studies (3,4) (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0937-App1.pdf). We found that weekly influenza activity had decreased to almost zero since March 2020, when PHSMSs against COVID-19 began (Figure). Before mandatory on-arrival quarantine of travelers started on September 26, 2022, only sporadic influenza-positive samples were detected by surveillance, all from travelers or children who had recently received live-attenuated influenza vaccine (5). After travel restrictions were removed, sporadic influenza detections increased, but overall
activity remained low. After mandatory indoor and outdoor mask wearing restrictions were lifted on March 1, 2023, influenza transmission increased substantially; the first influenza season after COVID-19 in Hong Kong started and peaked on April 9, ended on May 25, and lasted for 12 weeks (6).

Because various other PHSMs were implemented concurrently with the mask mandate, resurgence of influenza activity could not be attributed to relaxation of the mask mandate alone. Therefore, we used a previous approach that estimated the time-varying effective reproductive number ($R_t$) (7) and a multivariable log-linear regression model on $R_t$ that could allow for adjustment of other factors affecting influenza transmission, including depletion of susceptible persons, seasonal differences, and meteorologic predictors and preventive measures (Appendix). Because the predominating influenza strain in 2023 was influenza A(H1N1)pdm09, we identified previous influenza A(H1N1)pdm09 epidemics that had occurred during 2010–2020. To construct a preventive score, we used data from cross-sectional telephone surveys among the general adult population in Hong Kong from 2020 to 2023 as a proxy for the intensity of preventive measures, other than mask wearing, against COVID-19 (1). The preventive score included the average proportion of persons who avoided visiting crowded places, avoided going to healthcare facilities, avoided touching or use protective measures when touching public objects, and washed hands immediately after going out. Before 2020, the proportion of those preventive measures was established as baseline. When constructing a preventive score, we compared the Akaike information criterion of 4

**Table.** Effects of public health and social measures to protect against COVID-19 on $R_t$ for influenza, Hong Kong, 2010–2023*

<table>
<thead>
<tr>
<th>Model†</th>
<th>PHSM description</th>
<th>% Change in $R_t$ (95% CI)</th>
<th>$\Delta$AIC‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Mask</td>
<td>Avoid social gatherings. Wash hands after being outside. Avoid touching or use protective measures with shared objects.</td>
<td>−25 (−43 to −1)</td>
</tr>
<tr>
<td>Preventive score 1</td>
<td></td>
<td></td>
<td>−82 (−91 to −63)</td>
</tr>
<tr>
<td>Model 2</td>
<td>Mask</td>
<td>Avoid going out as much as possible. Wash hands after being outside. Avoid touching or use protective measures with shared objects.</td>
<td>−26 (−44 to −2)</td>
</tr>
<tr>
<td>Preventive score 2</td>
<td></td>
<td></td>
<td>−80 (−91 to −55)</td>
</tr>
<tr>
<td>Model 3 (main model)</td>
<td>Mask</td>
<td>Avoid going to crowded places. Avoid going to healthcare facilities. Avoid touching or use protective measures with shared objects.</td>
<td>−25 (−43 to −1)</td>
</tr>
<tr>
<td>Preventive score 3</td>
<td></td>
<td></td>
<td>−77 (−88 to −60)</td>
</tr>
<tr>
<td>Model 4</td>
<td>Mask</td>
<td>Avoid going to crowded places. Avoid going to healthcare facilities. Avoid touching or use protective measures with shared objects. Wash hands after being outside.</td>
<td>−24 (−43 to 0)</td>
</tr>
<tr>
<td>Preventive score 4</td>
<td></td>
<td></td>
<td>−81 (−90 to −62)</td>
</tr>
</tbody>
</table>

*AIC, Akaike information criterion; $R_t$, time-varying effective reproductive number.
†Models were adjusted for depletion of susceptible persons, between-season effects, and absolute humidity.
‡$\Delta$AIC$_{model i} = $AIC$_{model i} − $AIC$_{min}$. $\Delta$AIC$_{min} = $min(AIC$_{model i}$), $i = 1,...,4$.
combinations of those protective measures. Meteorologic variables provided by the Hong Kong Observatory (http://hko.gov.hk) were temperature, wind speed, and relative and absolute humidity. To quantify the effects of meteorologic variables, we fitted the models to data before the COVID-19 pandemic.

Among the 9 epidemics of 2010–2023, the estimated $R_t$ varied from 0.62 to 1.38 (median 1.02) (Appendix Figure 1). The estimated $R_t$ showed a decreasing pattern in each season, ranging from >1.2 at the beginning of an epidemic period to 0.8 at the end of an epidemic period. After model selection (Appendix), we found that a model of absolute humidity, mask wearing, and preventive score 3 (Table) explained 92% of the observed variance in estimated $R_t$ (Appendix Table 1). Changes in absolute humidity (Appendix Figure 2, panel A), the proportion of mask wearing, and preventive score 3 (Appendix Figure 2, panel B) strongly correlated with changes in $R_t$. After adjusting for other factors, such as depletion of susceptible persons, between-season effects, and absolute humidity, we found that mask wearing was associated with a 25% (range 1%–43%) reduction in $R_t$ and that other preventive measures (combined) were associated with a 77% (range 60%–88%) reduction (Table).

We found that that influenza increased after PHSMs were relaxed and influenza transmission increased shortly after the mask mandate was relaxed. Our results are consistent with those of several studies that found that PHSMs against COVID-19 may reduce influenza transmission (8) and that mask wearing may have a lower moderate protective effect against influenza virus transmission in the community (9,10).

A limitation of our analysis was that we used results of survey reports to generate a proxy of intensity of implemented PHSMs over time, which may not be accurate. Also, we used a proxy measure of influenza activity based on surveillance data, and the reliability of our analysis depended on the accuracy of this proxy. In addition, influenza vaccination coverage (Appendix Figure 5) was not included in the model because our model included the effect of vaccination via season-specific intercept. Nevertheless, our study results suggest that the resurgence of influenza after relaxation of PHSMs was most likely affected by the lifting of mask mandate and other PHSMs.

Acknowledgments
We thank Julie Au for technical assistance.

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Ms. Xiong is a PhD candidate at the School of Public Health, University of Hong Kong. Her research interests are infectious disease epidemiology and modeling and development of statistical approaches for infectious disease analysis.

References
SARS-CoV-2 Variants BQ.1 and XBB.1.5 in Wastewater of Aircraft Flying from China to Denmark, 2023

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Author affiliation: Statens Serum Institut, Copenhagen, Denmark

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We analyzed wastewater samples from 14 aircraft arriving in Denmark directly from China during January 9–February 12, 2023. Wastewater from 11 aircraft was SARS-CoV-2–positive by PCR; 6 predominantly contained BQ.1 and XBB.1 subvariants. Wastewater-based surveillance can contribute to public health monitoring of SARS-CoV-2 and other emerging infectious agents.

Relaxation of China’s zero-COVID policy in December 2022 led the European Centre for Disease Prevention and Control to recommend several nonpharmaceutical interventions to curb COVID-19 spread and monitor any emerging SARS-CoV-2 variants; those interventions included wastewater-based surveillance (1). We report results of subsequent wastewater surveillance of aircraft arriving at Copenhagen Airport in Copenhagen, Denmark, directly from Beijing or Shanghai, China.

1These first authors contributed equally to this article.
2These senior authors contributed equally to this article.

During weeks 2–6 of 2023 (January 9–February 12), a total of 14 aircraft arrived at Copenhagen Airport from China. A service truck extracted waste from the aircraft by using vacuum pressure, after which a rinsing program was performed, and the disinfectant Idu-Flight (Brenntag Nordic A/S, https://www.brenntag.com) was added to the waste tank. Wastewater samples were collected as grab samples from the service truck and immediately transported to Statens Serum Institut in Copenhagen for analysis.

The pH value of the sample material ranged from 9–10 because of the addition of Idu-Flight. Idu-Flight contains the active ingredients glutaraldehyde and benzalkonium chloride; the disinfectant is expected to negatively affect the stability of virus particles and hinder amplification of RNA sequences. We adjusted the samples to pH 7.5–8.5 by using HCl and homogenized them by vigorous vortexing. We split the 14 samples into a total of 43 aliquots and then centrifuged those at either 4,000 × g or 10,000 × g for 10 min to pellet solid material. For the first aliquot from aircraft AC1, we analyzed 10 mL of sample material without any centrifugation; for all other samples, we analyzed 10 mL of supernatant after centrifugation. We purified viruses by using NanoTrap Microbiome A particles (Ceres Nanosciences Inc., https://www.ceresnano.com) and RNA by using Maxwell RSC Cartridges (Promega Corporation, https://www.promega.com). We performed quantitative reverse transcription PCR (qRT-PCR) in technical triplicate by using the GoTaq Enviro kit (Promega) and the US Centers for Disease Control and Prevention N2 primer/probe for SARS-CoV-2 detection (Table; Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-0717-App1.pdf).

Of the 43 qRT-PCR reactions, 31 (72%) were positive for SARS-CoV-2, representing 11 aircraft. We conducted whole-genome sequencing of samples from those 11 aircraft by using the Illumina MiSeq platform (https://www.illumina.com) according to the ARTIC protocol; we generated 2 × 150-bp paired-end reads by using the ARTIC 4.1 primer scheme (2). Wastewater raw reads are available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena; accession no. PRJEB66221). We trimmed reads by using Trim Galore with default settings (3; https://zenodo.org/record/5127899). We removed human sequence reads by using the BWA-MEM alignment algorithm with default settings (H. Li, unpub. data, http://arxiv.org/abs/1303.3997) and the human genome reference build GRCh38. We then used BWA-MEM with default settings to map SARS-CoV-2 reads to the SARS-CoV-2 wild-type reference genome (GenBank accession no. MN908947.3). We performed
toward variants with immune evasive features, such as infection-naive population of China might not have passengers infected outside of China.

mation for passengers, the SARS-CoV-2 variants ob-

tions lacking. Because of the lack of supporting infor-
matic instead of those variants arising in China. Since January and February 2023, XBB has be-

were indeed circulating in China to the extent suggested by our analysis, their dominance in wastewater samples might have occurred because of a founder effect in selected communities instead of those variants arising in China. Since January and February 2023, XBB has become the dominant variant in sequence data from China (6). No new variants have been identified, but our study highlights the potential for wastewater-based surveillance to monitor virus spread among airline passengers in a cost-effective, anonymous, and noninvasive manner and to potentially identify circulating variants. This method can be rapidly modified to include other emerging infectious agents and can contribute substantially to future public health surveillance.

We thank Man-Hung Eric Tang and Jannik Fonager for their insights regarding the analyses and the study in general; Mohammad El-Najjar, Hannibal Morten Schultz, Randi Thogersen, Cecilie Muss, and Nadia Hamza for their thorough laboratory work and sample sequencing; and the staff at Copenhagen Airport for their cooperation.

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primer trimming by using iVar with a minimum read length of 30 nt (4) and estimated SARS-CoV-2 lineage abundance in each sample by using Freyja; depth cutoff was 10×, and the lineage abundance filter was 5% (5). We used a 50% coverage minimum across the genome as the threshold for lineage calling. We obtained sequencing results for 13 (42%) of 31 SARS-CoV-2-positive samples (Appendix Table).

We analyzed sequence reads for each sample aliquot and also after combining raw reads for each aircraft (Table; Appendix Table). When reads were combined for each aircraft, we found that the SARS-CoV-2 BQ.1 variant was dominant in wastewater of 1 aircraft, and XBB.1 variants were dominant in wastewater of 5 aircraft; the XBB.1.5 subvariant was dominant in 4 of those 5 aircraft (Table). The discovery of predominant XBB subvariants (dominant in Europe and the United States during the study period) in the aircraft samples contrasts with variant data uploaded to the GISAID database (https://www.gisaid.org) from China within the same time frame, which were mainly subvariants BA.5.2.48 and BF.7.14 (6).

For wastewater-based surveillance, limited information is generally available regarding the persons who contributed to the samples, and, consequently, data related to travel history and place of residence are lacking. Because of the lack of supporting information for passengers, the SARS-CoV-2 variants observed in wastewater-based surveillance of aircraft arriving in Copenhagen might have come from passengers infected outside of China.

In conclusion, our findings indicate that the large-

<p>|</p>
<table>
<thead>
<tr>
<th>Aircraft ID</th>
<th>Arrival week</th>
<th>Raw sequence reads</th>
<th>Mapped sequence reads</th>
<th>Genome (spike) coverage, † %</th>
<th>Pangolin lineage‡ (% abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>2</td>
<td>16,699,590</td>
<td>948,407</td>
<td>85.5 (49.5)</td>
<td>XBB.1.1 (21), BA.5 (9), XBB.2 (7), XBB.1 (7)</td>
</tr>
<tr>
<td>AC2</td>
<td>2</td>
<td>15,892,650</td>
<td>122,882</td>
<td>48.5 (17.6)</td>
<td>NA</td>
</tr>
<tr>
<td>AC3</td>
<td>3</td>
<td>14,467,192</td>
<td>725,320</td>
<td>93.1 (90.7)</td>
<td>BQ.1 (60)</td>
</tr>
<tr>
<td>AC4</td>
<td>4</td>
<td>13,463,062</td>
<td>5,584</td>
<td>10.3 (8.1)</td>
<td>NA</td>
</tr>
<tr>
<td>AC5</td>
<td>4</td>
<td>25,193,066</td>
<td>607,011</td>
<td>83.9 (88.7)</td>
<td>XBB.1.5 (96)</td>
</tr>
<tr>
<td>AC6</td>
<td>4</td>
<td>5,215,440</td>
<td>135</td>
<td>16.7 (0.0)</td>
<td>NA</td>
</tr>
<tr>
<td>AC7</td>
<td>5</td>
<td>15,691,594</td>
<td>960,609</td>
<td>82.9 (91.4)</td>
<td>XBB.1.5 (99)</td>
</tr>
<tr>
<td>AC8</td>
<td>5</td>
<td>2,749,174</td>
<td>102</td>
<td>0.0 (0.0)</td>
<td>NA</td>
</tr>
<tr>
<td>AC9</td>
<td>6</td>
<td>5,880,410</td>
<td>14,457</td>
<td>14.0 (9.1)</td>
<td>NA</td>
</tr>
<tr>
<td>AC10</td>
<td>6</td>
<td>13,244,030</td>
<td>1,306,993</td>
<td>98.1 (95.2)</td>
<td>XBB.1.5 (59), XBB.2 (18), XBB.1 (18)</td>
</tr>
<tr>
<td>AC11</td>
<td>6</td>
<td>1,132,399</td>
<td>1,127,995</td>
<td>98.1 (95.2)</td>
<td>XBB.1.5 (97)</td>
</tr>
<tr>
<td>EC</td>
<td>NA</td>
<td>4,050,388</td>
<td>370</td>
<td>0.5 (0.0)</td>
<td>NA</td>
</tr>
</tbody>
</table>

§Wastewater was collected from aircraft during January 9 (week 2)–February 12 (week 6), 2023. Samples from each aircraft were combined and reverse transcription quantitative PCR and sequencing were performed; only 1 sample each was collected from AC6, AC8, and AC9. Full table of results for each aliquot is available in the Appendix (https://wwwnc.cdc.gov/EID/article/29/12/231749-App1.pdf). EC, extraction control; ID, identification; NA, not applicable.

†Coverage percentages are provided for the full genome sequence and for the spike protein gene.

‡Lineages according to Pangolin software (https://cov-lineages.org).

§Water sample was extracted and sequenced as a control.

Table. Sequencing results for SARS-CoV-2–positive wastewater samples in study of SARS-CoV-2 variants BQ.1 and XBB.1.5 in wastewater of aircraft flying from China to Denmark, 2023*
**Systemic Erysipelas Outbreak among Free-Ranging Bottlenose Dolphins, San Diego, California, USA, 2022**

Kerri Danil, Kathleen M. Colegrove, Martha A. Delaney, Alexandrea Mena, Nancy Stedman, Elyse Wurster

Author affiliations: Southwest Fisheries Science Center, La Jolla, California, USA (K. Danil); University of Illinois, Brookfield, Illinois, USA (K.M. Colegrove, M.A. Delaney); SeaWorld, San Diego, California, USA (A. Mena), Busch Gardens, Tampa, Florida, USA (N. Stedman), Ocean Associates Inc., under contract to Southwest Fisheries Science Center, La Jolla (E. Wurster)

We diagnosed fatal *Erysipelothrix rhusiopathiae* sepsis in 3 stranded bottlenose dolphins (*Tursiops truncatus*) during summer 2022, in San Diego, California, USA. The previously undetected disease in this relatively small, regional population of dolphins most likely indicates an environmental or biological change in the coastal ocean or organisms.

Erysipelas is a disease of animals caused by the bacterium *Erysipelothrix rhusiopathiae*, which can be transmitted via exposure to feces, urine, saliva, and nasal secretions from infected animals and contaminated food, water, and soil (1). Human infection with this bacterium most often involves occupational exposure (1). In cetaceans, the disease is thought to be caused by ingesting infected fish, tooth raking from infected conspecifics, or infected wounds. Chronic cutaneous and acute fatal septicemic forms of the disease have been reported for captive and free-ranging cetaceans (2) but not for free-ranging cetaceans along the Pacific Coast of the United States.

Two stocks of bottlenose dolphins (*Tursiops truncatus*) inhabit the waters of California, USA: coastal and offshore. The coastal population comprises ≈500 dolphins that range from San Francisco, California, USA, to San Quintin, Mexico (latitudinal distance = 802 km), with little site fidelity (3). In southern California, coastal bottlenose dolphins are typically found within 500 meters of the land.

During summer 2022 (June–September), 3 coastal bottlenose dolphins, of mixed sex and age class, were found stranded within 46 km of each other in San Diego, California, USA; we diagnosed sepsis caused by *E. rhusiopathiae*. The diagnoses coincided with increased strandings for this species in the region. In 2022, a total of 8 bottlenose dolphins were stranded, compared with a 20-year average of 4.35 per year (K. Danil, unpub. data; calculated by using Southwest Fisheries Science Center stranding records).

We determined cause of death for 6 of the 8 dolphins: 3 systemic erysipelas, 1 brucellosis, 1 trauma, and 1 malnutrition (Table). Gross necropsy findings for the 3 with erysipelas included open rake wounds (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0811-App1.pdf), mottled livers, distended urinary bladders, empty stomachs, and pulmonary edema; 2 dolphins also had ascites and icterus. Histopathologic examination for the 3 dolphins with erysipelas indicated vasculitis associated with multiorgan inflammation, necrotizing adenitis and nephritis for 1, and gastroenteritis for 1. Intracellular bacteria were identified (Figure), and *E. rhusiopathiae* were cultured...
from ≥2 organs from all 3 dolphins (Table). We confirmed the identity of all colonies of interest by using biochemical testing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Overall, the gross and histopathologic findings were consistent with other reports of *E. rhusiopathiae* infection in cetaceans (2).

For the past 20 years, histopathology and microbiology have been used to determine marine mammal cause of death in the San Diego region. Lack of *erysipelas* detection during that time suggests that the recent cluster indicates emerging *erysipelas* in cetaceans of this region. Similarly, a recent large mortality event of harbor porpoises (*Phocoena phocoena*) in the Netherlands was attributed to *E. rhusiopathiae* (3), which had not been previously detected in that area (4). The close temporal and geographic proximity of the affected dolphins in San Diego suggests that an *erysipelas* outbreak may have led to the increased coastal bottlenose dolphin deaths in this region. Although the causes of this outbreak are unclear, possible explanations include a changing environment, poor water quality, increased susceptibility to *E. rhusiopathiae* via emergence of a more pathogenic strain, or host immunosuppression in coastal bottlenose dolphins.

Links between environmental conditions and exposure to *E. rhusiopathiae* in other mammals have been found, although the mechanism is unclear (5). Similarly, short- and long-term ocean warming along the California coast could affect bacterial growth conditions or bottlenose dolphin prey. A change in prey could influence exposure if the presence, abundance, or pathogenicity of *E. rhusiopathiae* varies by fish species. In southern San Diego, untreated wastewater effluent from the Tijuana River Estuary and a wastewater treatment plant in Tijuana, Mexico, has resulted in poor ocean water quality and frequent beach closures (6). During a 2019–2020 winter study, *Erysipelothrix* spp. were detected by molecular genetic techniques in low numbers in the Tijuana River Estuary (7). However, it is unknown whether *E. rhusiopathiae* was present during the 2022 outbreak. It is also unknown whether coastal bottlenose dolphins have suppressed immune systems that may make them more susceptible to infection with *Erysipelothrix* spp. bacteria. Recorded concentrations of DDT compounds are higher among California coastal bottlenose dolphins than among any cetacean in the world (8), and halogenated organic compound load (e.g., from DDT) has been correlated with endocrine disruption in that population (9), which is relevant because endocrine function is closely tied to immune function (10).

**Table.** Characteristics of *Tursiops truncatus* dolphins stranded in San Diego, California, USA, 2022

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Latitude and longitude</th>
<th>Decomposition status</th>
<th>Sex</th>
<th>Strand date</th>
<th>Age class</th>
<th><em>Erysipelothrix rhusiopathiae</em> culture</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>KXD0391</td>
<td>32.8764, −117.2513</td>
<td>Fresh</td>
<td>F</td>
<td>May 16</td>
<td>Neonate</td>
<td>Spleen −, lung −, liver −</td>
<td><em>Brucella</em> infection</td>
</tr>
<tr>
<td>KXD0393</td>
<td>33.05023, −117.2995</td>
<td>Fresh</td>
<td>F</td>
<td>Jun 26</td>
<td>Juvenile</td>
<td>Brain +, spleen +</td>
<td><em>Erysipelothrix</em> sepsis Trauma</td>
</tr>
<tr>
<td>KXD0394</td>
<td>32.6404, −117.146</td>
<td>Moderate</td>
<td>M</td>
<td>Jun 28</td>
<td>Neonate</td>
<td>NA</td>
<td><em>Erysipelothrix</em> sepsis Sepsis</td>
</tr>
<tr>
<td>KXD0395</td>
<td>32.9055, −117.2555</td>
<td>Fresh</td>
<td>F</td>
<td>Jul 2</td>
<td>Adult</td>
<td>Kidney +, spleen +</td>
<td>Malnutrition</td>
</tr>
<tr>
<td>WC-TT-2201B</td>
<td>32.5789, −117.1323</td>
<td>Fresh</td>
<td>F</td>
<td>Aug 29</td>
<td>Neonate</td>
<td>Spleen −</td>
<td>Unknown, no necropsy</td>
</tr>
<tr>
<td>KXD0399</td>
<td>32.7095, −117.2349</td>
<td>Advanced</td>
<td>F</td>
<td>Sep 11</td>
<td>Adult</td>
<td>NA</td>
<td>Unknown, no necropsy</td>
</tr>
<tr>
<td>WC-TT-2202B</td>
<td>32.6398, −117.1463</td>
<td>Fresh</td>
<td>M</td>
<td>Sep 12</td>
<td>Calf</td>
<td>Brain +, spleen +, lung +</td>
<td><em>Erysipelothrix</em> sepsis Sepsis</td>
</tr>
<tr>
<td>KXD0400</td>
<td>32.8606, −117.2559</td>
<td>Advanced</td>
<td>F</td>
<td>Sep 15</td>
<td>Juvenile</td>
<td>NA</td>
<td>Unknown, no necropsy</td>
</tr>
</tbody>
</table>

*Boldface indicates *erysipelas* cases. NA, not applicable; +, positive culture result; −, negative culture result.*

Figure. Section of kidney with neutrophilic nephritis associated with histiocytic bacterial rods (arrows) consistent with *Erysipelothrix* infection in specimen KXD0395 from study of systemic *erysipelas* outbreak among free-ranging bottlenose dolphins, San Diego, California, USA, 2022. Hematoxylin and eosin stain; scale bar indicates 20 microns.
If erysipelas outbreaks continue, they could threaten this relatively small population of dolphins. In addition, emergence of *E. rhusiopathiae* has potential health implications for persons who recreate in these waters or work with fish, and for free-ranging marine mammals or other animals that prey on fish in this region.

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We thank Brittany Hanser, Alaina Harmon, Madilyn Pardini, Melanie Peel, Zoe Prescott, and Jessica Ruth for necropsy support. We also thank Deborah Fauquier for providing logistical and scientific support and and Heather Fritz for *Erysipelothrix* identification and consultation. Thanks to Judy St. Leger for conversations about erysipelas and histopathology.

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Mrs. Danil is a research biologist at the National Oceanic and Atmospheric Administration Southwest Fisheries Science Center. Her research interests include the interplay of cetacean life history, health, and the environment.

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**OXA-48–Producing Uropathogenic *Escherichia coli* Sequence Type 127, the Netherlands, 2015–2022**

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During 2015–2022, a genetic cluster of OXA-48–producing uropathogenic *Escherichia coli* sequence type 127 spread throughout the Netherlands. The 20 isolates we investigated originated mainly from urine, belonged to Clermont phylotype B2, and carried 18 genes encoding putative uropathogenicity factors. The isolates were susceptible to first-choice antimicrobial drugs for urinary tract infections.

We recently described OXA-48 carbapenemase-producing *Escherichia coli* sequence type (ST) 38 with putative uropathogenicity factors (I). Here we report a genetic cluster of 20 OXA-48–producing uropathogenic *Escherichia coli* (UPEC) ST127 isolates in the Netherlands.

Medical microbiology laboratories in the Netherlands are requested to submit isolates with suspected carbapenemase production to the National Institute for Public Health and the Environment (RIVM) as part of the carbapenemase-producing *Enterobacterales* Public Health and the Environment (RIVM) as part of the carbapenemase surveillance program. For all isolates, we perform meropenem Etest, carbapenem inactivation method, next-generation sequencing (NGS; Illumina, https://www.illumina.com), and long-read sequencing (Oxford Nanopore Technologies, https://www.nanoporetech.com). We use NGS data to analyze the Clermont phylotype (2), core-genome single-nucleotide polymorphisms, classical multilocus sequence typing (MLST) STs, and in-house *E. coli* whole-genome MLST (wgMLST) types (I,3). We also evaluated presence of antimicrobial resistance genes (AMRfinder, https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder), plasmid replicons (PlasmidFinder, https://cge.food.dtu.dk/services/PlasmidFinder), and 31 previously described putative uropathogenicity factors (PUFs) using an in-house PUFfinder (4). For identity/query ≥90%, we scored the PUF gene as present.

During January 1, 2015–December 31, 2022, we sequenced 799 carbapenemase-producing *E. coli* by using NGS; 258 (32%) carried a *bla*<sub>OXA-48</sub> gene, of which 24 were ST127. According to wgMLST, 20 of the *bla*<sub>OXA-48</sub>–carrying ST127 isolates formed a genetic cluster (Appendix 1 Figure, panel A) and were sent to the RIVM during October 2015–December 2022 (Appendix 1 Figure, panel B). Allelic distance in the cluster was 3–20, and isolates differed by 3–46 core-genome single-nucleotide polymorphisms (Appendix 2 Table 2, https://wwwnc.cdc.gov/EID/article/29/12/23-1114-App2.pdf). When we compared the 20 cluster isolates with 603 international *E. coli* ST127 isolates (Enterobase, https://enterobase.warwick.ac.uk), they clustered with 3 isolates: Ireland (2016), United States (2019), and Spain (2019) (Appendix 2 Table 3). All were sensitive to meropenem (European Committee on Antimicrobial Susceptibility Testing, https://www.eucast.org); MICs were 0.125–0.38 mg/L (5). All grew on OXA-48 agar but not on carbapenem agar (CHROMID OXA-48/CHROMID CARBA; bioMérieux, https://www.biomerieux.com) and produced carbapenemase according to the carbapenem inactivation method. Nanopore sequencing yielded 10/20 circular assemblies, which revealed a chromosomal copy of the *mdf(A)*– and the *bla*<sub>OXA-48</sub> genes. The *bla*<sub>OXA-48</sub> gene is flanked by IS1/tnp-IS1B and inserted in a variable ≈148-kb region of the chromosome (Appendix 1 Figure, panel C; Appendix 2 Tables 1, 4). Of the 20 isolates, 18 lacked plasmid replicons.

The median age of the 11 male and 9 female patients was 57 (range 3–87) years; patients lived throughout the Netherlands (Appendix 1 Figure, panel D). Cultures were submitted by general practitioners (8/20) and hospitals (12/20). Two patients were recently hospitalized in Morocco; no travel history was reported for the other patients, although 1 was born in Morocco and 1 in Turkey.

Most isolates were from urine (12/20), followed by perineal/rectal swab samples (4/20), blood (3/20), and wounds (1/20). Of the 20 cultures, 12 were diagnostic, 5 were screening, and 3 were for unknown purpose. Two patients had recurrent urinary tract infections (UTIs). All isolates were type O6:H31 and Clermont phylotype B2, the most common Clermont phylotype associated with UPEC in the United States and Europe (4,6). A variety of PUFs were detected in cluster isolates associated with UPEC, (Appendix 2 Table 5, Appendix 1 Figure, panel E), including adhesins (e.g., sfaH, pilin *papGII/papGIII*), toxins (e.g., α-hemolysin, cytotoxic necrotizing factor-1, and *E. coli* uropathogenic-specific protein) (4,7,8). Cluster isolates carried significantly more (mean 18) PUFs, than the other *E. coli* isolates from CPE surveillance (mean nonurine isolates, 7; urine isolates, 9; previously reported OXA-244 *E. coli* ST38 isolates, 8; p<0.001 by Mann-Whitney U-test) (1). We identified additional uropathogenicity determinants curli, type-1 fimbriae, S-fimbriae, flagella, and group 2 capsule genes but not group 3 capsule genes. Eighteen isolates phenotypically produced hemolysin, visible as β-hemolysis on blood agar (Appendix 1 Figure, panel F), in line with in silico genetic analyses (Appendix 1 Figure, panel E).

Antimicrobial susceptibility pattern was known for 13 isolates in the Infectious Diseases Surveillance Information System–Antimicrobial Resistance in the...
Netherlands (https://www.rivm.nl/isis-ar). All were phenotypically resistant to penicillins/penicillin combinations (e.g., amoxicillin/clavulanic acid and piperacillin/tazobactam) but susceptible to oral first-choice antimicrobial drugs for UTIs in the Netherlands (e.g., nitrofurantoin, fosfomycin, ciprofloxacin, sulfamethoxazole/trimethoprim) (Appendix 1 Figure, panel E). Prevalence of UPEC in the Netherlands is most likely underestimated because general practitioners in the Netherlands usually send cultures only when treatment with first-choice drugs fails. Although UTIs are not known to be contagious, *E. coli* can spread and cause UTI outbreaks (caused by a specific *E. coli* strain in several communities), for which an association with food has been suggested (9). A New Zealand study described an outbreak in which MLST identified 77 multidrug-resistant *E. coli* isolates (10).

We demonstrated ongoing dissemination of OXA-48–producing and hemolysin-producing UPEC ST127 from Clermont phylotype B2 with 18/31 PUFs in patients across the Netherlands with no direct epidemiologic link. The origin of the cluster is unknown, but international spread is possible. Low-level resistance and growth only on OXA-48 agar suggests that this carbapenemase-producing UPEC may be missed and the actual size of this cluster may be underestimated.

**Acknowledgments**

We thank all members of the CPE Surveillance Study Group and the medical microbiology laboratories in the Netherlands for submitting *E. coli* isolates to RIVM for the national CPE surveillance program. We also thank the Municipal Health Services for the epidemiologic data.

Ethics approval was not required because this study was based on genomic and phenotypic surveillance data only; samples from which the isolates were cultured were collected as part of routine healthcare. Sequence data are available in the National Center for Biotechnology Information Sequence Read Archive (BioProject nos. PRJEB35685 and PRJNA980147) (Appendix 2 Table 1).

**About the Author**

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**References**


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Genome-Based Characterization of *Listeria monocytogenes*, Costa Rica

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Core-genome MLST (cgMLST) further identifies sublineages (SLs) and cgMLST types (CTs) (2). Major CCs and SLs are distributed globally and can be heterogeneous in terms of virulence; isolates from serogroup IVb (lineage I) often cause the most severe infections (2–4).

Pathogen surveillance using whole-genome sequencing (WGS) provides unprecedented resolution for identifying case clusters and contamination sources and for predicting strain virulence and antimicrobial resistance, which can aid in risk assessment (2,5). Previous studies confirmed *L. monocytogenes* in various foods in Costa Rica; reported contamination levels were 5%-20% in processed meat products and fresh cheeses (6,7). Because listeriosis is not a notifiable disease in Costa Rica, its prevalence is unknown, and diversity of *L. monocytogenes* circulating in the country is unclear.

To clarify the diversity of and potential public health risk from circulating strains, we used WGS to characterize 92 isolates recovered during 2009–2019 from 16 clinical, 67 food, and 9 production environment samples in Costa Rica (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0774-App1.pdf). When location data were available, isolates were from urban areas, including the capital city San José, and from rural areas where fresh cheese production is prevalent, including Alajuela, Naranjo, San Ramón, Vara Blanca, Upala, and Turrialba. Turrialba region accounts for 70% of fresh cheese produced in Costa Rica (Figure; Appendix).

We found that isolates from lineage I (95%, n = 88) and lineage II (5%, n = 4) were unevenly distributed into 12 different SLs and CCs (Figure; Appendix Figure 1). Those isolates included a new lineage I sublineage, designated SL1079 (new MLST singleton ST1079), which was identified in an isolate from shrimp (cgMLST type L1-SL1079-ST1079-CT1669). That isolate had an atypical genoserogroup IIb profile, designated IIb-v1, that differed from the classic IIb profile by the presence of lmo0737. WGS confirmed the presence of lmo0737 and flanking genes lmo0733–39, typically found in lineage II isolates from serogroups IIa and IIc but only occasionally found in lineage I serogroup IVb-v1 (8). Of note, 80% of isolates investigated from both clinical and food-associated sources were from sublineages SL2/CC2 (66%, n = 61) and SL3/CC3 (14%, n = 13). SL2/CC2 (serogroup IVb) and SL3/CC3 (serogroup IIb) isolates are found worldwide and are associated with invasive infections (2–4). However, they are rarely the most prevalent genotypes (2,3). Available data from other countries in Central America confirmed...
Figure. Single linkage dendrogram of 92 isolates generated for genome-based characterization of *Listeria monocytogenes*, Costa Rica. Dendrogram is based on core-genome multilocus sequence typing (cgMLST) allelic profiles (1,748-locus scheme). Branches are colored according to lineages: L1, red; L2, blue. Branches are labeled according to lineages, sublineages, and clonal complexes. Information on isolates’ serogroup, and resistance profiles are provided in the columns. Colors in location column correspond to dots on map. Gray bars indicate clusters of isolates with ≤7 allelic differences out of 1,748 cgMLST loci. Presence of selected virulence and resistance genetic traits in each isolate is represented by dark blue boxes and empty boxes denote genes with premature stop codons. More details are provided in Appendix Figure 1 (https://wwwnc.cdc.gov/EID/article/29/12/23-0774-App1.pdf). CC, clonal complex; L, lineage; LIPI, listeria pathogenicity island; SL, sublineage.
Markers of tolerance to disinfectants included qacA encoding for tolerance to disinfectants or stress. In this study, 90% of isolates carried >1 genetic element. In this adverse environmental conditions (Table; Figure; Appendix Figures 1–3). Eight isolates were cgMLST type L1-SL2-ST2-CT2715, which accounted for 25% of clinical cases and spanned 9 years (Table).

Most human cases were associated with dairy products (Table). However, tracing to confirm the source of infection was not possible because most production is conducted by local farmers, often without traceability or attribution to the site of production.

Fresh cheese production is an economic staple in Costa Rica, and previous studies have reported L. monocytogenes detection in those products (7). Results from this study also show detection of identical strains of cgMLST type L1-SL2-ST2-CT2715 along the same production line, from raw materials to the final product, suggesting inadequate sanitation contributes to contamination (9).

L. monocytogenes is problematic for the food industry because it can survive and multiply under adverse environmental conditions (10). In this study, 90% of isolates carried ≥1 genetic element encoding for tolerance to disinfectants or stress. Markers of tolerance to disinfectants included qacA (51%, n = 47), bcrABC (23%, n = 21), and emrC (1%, n = 1). In addition, isolates had stress survival islet (SSI) genes, including SSI-1, conveying tolerance to low pH and high salt concentrations (21%, n = 19), and SSI-2 conveying tolerance to high pH and oxidative stress (1%, n = 1), as well as Listeria genomic island (LGI) genes, including LGI-2 (50%, n = 48) and LGI-3 (1%, n = 1) conveying tolerance to metals. Those tolerances can make L. monocytogenes elimination from production sites more difficult.

This study provides insight into the diversity of L. monocytogenes strains circulating in Central America and can aid national reference institutions in promoting regulatory changes to guarantee mandatory listeriosis reporting. In addition, institutions should establish mechanisms to provide low-cost microbiologic analysis. We also recommend regular sampling of risk products and training of artisanal processors.

In conclusion, strengthened WGS surveillance in Costa Rica could assist in controlling L. monocytogenes and provide food producers with information on strain diversity and effective means of eradication. WGS surveillance also would enable authorities to detect outbreaks and trace sources of contamination.

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This work includes multilocus sequence typing profiles publicly available on BIGSdb-Listeria (https://bigedb.pasteur.fr/listeria).

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Human Taenia martis Neurocysticercosis, Switzerland

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Neurocysticercosis is almost exclusively caused by Taenia solium tapeworms. We describe a case of neurocysticercosis in Switzerland caused by infection with Taenia martis, the marten tapeworm, and review all 5 published cases of human infection with the marten tapeworm. In epidemiologically nonplausible cases of neurocysticercosis, zoonotic spillover infections should be suspected.
Neurocysticercosis is a zoonotic, parasitic, central nervous system infection almost exclusively caused by the larvae of *Taenia solium*, the pork tapeworm (1). In a few exceptional cases, neurocysticercosis in humans is not caused by *T. solium* but by other zoonotic *Taenia* species, representing rare spillover infections from distant ecologic niches (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-0697-App1.pdf). In this article, we describe a rare case of *T. martis* neurocysticercosis in a woman in Switzerland.

A woman 55 years of age sought care at the emergency department of the Cantonal Hospital of Lucerne (Lucerne, Switzerland) because of a 3-week history of progressive transient numbness and convulsions of her left hand. Her medical history was unremarkable. Clinical examination revealed disorientation to time, a pronator drift of the left arm, hypoesthesia of the left extremities, and a tactile neglect toward the left side. A comprehensive metabolic panel and a complete blood count showed no major abnormalities. Computed tomography of the brain revealed a 12 × 14 mm mass in the right postcentral gyrus with strong ring-enhancement and perifocal edema (Appendix Figure 1). We started the patient on levetiracetam and admitted her for further investigation.

Results of computed tomography of the thorax and abdomen were unremarkable. Magnetic resonance imaging (MRI) (Appendix Figure 2) showed no restriction on diffusion-weighted imaging. After starting the patient on dexamethasone, we removed the lesion through a right parietal craniotomy. Postoperative MRI confirmed complete resection. We gradually discontinued dexamethasone and discharged the patient 6 days after the operation.

The specimen consisted of a cyst with a thick wall and with no macroscopically discernible content. Histologic analysis revealed a singular membrane, compatible with a helminthic parasite, and an abscessing inflammation at the border (Figure). Results of immunohistochemical tests, eubacterial 16S PCR, and whole-genome sequencing were negative, as was serologic screening for *Echinococcus* spp. However, Western blot for cysticercosis, which uses *T. solium* IgG (LDBIO Diagnostics, https://www.ldbiodiagnostics.com), revealed a weak band pattern suggestive of an infection with *T. solium*. We contacted the Swiss Tropical and Public Health Institute, which raised the issue of missing epidemiologic plausibility, given that the patient had never traveled to a *T. solium*-endemic area. The possibility of zoonotic spillover infection was considered and MRI of the whole spine, ocular ultrasound, and stool investigations recommended to exclude additional lesions and taeniasis. We analyzed the resected neurocysticercus by using a pan-helminthic PCR and sequencing of the cestode- and nematode-specific cytochrome c oxidase subunit 1 (*cox1*) gene (2), which revealed 100% sequence identity with *Taenia martis*, the marten tapeworm (GeneBank accession no. OQ536306).

Because of the unclear date of infection, the unknown proliferation rate of the parasite in humans, and the possibility of undetectable lesions, we treated the patient with albendazole and praziquantel, analogous to treatment for *T. solium* neurocysticercosis, assuming similar susceptibility of the parasite (3). Three months after the operation, the patient showed no neurologic deficits and no new focal seizures.

The definitive hosts of *T. martis* tapeworms are martens and other mustelids. *T. martis* tapeworms

Figure. Histologic sections of resected *Taenia martis* metacestode from a patient in Switzerland. A) Cross-section through the excised tissue revealed strong infiltration surrounding a structure suggestive of parasitic origin. Boxed areas are shown in a higher magnification in panels B and C. B, C) The metacestode’s cyst wall, showing the warty appearance (B) characteristic of tapeworm metacestodes’ tegument (3,4). Hematoxylin and eosin stain. Scale bars indicate 200 μM (A), 10 μM (B), and 100 μM (C).
have been found in the intestines of 36% of stone martens (Martes foina) in southwest Germany but also parasitize other mustelids (4,5). Stone martens occur throughout Europe and Central Asia. The natural intermediate hosts of T. martis tapeworms are small rodents, which develop cysticerci in the pleural and peritoneal cavities. The infection in this patient suggests an accidental fecal–oral transmission, although it remains unclear when and how she came into contact with marten droppings.

Diagnosed human infections with T. martis tapeworms are limited to 5 cases reported from Germany and France (Table; Appendix Figure 3) (6–10). Those cases were 2 peritoneal infections, 2 eye infections, and 1 central nervous system infection, all with single lesions occurring in immunocompetent women. Of note, an increased susceptibility to various Taenia spp. tapeworms dependent on sex and hormone status has been described in animals (5). All 6 patients, including ours, lived in rural villages, 5 (including ours) grew their own vegetables, and at least 3 (including ours) had frequent marten sightings around their homes. Similar to what other authors reported, we did find cross-reactivity with a T. solium–specific assay.

All but 1 of the patients described in the published cases received antiparasitic treatment; the only untreated case was in a patient who refused medical treatment (10). None of the published cases reported recurrence or emergence of additional lesions.

Neurocysticercosis may be caused by Taenia spp. other than T. solium tapeworms. Cases in which an infection with T. solium is epidemiologically not plausible should be investigated for zoonotic spillover infections (in Central Europe, specifically infection with T. martis should be considered). Such cases are probably underrepresented in the literature, given the high prevalence of stone martens, their high infection rate with T. martis tapeworms, and the possibility of false positives of available, cross-reactive T. solium– and Echinococcus spp.–specific serologic assays. If adequate material is available, a panhelminthic PCR with cox1 sequencing is highly recommended.

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### References

Highly pathogenic avian influenza (HPAI) H5 viruses of the goose/Guangdong lineage have been categorized into multiple clades (0–9) and subclades. Viruses belonging to H5 clade 2.3.4.4 are differentiated into 8 subclades (a–h) and are of high concern because of spillover events into mammals and direct mammal-to-mammal transmission reported in Spain (1,2). HPAI H5N1 virus subclade 2.3.4.4b has been circulating in Africa, Asia, and Europe since ≈2020 (3,4). Subsequently, this subclade was identified in North America and Canada in late 2021; Colombia, Venezuela, Peru, Ecuador, and Chile during October–December 2022; and in Bolivia, Argentina, and Uruguay during January–February 2023 (1).

By November 2022, ≈300 dead Peruvian pelicans (Pelecanus thagus) and 24 dead blue-footed boobies (Sula nebouxii) were found on the northern coast of Peru (5). On November 23, 2022, the National Agrarian Health Service of Peru (Servicio Nacional de Sanidad Agraria del Peru) and the US Naval Medical Research Unit SOUTH reported HPAI H5N1 virus was present in Peru (6). Subsequently, we sequenced 18 additional virus samples positive for hemagglutinin (HA) subtype 5 (H5) that were collected from 3 Peruvian pelicans, 12 chickens (Gallus gallus domesticus), 2 Neotropic cormorans (Nannopterum brasilianum), and 1 lion (Panthera leo, from a zoo). We extracted viral RNA from respiratory tissue or environmental fecal samples. We collected samples from birds during November–December 2022 from northern and central coasts of Peru and the sample from the lion in February 2023 from Junin (Andean region) (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/29/12/23-0505-App1.xlsx).

We amplified influenza A virus genomes by using a modified protocol (7). We prepared libraries by using the Nextera XT DNA Library Preparation Kit (Illumina, https://www.illumina.com) and sequenced them by using the MiSeq Reagent Kit v3 (600-cycle paired-end) on the MiSeq platform (Illumina). We trimmed raw reads, removed host sequences, and then de novo assembled the filtered reads. We identified the resulting contigs as H5N1 by using a BLASTn search (https://blast.ncbi.nlm.nih.gov). We deposited all obtained sequences in GenBank (accession nos. OQ547312–451).

We performed phylogenetic analysis to classify subclades by using the maximum-likelihood method. We retrieved H5 sequences from HPAI clade 2.3.4.4 and low pathogenicity avian influenza viruses published in GISAID (https://www.gisaid.org) and GenBank during 2014–2023 (until July 20, 2023). The phylogenetic tree of HA sequences placed H5N1

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Highly Pathogenic Avian Influenza A(H5N1) from Wild Birds, Poultry, and Mammals, Peru

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We identified highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b in wild birds, poultry, and a lion in Peru during November 2022–February 2023 and markers associated with transmission adaptation and antiviral drug resistance. Continuous genomic surveillance is needed to inform public health measures and avoid mass animal deaths.
**Figure.** Phylogenetic analysis of highly pathogenic avian influenza A(H5N1) from wild birds, poultry, and mammals, Peru. Maximum-likelihood method was used for phylogeny of 101 hemagglutinin H5 sequences from avian influenza viruses. Red lines indicate clustering of strains from Peru and sequences from this study; bold font indicates the sequences from this study. Dark blue lines indicate other strains from South and North America. Non–goose/Guangdong lineage virus strains from Eurasia were outgroups. Phylogenetic tree was generated and edited with MEGA X software (https://www.megasoftware.net). Sequences were aligned by using the MUSCLE program in the AliView alignment viewer and editor (https://www.ormbunkar.se/aliview). We used general time reversible and gamma distribution models; robustness of tree topology was assessed with 1,000 bootstrap replicates. Only bootstrap values >70% are shown. Scale bar indicates nucleotide substitutions per site.
strains from North, Central, and South America into different groups within subclade 2.3.4.4b. We identified 6 subclades comprising sequences from 1–5 countries (Venezuela, Colombia, Ecuador, Mexico/Honduras/Costa Rica/Panama/Colombia, Costa Rica/Panama/Colombia, and Ecuador/Peru/Chile) and 1 sequence from Colombia that did not cluster with other strains from South America. Our results suggest that the strains from South America were not monophyletic and represented 7 independent virus introduction events (Figure), complementing a previous report (8).

We also compared available amino acid sequences of virus proteins among strains from South America to identify differences among subclades (Appendix Table 2). We identified several amino acid changes that were shared among members of the same subclade (Appendix Table 3). Those changes were consistent with our HA phylogenetic analysis, supporting the hypothesis that independent virus introduction events occurred in South America.

We performed molecular marker analysis to identify specific amino acid mutations associated with HPAI adaptation, transmission, and antiviral drug resistance, such as those in neuraminidase (NA), matrix protein 2, and polymerase acidic protein (9). We identified 21 molecular markers involved in H5N1 pathogenicity that were present in all analyzed sequences from South America and 7 markers that were found in some sequences (Table). However, 2 mutations in the polymerase basic 2 protein (Q591K and D701N) associated with mammal adaptation were identified only in sequences from sea lions in Peru and from 1 human case in Chile. The T271A mutation in polymerase basic 2 protein linked to mammal adaptation and S369I and I396M mutations in NA that were observed in the mink outbreak in Spain (2) were not found in sequences from South America. We did not find amino acid mutations related to resistance to the antiviral drugs oseltamivir, zanamivir and peramivir (in NA), amantadine and rimantadine (in matrix protein 2), or baloxavir (in PA). We only

Table. Summary of molecular markers identified in influenza virus strains from South America in study of highly pathogenic avian influenza A(H5N1) from wild birds, poultry, and mammals, Peru*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation/motif</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>D9N†</td>
<td>Increases virulence in mice</td>
</tr>
<tr>
<td></td>
<td>L89V, G309D, T339K, R477G, I495V, K627E, A676T, Q591K‡</td>
<td>Increases polymerase activity in mammalian cell lines and increases virulence in mice</td>
</tr>
<tr>
<td></td>
<td>D701N‡</td>
<td>Increases polymerase activity in mammalian and avian cell lines, increases replication in mammalian cell lines, increases virulence in mice and contact transmission in guinea pigs, increases virulence in mice</td>
</tr>
<tr>
<td>PB1</td>
<td>D3V</td>
<td>Increases polymerase activity and viral replication in avian and mammalian cell lines</td>
</tr>
<tr>
<td>PB1-F2</td>
<td>N66S</td>
<td>Increases polymerase activity and virulence in mammal cell lines</td>
</tr>
<tr>
<td>PA</td>
<td>N383D</td>
<td>Increases polymerase activity in mammalian and avian cell lines</td>
</tr>
<tr>
<td>HA</td>
<td>D94N, S133A, S154N, T156A, S107R, T108I, K218Q, S223R, 321-329 (PLR(EorG)KRRKR)</td>
<td>Increases virus binding to α2–6 receptor, increases virus binding to α2–6, increases transmission in guinea pigs, increases virulence in chickens and mice and the pH of fusion, increases virus binding to α2–3 and α2–6 receptors</td>
</tr>
<tr>
<td>NP</td>
<td>M105V‡</td>
<td>Increases virulence in chickens</td>
</tr>
<tr>
<td></td>
<td>I109T#</td>
<td>Increases polymerase activity and viral replication in chickens (but not ducks), increases virulence in chickens</td>
</tr>
<tr>
<td>M1</td>
<td>N30D</td>
<td>Increases polymerase activity and viral replication in avian cells and virulence in chickens</td>
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<tr>
<td></td>
<td>I43M</td>
<td>Increases virulence in mice, chickens and ducks</td>
</tr>
<tr>
<td></td>
<td>T215A</td>
<td>Increases virulence in mice</td>
</tr>
<tr>
<td>M2</td>
<td>I27A**</td>
<td>Increases resistance to amantadine and rimantadine</td>
</tr>
<tr>
<td>NS1</td>
<td>P42S</td>
<td>Increases virulence and decreases the antiviral response in mice</td>
</tr>
<tr>
<td></td>
<td>C138F</td>
<td>Increases replication in mammalian cell and decreases the interferon response</td>
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<tr>
<td></td>
<td>V149A</td>
<td>Increases virulence and decreases the interferon response in chickens</td>
</tr>
<tr>
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<td>L103F, 1106M</td>
<td>Increases virulence in mice</td>
</tr>
<tr>
<td></td>
<td>K55E, K66E, C138F</td>
<td>Enhances replication in mammalian cells and decreases the interferon response</td>
</tr>
</tbody>
</table>

* Molar markers of influenza virus strains were identified as previously described (9). HA, hemagglutinin; HPAIV, highly pathogenic avian influenza virus; M1, matrix protein 1; M2, matrix protein 2; NP, nucleoprotein; NS1, nonstructural protein 1.
† Only in 2 sequences from pelicans (GISAID [https://www.gisaid.org] accession nos. EPI_ISL_17099964, EPI_ISL_17165223).
‡ Only in 2 sequences from 2 sea lions in Peru and 1 human case in Chile.
§ Only in sequences from a wild bird in Peru (GISAID accession no. EPI_ISL_17660074).
¶ Mutation sequences from Venezuela and Colombia (Choco) have M rather than V.
# Only in sequences from Colombia (Choco).
** Only in 1 sequence from a wild bird in Peru (GISAID accession no. EPI_ISL_17777528).
found the H252Y mutation in NA associated with moderately reduced susceptibility to oseltamivir (10).

In conclusion, HPAI H5N1 virus clade 2.3.4.4b was identified in samples collected in Peru from wild birds, poultry, and a lion during November 2022–February 2023. According to phylogenetic analysis, the multiple cluster distribution revealed independent introductions of HPAI H5N1 clade 2.3.4.4b viruses into South America from North and Central America. Four introductions occurred in Colombia, 2 in Ecuador, and 1 in Venezuela/Peru. In addition, strains from Peru were closely related to those from Ecuador and Chile. Finally, we describe the presence of previously reported mutations that might have public health implications because of their associations with increased virulence and virus replication and mammal host adaptation along with reduced susceptibility to oseltamivir. Continuous genomic surveillance is needed to identify markers associated with mammal adaptation and potential human-to-human transmission, to inform public health measures, avoid mass animal deaths, and to protect human populations.

Acknowledgments
We thank team members from Servicio Nacional de Sanidad Agraria del Peru who were engaged in field collection and laboratory testing; Yoselín Vasquez, Christian Albajar, and Claudia Guezala for fieldwork support; Carolina Guevara and Maria Silva for laboratory guidance; and Paul Graf for his expert advice and insightful review.

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Ethics approval was not necessary for this study because activities were performed as part of a public health response to avian influenza outbreaks by Servicio Nacional de Sanidad Agraria del Peru and the US Naval Medical Research Unit SOUTH. Environmental fecal samples used for sequencing were collected under Directive Resolution no. 392-2018-MINAGRI-SERFOR-SERFOR-DGGSPFFS of the General Directorate of Sustainable Management of Forest and Wildlife Heritage, National Forest and Wildlife Service, Peru, in cooperation with the Faculty of Veterinary Medicine, Universidad Nacional Mayor de San Marcos.

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Novel Ozark Orthohantavirus in Hispid Cotton Rats (Sigmodon hispidus), Arkansas, USA

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We report a novel orthohantavirus, putatively named Ozark orthohantavirus, in hispid cotton rats captured within the Ozark Plateau in Arkansas, USA. This virus phylogenetically clusters with other orthohantaviruses that cause severe human disease. Continued orthohantavirus surveillance and virus sequencing are needed to address the potential public health threat of this virus.

Orthohantaviruses (family Hantaviridae, genus Orthohantavirus) are a group of zoonotic viruses primarily found in murid rodents; many of the viruses are pathogenic in humans (1). Pathogenic orthohantaviruses in the Americas are hosted by rodents in subfamilies Sigmodontinae and Neotominae and cause hantavirus cardiopulmonary syndrome (HCPS) in humans, which has a 30%-40% case-fatality rate (2,3). Although several pathogenic orthohantaviruses have been identified in the Americas, the specific etiologic virus is unknown for many HCPS cases (2).

We report a novel orthohantavirus species, putatively named Ozark orthohantavirus or Ozark virus (OZV), in hispid cotton rats (Sigmodon hispidus) in Arkansas, USA. Hispid cotton rats are a reservoir host of a notable pathogenic orthohantavirus, Black Creek Canal virus (BCCV) (4), in the United States and have also been identified as the host of the proposed Mule-shoe virus (5). Despite the wide distribution of hispid cotton rats in North America (22 US states and northern Mexico), previously published orthohantavirus surveillance and detection in this rat species has been limited to only Florida and Texas in the United States.

We analyzed frozen lung tissue samples collected from euthanized hispid cotton rats previously captured during 2020 and 2021 in the Ozark Plateau region of Arkansas, USA (6). Of 338 rat samples previously tested, 26 (7.7%) were orthohantavirus-seropositive; seropositive rats had been captured in 5 distinct grassland sites (6).

We performed homogenization, filtration, and nuclease pretreatment of available lung tissue samples from 13 orthohantavirus-seropositive rodents captured in 3 of the 5 unique grassland sites (Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-0549-App1.pdf) (7,8). We then extracted RNA by using Invitrogen TRIzol (Thermo Fisher Scientific, https://www.thermofisher.com) according to manufacturer guidelines. We used the NEBNext rRNA Depletion Kit (human/mouse/rat) to remove host rRNA, then the NEBNext Ultra II RNA Library Prep Kit (both from New England Biolabs, https://www.neb.com) to construct libraries. We performed next-generation sequencing by using the Illumina NovaSeq system (https://www.illumina.com). We quality filtered and de novo assembled the raw data and annotated the contigs by using LazyPipe (9).

We obtained complete genome sequences of OZV coding regions for small (S), medium (M), and large (L) segments from 2 rat samples and partial genome sequences from 6 other rat samples that included 3 additional complete S and 4 additional complete M segment sequences (Appendix Table). We used Open Reading Frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder) to detect ORFs and the Expasy translate tool (https://www.expasy.org) to translate ORFs to amino acid sequences. We compared corresponding nucleic acid and protein phylogeny of
each OZV genome segment with BCCV and other related orthohantavirus sequences obtained from GenBank by using IQ-TREE2 (http://www.iqtree.org). We then used the Sequence Demarcation Tool version 1.2 program (http://web.cbio.uct.ac.za/~brejnev/) to compare protein sequence pairwise identities of each OZV segment with those of closely related orthohantaviruses. Finally, we performed pairwise evolutionary distance (PED) analyses by using TREE-PUZZLE version 5.2 (http://www.tree-puzzle.de) with a PED cutoff value of 0.1 for species classification (10).

OZV nucleotide sequences most closely clustered with other sigmodontine-borne orthohantaviruses, particularly BCCV and Bayou virus (BAYV), which are pathogenic to humans, and Catacamas virus (CATV), which is not known to cause human infections (2). OZV S segment contig lengths were 1,988 and 1,884 nt and were 80.84% similar to BCCV, 81.15% similar to BAYV, and 80.93% similar to CATV S gene segments (Appendix Figures 1). OZV M segment contig lengths were 3,690 and 3,709 nt and were 77.91% similar to BCCV and 78.11% similar to BAYV (Appendix Figures 2). OZV L segment contig lengths were 6,523 and 6,462 nt and were 80.32% similar to BCCV, 80.16% similar to BAYV, and 80.01% similar to CATV (Appendix Figures 3). Pairwise relationships for protein sequences among OZV and related viruses were similar to those observed for nucleotide sequences (Figure, https://wwwnc.cdc.gov/EID/article/29/12/23-0549-F.htm; Appendix Figures 4–6). PED results for sigmodontine- and neotomine-borne orthohantaviruses indicated that OZV is a novel species with a PED value >0.1 and is closely related to BCCV, BAYV, and CATV (Appendix Figure 7).

OZV is the second definitive orthohantavirus species identified in hispid cotton rats. This discovery also expands the geographic distribution of orthohantavirus-carrying hispid cotton rats in the United States, previously limited to Florida and Texas; because of OZV’s similarity to BCCV and BAYV, which cause severe disease, this discovery provides crucial public health information. OZV identification also informs broader orthohantavirus evolution, especially for within-host evolution and divergence. Although uncommon, multiple orthohantaviruses in a single reservoir host species have been observed, particularly in cricetid-borne orthohantaviruses in the Americas (3).

In conclusion, hispid cotton rats are primarily found in grassland and agricultural habitats, and their range comprises the entire state of Arkansas. At least 1 HCPS case has been recorded in Arkansas; because of its close phylogenetic relationship with known human pathogens, OZV should be considered a potential cause of future HCPS cases in Arkansas, surrounding states, and other areas that harbor hispid cotton rats. Continued surveillance is needed to address the potential public health threat of OZV throughout the distribution range of the hispid cotton rat host.

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Dr. Mull is an assistant professor at Shawnee State University in Ohio, USA. His research interests focus on wildlife ecology and understanding pathogen dynamics, particularly rodentborne zoonotic viruses.

References
Food safety is an unquestioned global public health imperative. Strict controls on food before release to markets are intended to prevent disease caused by agents of infectious diseases, including parasites such as human anisakiasis. Anisakiasis, an emerging zoonosis, is caused mainly by marine nematodes of the genus *Anisakis*. They mature in dolphins and whales, but their third-stage larvae, which reside in the flesh, gonads, and body cavities of marine fish, can infect humans who consume raw or undercooked fish (1). Most human cases are reported in Japan, Spain, and South Korea (1).

Zoonotic diseases caused by marine parasites have been largely confined to coastal regions, but surveillance by health authorities in landlocked countries is lacking. However, global trade and the increasing popularity of raw fish consumption have contributed to emergence of that disease. We provide data on extensive infection of fish products with *Anisakis* larvae in Slovakia, a landlocked country in central Europe. We also report seropositive cases in a group of volunteers regularly eating fish products. We conducted this study in accordance with ethics standards in the 2013 revision of the Declaration of Helsinki of 1975. It was approved by the ethics committee of the Institute of Parasitology, Slovak Academy of Sciences (EC/01/2018; December 14, 2018).

We examined 100 frozen Atlantic herring (*Clupea harengus*) provided by a fish product supplier and 18 packages of ready-to-eat pickled herring from local supermarkets for anisakid larvae. We found 4,163 larvae in frozen Atlantic herring at an intensity of infection of 2–368 (mean 42) larvae/fish (Figure, panels A–C, E–H). Although we found most larvae in the abdominal cavity, we also found them in the muscles of 1/3 and the gonads of 1/5 of fish we examined. Although all larvae were dead, even dead parasites or their residues in contaminated fish products can cause allergic reaction in sensitized persons (2,3). In addition, we found anisakids in 1/3 of ready-to-eat pickled herring (1–9 larvae/fish) (Figure, panel D).

We morphologically identified larvae as *Anisakis* spp. and used several larvae for genotyping based on the ≈800 bp–long internal transcribed spacer region. Sequences of all isolates were identical to those of *A. simplex* sensu stricto (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0674-App1.pdf), considered the predominant agent of human anisakiasis (4).

We used a Trisakis 170, *A. simplex* IgE-ELISA kit (Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain) to test IgE sensitization to *Anisakis* spp. in a human population. The kit detects IgE to the recombinant allergens r-Ani s 1 and t-Ani s 7 in human serum, which we collected in 2020 from 91 volunteers who regularly ate fish products. The allergen tests are highly specific (up to 100%), as deduced also from primary amino acid sequences of both allergens that have no significant homologies with other allergens to which humans are known to be sensitized. Sensitivity reaches 61.1% for Ani s 1 and 93.9% for Ani s 7; many researchers consider serum truly
positive only when testing positive to Ani s 1 or Ani s 7 (5,6). Moreover, Ani s 7 and, probably, Ani s 1 are excretory-secretory allergens that are recognized by the host immune system only in the course of an Anisakis infection (5). Of 91 serum samples collected in 2020, sensitization to Anisakis allergen t-Ani s 7 was detected in 2 (2.2%) samples. The positive case-patients, both women, had no clinical symptoms of sensitization to Anisakis but had experienced allergy symptoms on several occasions in the past.

Any report of sensitization to Anisakis in Slovakia is unusual in that it is a landlocked country. Given the low level of raw fish consumption in Slovakia, the 2.2% rate of detected positivity was relatively high. In a similar study in Norway, 0/993 blood donors and 1/414 (0.2%) patients with high IgE levels tested positive for anisakiasis (6). In a study in Croatia in which the same ELISA method was used as in our study, 3.5% positivity was found in persons living on islands, but only 1.5% in persons in urban areas on the coast (7). A 15.4% positivity rate was detected in an adult population in Spain (8), whereas seroprevalence was only 0.4% in blood donors in Galicia, in northwestern Spain (9). In the future, IgE for Ani s 1, Ani s 4, Ani s 5, and Anis s 9, heat-resistant allergens that cause most clinical episodes of the allergic form of anisakiasis, should be evaluated.

Monitoring fish products intended for human consumption for parasites currently receives insufficient attention. Although all parasites we found were dead, frequent presence of Anisakis spp. in herring poses a potential risk to sensitive persons who might suffer a hyperallergic reaction. In addition, some Anisakis larvae can survive freezing (10), so risk of infection...
remains even in fish products frozen for a short time. In conclusion, the results of our study signal the need for health authorities to closely monitor marine parasites with zoonotic potential, even in inland areas.

Acknowledgements

We thank fish product supplier who generously provided frozen herrings for parasitologic examination. This study was supported by the Slovak Research and Development Agency (APVV SK-CZ-RD-21-0078 to M.O.) and Ministry of Education, Youth and Sports of the Czech Republic (LUASK22045 to T.S.).

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References


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Detection of African Swine Fever Virus from Wild Boar, Singapore, 2023

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We detected African swine fever virus (ASFV) from a wild boar in Singapore. In ≤72 hours, we confirmed and reported ASFV p72 genotype II, CD2v serogroup 8, and IGR-II variant by using a combination of real-time PCR and whole-genome sequencing. Continued biosurveillance will be needed to monitor ASFV in Singapore.

African swine fever (ASF), a nonzoonotic, World Organisation for Animal Health (WOAH) notifiable disease, is a devastating hemorrhagic infectious disease of domestic (Sus domesticus) and wild (Sus scrofa) swine populations (1). ASF was identified in Kenya in 1921 and subsequently spread through >50 countries (1). ASF is known to be spread by Ornithodorus soft ticks, vectors of ASF virus (ASFV), as well as contact with infected swine or contaminated vehicles or equipment and by consuming of infected carcasses (1). ASF often is associated with high illness and death rates in suids. However, wild suid species in Africa, such as warthogs (Phacochoerus aethiopicus), act as reservoir hosts for the virus but reportedly
**Figure.** Phylogenetic analysis of African swine fever virus detected in a wild boar, Singapore, 2023. A) Analysis of p72 genotype. Roman numerals to the right indicate the respective genotypes; 10 of 24 known genotypes are shown. B) Analysis of CD2v serogroups constructed by using the maximum-likelihood method and Tamura-Nei model with 1,000 bootstrap values in MEGA X software (https://www.megasoftware.net). Only bootstrap values >70% are shown. Black squares indicate sample from this study (Singapore/NParks/A-MAM-2023-02-00021; GenBank accession number OR135685). GenBank accession numbers are provided for all reference sequences. Scale bars indicate nucleotide substitutions per site.
remain asymptomatic (1). The occurrence of ASF in affected countries has caused substantial economic losses in the swine industry, amounting to billions of US dollars globally (2).

ASF is caused by a large, enveloped DNA virus ≈200 nm in diameter, and ASFV is the only member of the family Asfarviridae. The ASFV genome contains 170–193 kb of double-stranded DNA (1); the observed range in genome size is primarily due to gain or loss of gene copies belonging to multigene families and variation within the number of tandem repeats in noncoding regions of the ASFV genome (3).

The absence of a safe and effective vaccine against ASFV and limited information on the spatial distribution of wild boar in Asia has restricted effective disease control measures and outbreak management (4). We report detection of ASFV in a wild boar in Singapore.

A wild boar (Sus scrofa) carcass was found in the northwestern part of the Singapore main island on February 5, 2023, and was submitted to the Centre for Animal and Veterinary Sciences for disease investigation. The extensive spread of ASF within the region (5,6), coupled with the necropsy findings of hemothorax, hemoperitoneum, and widespread subcutaneous and pulmonary hemorrhage within the carcass, prompted us to include ASF as one of the key differential diagnoses.

We obtained samples from 7 organs (liver, lung, heart, spleen, lymph nodes, kidney, and tonsil) and 2 fluid samples (abdominal and thoracic fluids) from the carcass for virological analysis. In addition, we collected 2 adult ticks (1 male and 1 female) from the carcass and identified them as Dermacentor auratus ticks by DNA barcoding (7). As part of the disease investigation, we removed the residual host tissue from the ticks’ mouthparts before using the whole tick for nucleic acid extraction using the DNeasy Blood and Tissue Kit (Qiagen, https://www.qiagen.com).

We extracted viral DNA from the 7 organs and 2 fluid samples by using the IndiMag Pathogen Kit (Indiical Bioscience GmbH, https://www.indical.com), according to the manufacturer’s guidelines. We detected ASFV from the extracted nucleic acids from all 11 (9 suid and 2 tick) samples by real-time PCR (1); cycle threshold values were 19.82–33.83.

We constructed an ASFV-positive library by using the LSK109 Ligation Sequencing Kit (Oxford Nanopore Technologies, https://nanoporetech.com) and Illumina DNA Prep Kit (Illumina, https://www.illumina.com). Then we performed whole-genome shotgun sequencing by using an R9.4.1 flow cell on the MinION (Oxford Nanopore Technologies) platform and the iSeq 100 (Illumina) platform, according to the manufacturers’ specifications. We were able to retrieve full-length sequences from the wild boar samples but not from the tick samples. The overall sequence similarity from both platforms, based on an ungapped alignment to the reference ASFV sequence Georgia 2007/1 (GenBank accession no. FR682468.2), was 99.89%. We used SAMtools consensus version 1.17 and the default Bayesian counting (8) to merge the final ASFV sequence from both sequencing technology platforms. The merged sequence had 99.57% coverage and a mean depth of 14.49. We deposited the full-length ASFV genome (190,148 nt) from this study into GenBank (accession no. OR135685).

Genotyping of the p72 gene and serotyping with 90 nt from the EP402R gene have been used to characterize ASFV strains to provide possible viral origins and differentiation between closely related strains (9). The Singapore ASFV strain was classified as genotype II, based on monophyly (Figure 1, panel A), and serogroup 8 (Figure 1, panel B). Compared with the reference sequence, FR682468.2, the Singapore strain also showed insertion of an additional 10-bp tandem repeat sequence (5’-GGAATATATA-3′) between the intergenic region of the I73R and I329L gene (10), which is consistent with ASFV sequences reported in the region as IGR-II variant (5,6).

The combination of real-time PCR and high-throughput sequencing enabled rapid confirmation of the ASFV in Singapore within 72 hours of detection of the index carcass. We subsequently notified ASFV detection to WOAH on February 7, 2023, and Singapore initiated islandwide ASF control and management measures.

In conclusion, we detected ASFV in a wild boar in Singapore. How and when the virus was introduced into the local wild boar population and the significance of the D. auratus tick in ASFV transmission in Singapore remain to be determined. Further studies are ongoing to elucidate the effects of this ASF incursion to the local wild boar populations. Continued biosurveillance will be needed to monitor ASFV in swine in Singapore.

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Substantial Diversity in Cocirculating Omicron Lineages in Hospital Setting, Porto Alegre, Brazil

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We describe substantial variant diversity among 23 detected SARS-CoV-2 Omicron lineage viruses cocirculating among healthcare workers and inpatients (272 sequenced samples) from Porto Alegre, Brazil, during November 2022–January 2023. BQ.1 and related lineages (61.4%) were most common, followed by BE.9 (19.1%), first described in November 2022 in the Amazon region.

When SARS-CoV-2 variants of concern were first described, the epidemiologic situation was characterized by sequential waves of Alpha, Beta, Gamma, and Delta variants, with relatively few other variants cocirculating with the dominant variant of concern of each wave (1). The epidemiologic situation shifted with the emergence of the Omicron variant (B.1.1.529) in November 2021 (2). Distinct Omicron lineages rapidly emerged, causing successive, relatively narrow waves of infection associated with novel lineages that had pronounced immune escape and increased transmissibility (3).

The Global Action in Healthcare Network—Healthcare-associated Infection (GAIHN-HAI) module is a multinational network of healthcare facilities and laboratories developed by the Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases, US Centers for Disease Control and Prevention (Atlanta, GA, USA), to address emerging infectious disease threats in healthcare settings. The network began genomic surveillance of SARS-CoV-2 lineages affecting healthcare...

1Team members are listed at the end of this article.
workers (HCWs) and inpatients in Brazil in November 2022. We present initial findings from a tertiary-care COVID-19 reference hospital in Porto Alegre, the capital of the southernmost state of Brazil.

We conducted this surveillance study based on data from Hospital Moinhos de Vento, the first facility to join the Brazil GAIHN-HAI network. We obtained demographics and exposure risk factors by participant interview and invited into the study all HCWs and inpatients ≥18 years of age with COVID-19 diagnosed by real-time reverse transcription PCR that had a cycle threshold <30 in any probe. We performed whole-genome sequencing (Appendix, https://wwwnc.cdc.gov/EID/article/29/12/23-0880-App1.pdf) and submitted all viral genome sequences to GISAID (http://www.gisaid.org) (Appendix Table 1) (4).

During November 2022–January 2023, we collected 552 deduplicated SARS-CoV-2 real-time reverse transcription PCR–positive specimens (360 [65.2%] HCWs, 192 [34.8%] inpatients). Of the 552 specimens, we excluded 124 (22.5%) because cycle threshold was >30 and 156 (28.3%) because we were unable to obtain consent for, resulting in 272 (49.3%) samples sequenced and available for analysis. Analyzed samples consisted of 182 (66.9%) samples from HCWs and 90 (33.1%) from inpatients.

We identified 23 distinct lineages, all belonging to the Omicron variant (Table; Appendix Figure). BQ.1 and related lineages were most prevalent (61.4%), followed by BE.9 (19.1%) and others (19.5%). We detected BE.9 first, in epidemiologic week 45 of 2022, and that lineage remained cocirculating with BQ.1 in a subdominant proportion of cases throughout the study period (Appendix Figure). We also noted genetic relatedness of other Omicron lineages (Figure).

We noted no difference in the distribution of lineages between HCWs and inpatients. Compared with other lineages, BE.9 was more common in female patients (p = 0.027), younger patients (p = 0.017), and patients reporting previous contact with a person infected with SARS-CoV-2 (ps0.001). We noted vaccination status and report of a previous infection were similar among participants, regardless of lineage (Appendix Table 2).

Our findings from a select population of HCWs and inpatients from a hospital in southern Brazil revealed the cocirculation of 23 Omicron lineages over a relatively short (11-week) period. Although we observed BQ.1-related lineages most frequently, consistent with this lineage’s recent predominance both globally (5) and in Brazil (3), the number of subvariants we observed represents a departure from the observed serial dominance common with earlier variants of concern (1,6). Generalizability of our findings is limited, but our observations are consistent with other global findings suggesting that Omicron has diversified to include multiple lineages, with adequate fitness allowing them to cocirculate among humans (2,3,7,8). One proposed explanation is that population-level variation in vaccination and previous infection has led to heterogeneous

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<tr>
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<td>0.4</td>
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<td>0.4</td>
<td>60.3</td>
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</tr>
<tr>
<td></td>
<td>BQ.1.1.24</td>
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<td>61.0</td>
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<tr>
<td></td>
<td>BQ.1.1.4</td>
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<td>0.4</td>
<td>61.4</td>
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<tr>
<td>BE.9, n = 52 (19.1%)</td>
<td>BE.9</td>
<td>52</td>
<td>19.1</td>
<td>80.5</td>
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<tr>
<td>BA.5, n = 15 (5.5%)</td>
<td>BA.5.3.1</td>
<td>6</td>
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<tr>
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<td>BA.5.1.27</td>
<td>2</td>
<td>0.7</td>
<td>86.0</td>
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<tr>
<td>Other lineages, n = 38 (14%)</td>
<td>BE.10</td>
<td>14</td>
<td>5.1</td>
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<tr>
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<td>XBB.1.5</td>
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<td>97.8</td>
</tr>
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<td>BA.4.6</td>
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<td></td>
<td>BN.1.3.1</td>
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immunologic background, leading to cocirculation of distinct lineages with varying proficiency for natural and induced immunogenic escape (3,7). However, recent research suggests that immune imprinting induces Omicron receptor-binding protein mutation convergence (9).

One unexpected finding of this study was the relatively high proportion of BE.9 lineages we observed. BE.9 was first described in November 2022 in the northern Brazil state of Amazonas (7) and is characterized by a large, 244-nt deletion in the open reading frame (ORF) 7a gene at the position 27508–27751 and by mutations at spike:K444T, spike:N460K, spike:Y144del, ORF1a:V84del, and ORF1a:M85del (https://github.com/cov-lineages/pango-designation/issues/1302). Since its initial
description in Brazil, BE.9 has been reported in other countries, but only a small portion of those identified sequences have been submitted to GISAID (4). As for BE.9, most BE.10 cases (~75%) reported in GISAID are from Brazil (4). In contrast to other countries, where the recombinant XBB.1.5 has been circulating since August 2022 (10), we only detected XBB.1.5 in the study population starting January 19, 2023.

In conclusion, SARS-CoV-2 genomic surveillance at a hospital in southern Brazil found substantial diversity of Omicron lineages among HCWs and inpatients. Findings are specific to this facility and not generalizable to other hospitals or the population of Brazil. As countries globally adapt their national SARS-CoV-2 testing strategies to current COVID-19 epidemiology, they should consider focusing SARS-CoV-2 genomic surveillance strategies, along with infection trend monitoring on smaller, targeted populations such as HCWs and inpatients, to identify unusual epidemiologic events, characterize unusual viral transmission chains, and guide facility-level response measures.

The US Centers for Disease Control and Prevention GAHN-HAI team comprises Matthew Westercamp, Valery Tashayev, Morgane Donadel, Reed Magleby, Emily Petersen, and Garrett Mahon.

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References


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Tuberculosis in Lemurs and a Fossa at National Zoo, Madagascar, 2022

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We diagnosed Mycobacterium tuberculosis in captive lemurs and a fossa in Antananarivo, Madagascar. We noted clinical signs in the animals and found characteristic lesions during necropsy. The source of infection remains unknown. Our results illustrate the potential for reverse zoonotic infections and intraspecies transmission of tuberculosis in captive wildlife.

In 2020, the World Health Organization estimated that in Madagascar there were 238 cases of Mycobacterium tuberculosis complex infection per 100,000 persons, but fewer than half of infected persons were given the appropriate diagnosis and notification from public health authorities. Madagascar healthcare and wildlife protection sectors have low efficacy because government programs and infrastructure often face insufficient funding, ineffective initiative implementation, and corruption (1).

Captured wild animals experience high levels of stress and are often malnourished, which leads to immunosuppression (2). Most of the zoos in Madagascar, including the country’s national zoo, the Botanical and Zoological Park of Tsimbazaza (PBZT), hold wild-captured wildlife and have substandard captive care compared with zoos in developed countries (3,4). Humans, who may harbor pathogens, and animals at captive facilities in Madagascar are in close contact, creating an ideal setting for zoonotic and reverse zoonotic disease transmission (5).

We previously reported M. tuberculosis (lineage 3 with streptomycin resistance) infection in a wild-captured, pet ring-tailed lemur (Lemur catta) in Madagascar (6). This disease has not been reported in wild lemurs, although few populations or species have been screened (7). In this report, we document M. tuberculosis infection in L. catta and several other threatened species of lemurs, as well as in a fossa (Cryptoprocta ferox), in Madagascar (Table; Appendix Table, https://wwwnc.cdc.gov/EID/article/29/12/23-1159-App1.pdf).

Animals that tested positive for M. tuberculosis (n = 10) were housed at the PBZT in Antananarivo, Madagascar. Infected animals displayed clinical signs for extended periods, including lethargy, anorexia, vomiting, and fever, leading to death. Necropsies of 4 animals revealed massive lymphohematogenous dissemination in black and white ruffed lemurs (Varecia variegata), including nodules, lesions, and white foci noted in lungs, kidneys, spleen, and mesentery (Figure). Caseous, inflamed, and necrotic lymph nodes, and hemorrhages were also present (Appendix Figure 1). Infected, living animals (n = 6) were sampled by using oropharyngeal swabs or bronchoalveolar lavage. No information regarding the presence of M. tuberculosis in PBZT staff could be obtained.

We confirmed M. tuberculosis infection by PCR on the clinical samples using the GeneXpert MTB/RIF Ultra assay (Cepheid, https://www.cepheid.com) and culture on BACTEC-MGIT liquid media (8). We conducted whole-genome sequencing on extracted DNA from culture isolates by using MinION (Oxford Nanopore Technologies; https://nanoporetech.com) and NovaSeq PE150 (Illumina, https://www.illumina.com/) sequencing platforms. We mapped decontaminated sequencing reads to the M. tuberculosis H37Rv reference genome (accession no. NC_000962.3). Lineage typing based on single-nucleotide polymorphisms revealed all isolates cluster to lineage 4.3.3. (Euro-American lineage, Latin American sublineage) (9) and have a maximum distance of 2 single-nucleotide polymorphisms (Appendix Figures 2, 3). GeneXpert MTB/RIF Ultra and sequencing resistance prediction from cultures of clinical isolates did not show any rifampin resistance.

We deposited all the sequence data used in this study into the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA659624). We processed BACTEC-MGIT cultures at...
the National Reference TB Laboratory at the Institut Pasteur de Madagascar in Antananarivo. For every clinical isolate batch on BACTEC-MGIT culture, we used a positive-control mycobacteria growth indicator tube containing the laboratory reference stain h37Rv and a negative-control tube with the decontamination phosphate buffer. The National Reference TB Laboratory is externally certified twice annually for quality assurance and competency testing on BACTEC-MGIT culture.

Lineage 4 is both the most geographically widespread tuberculosis lineage and the most prevalent in persons residing in Antananarivo (10). Primates and other wildlife can contract Mycobacterium from

<table>
<thead>
<tr>
<th>Animal</th>
<th>Species (animal number)</th>
<th>Sex</th>
<th>Birth location</th>
<th>Date of birth</th>
<th>Date of death</th>
<th>Sample method</th>
<th>M. tuberculosis detected by GeneXpert</th>
<th>M. tuberculosis sublineage</th>
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<tbody>
<tr>
<td>Fossa</td>
<td>Cryptoprocta ferox</td>
<td>M</td>
<td>Wild</td>
<td>Unknown</td>
<td>2022 Aug</td>
<td>Necropsy</td>
<td>Low</td>
<td>4.3.3</td>
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<tr>
<td>Lemur</td>
<td>Eulemur flavifrons</td>
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<td>Unknown</td>
<td>Unknown</td>
<td>Alive in 2022 Oct</td>
<td>Swab</td>
<td>Trace</td>
<td>4.3.3</td>
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<tr>
<td></td>
<td>E. fulvus</td>
<td>M</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Alive in 2022 Oct</td>
<td>Swab</td>
<td>Trace</td>
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</tr>
<tr>
<td></td>
<td>E. rufus</td>
<td>F</td>
<td>Unknown</td>
<td>Unknown</td>
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<td>Trace</td>
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</tr>
<tr>
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<td>F</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Alive in 2022 Oct</td>
<td>Swab</td>
<td>Trace</td>
<td>4.3.3</td>
</tr>
<tr>
<td></td>
<td>Propithecus coquereli</td>
<td>M</td>
<td>Wild</td>
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<td>Low</td>
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<tr>
<td>Varecia variegata (1)</td>
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<td>PBZT</td>
<td>2019 Oct 7</td>
<td>2022 Jul 24</td>
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<td>High</td>
<td>4.3.3</td>
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<td>4.3.4</td>
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<tr>
<td>V. variegata (3)</td>
<td>M</td>
<td>Palmarium</td>
<td>2015</td>
<td>2022 Sep 12</td>
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<td>High</td>
<td>4.3.3</td>
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</tbody>
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*The National Reference TB Laboratory at the Institut Pasteur de Madagascar in Antananarivo is externally certified twice annually for quality assurance and competency testing on BACTEC-MGIT culture.*

**Table.** Details on Mycobacterium tuberculosis complex–positive animals at PBZT, Madagascar, 2022

**Figure.** Necropsy images of 2 lemurs (detailed in the Table), both Varecia variegata, from a study of Mycobacterium tuberculosis complex–positive animals at the Botanical and Zoological Park of Tsimbazaza, Madagascar, in 2022. A) Body cavity with nodules and white spots in liver (animal 3). B) Tracheobronchial caseation of lymph nodes (animal 1). C) Tracheobronchial caseation of lymph nodes (animal 1). D) Black spots in stomach mucosa (animal 3). Scale bars indicate 1 cm.
humans (5). Given that the whole genome sequences from animals in the PBZT have a low maximum distance (2 single-nucleotide polymorphisms) and that the human isolates with the same sublineage L 4.3.3 were found in close proximity to the PBZT around the same time period, there is a possibility that an infected human transmitted the disease to multiple animals. However, it is also possible that interspecies transmission might have occurred, although it is not known whether lemurs or fossas are reservoir hosts for *M. tuberculosis*. If they are, transmission to wild lemurs and other endemic wildlife could pose a threat (6) because captive wild animals are sometimes released into forests or live near wild populations (2). Moreover, immunocompromised humans may be at risk for *M. tuberculosis* latent or active infection if they are close to diseased animals, such as those at PBZT.

The threat of TB transmission between humans and endangered wildlife, such as lemurs, invokes the need for changes that minimize interactions between humans and wildlife, reducing the chance of new disease outbreaks (5,6). Recommendations to protect Madagascar wildlife should include no wild capture of animals for captivity, no breeding of wildlife in substandard captive conditions, improved captive care that is comparable to international standards, and humane euthanasia of animals with communicable diseases or disease exposure. We also recommend annual testing (and negative results) for communicable diseases in humans who work in proximity to wildlife and no-contact restrictions for the public and wildlife. Those recommendations are consistent with the guidelines of the American Association of Zoo Veterinarians.

The University of San Diego provided ethical oversight (no. IACUC 0619–01).

**About the Author**

Dr. LaFleur is an associate professor at the University of San Diego, California, USA, and the founder and director of Lemur Love, a US-based nonprofit organization conducting research, conservation, and small-scale development in Madagascar. Her research examines the ecology of wild ring-tailed lemurs and the legal and illegal trades of wild-captured lemurs in Madagascar. She is additionally interested in animal welfare and zoonotic diseases of captive wildlife.

**References**


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• Efficacy of Unregulated Minimum Risk Products to Kill and Repel Ticks
• Auritidibacter ignavus, an Emerging Pathogen Associated with Chronic Ear Infections
• Incidence of Legionnaires’ Disease among Travelers Visiting Hotels in Germany, 2015–2019
• Costs of Digital Adherence Technologies for Tuberculosis Treatment Support
• Effectiveness of Vaccines and Antiviral Drugs in Preventing Severe and Fatal COVID-19, Hong Kong
• Doxycycline Prophylaxis for Skin and Soft Tissue Infections in Naval Special Warfare Trainees, United States
• Delayed Plasmodium falciparum Malaria in Pregnant Patient with Sickle Cell Trait 11 Years after Exposure, Oregon, United States
• Excess Deaths Associated with Rheumatic Heart Disease, Australia, 2013–2017
• Increased Peripheral Venous Catheter Bloodstream Infections during the COVID-19 Pandemic, Switzerland
• Emergence of Novel Norovirus GII.4 Strains on 3 Continents

• Avian Influenza A(H5N1) Neuraminidase Inhibition Antibodies in Healthy Adults after Exposure to Influenza A(H1N1)pdm09
• Macacine alphaherpesvirus 1 (B Virus) Infection in Humans, Japan, 2019
• Estimation of Incubation Period of Mpox during 2022 Outbreak in Pereira, Colombia
• Reemergence of Human African Trypanosomiasis Caused by Trypanosoma brucei rhodesiense, Ethiopia
• Respiratory Viruses in Wastewater Compared with Clinical Samples, Leuven, Belgium
• Shiga Toxin–Producing Escherichia coli Diagnoses from Health Practitioners, Queensland, Australia
• Use of Doxycycline to Prevent Sexually Transmitted Infections, According to Provider Characteristics
• Mycobacterium senegalense Infection in Kidney Transplant Patient with Diabetes, Memphis, Tennessee, USA
• Emergence of Dengue Virus Serotype 2 Cosmopolitan Genotype, Colombia

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Article Title
Invasive Nocardia Infections across Distinct Geographic Regions, United States

CME Questions

1. Which one of the following statements regarding Nocardia species is most accurate?
   A. It is gram-negative
   B. It does not infect immunocompetent adults
   C. The main route of exposure is direct contact with the skin
   D. The most common sites of extrapulmonary involvement include the brain and skin

2. Which one of the following statements regarding the types of infection with Nocardia in the current study is most accurate?
   A. Less than one quarter of patients had isolated pulmonary involvement
   B. Disseminated infection was approximately twice as common among transplant recipients vs nontransplant recipients
   C. Half of transplant patients had central nervous system involvement
   D. The most common isolate among all patients was N. brasiliensis

3. Which one of the following Nocardia species was most associated with a higher risk for skin or soft tissue disease among transplant recipients in the current study?
   A. N. brasiliensis
   B. N. farcinica
   C. N. cyriacigeorgica
   D. N. nova complex

4. Which one of the following statements regarding treatment and outcomes of Nocardia infection in the current study is most accurate?
   A. Nocardia susceptibility to trimethoprim/sulfamethoxazole was approximately 30%
   B. Nocardia susceptibility to fluoroquinolones was high for every species
   C. Survival was reduced by 10% among transplant patients vs nontransplant patients
   D. Infection with N. farcinica was associated with a significantly higher mortality rate vs other Nocardia species
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Article Title
Risk Factors for Enteric Pathogen Exposure among Children in Black Belt Region of Alabama, USA

CME Questions

1. Which one of the following statements regarding demographic and sanitation data from the current study by Capone and colleagues is most accurate?
   A. More than 90% of children in the study identified as Black
   B. The study sample was taken from rural and urban communities from 6 states across the US South
   C. Most families reported straight piping wastewater directly onto their property
   D. Most participants reported less than 2 hours of screen time per day

2. What percentage of the study cohort had a positive test for a stool pathogen in the current research?
   A. 6%
   B. 26%
   C. 48%
   D. 70%

3. Which one of the following enteric pathogens was most commonly encountered in the current study sample?
   A. Clostridioides difficile
   B. Helicobacter pylori
   C. Salmonella spp.
   D. Blastocystis spp.

4. Which one of the following variables was the most significant risk factor for a positive polymerase chain reaction (PCR) test for an enteric pathogen in the current study?
   A. Straight pipe of wastewater into yard
   B. Presence of a septic tank
   C. Higher durations of screen time
   D. Not paying a water bill