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Zoonotic Infections
April 2022

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Shewanella spp. Bloodstream Infections in Queensland, Australia

Kevin B. Laupland, Adam G. Stewart, Felicity Edwards, David L. Paterson, Sonali Coulter, Claire Heney, Narelle George, Patrick Harris

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 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. Medscape, LLC designates this Journal-based CME activity for a maximum of 1.00 AMA PRA Category 1 Credit(s)™. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

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Release date: March 17, 2022; Expiration date: March 17, 2023

Learning Objectives

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

• Assess the incidence and epidemiology of Shewanella species bloodstream infections, based on findings of a population-based surveillance study in Queensland, Australia, during 2000–2019

• Evaluate the risk factors for development of Shewanella species bloodstream infections and clinical course, based on findings of a population-based surveillance study in Queensland, Australia, during 2000–2019

• Determine the clinical and public health implications of the incidence and epidemiology of and risk factors for development of Shewanella spp. bloodstream infections, based on findings of a population-based surveillance study in Queensland, Australia, during 2000–2019.

CME Editor

Thomas J. Gryczan, MS, Technical Writer/Editor, Emerging Infectious Diseases. Disclosure: Thomas J. Gryczan, MS, has disclosed no relevant financial relationships.

CME Author

Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. Disclosure: Laurie Barclay, MD, has disclosed the following relevant financial relationships: owned stock, stock options, or bonds for AbbVie (former).

Authors

Kevin B. Laupland, PhD; Adam G. Stewart, MBBS; Felicity Edwards, BHlthSc; David L. Paterson, MBBS; Sonali Coulter, PhD; Claire Heney, MBBCh; Narelle George, MSc; and Patrick Harris, PhD

Author affiliations: Royal Brisbane and Women’s Hospital, Brisbane, Queensland, Australia (K.B. Laupland, A.G. Stewart, D.L. Paterson); Queensland University of Technology, Brisbane (K.B. Laupland, F. Edwards); University of Queensland, Brisbane (A.G. Stewart, D.L. Paterson, P. Harris); Medication Services Queensland, Brisbane (S. Coulter); Pathology Queensland, Brisbane (C. Heney, N. George, P. Harris)

DOI: https://doi.org/10.3201/eid2704.212193
The epidemiology of bloodstream infections caused by *Shewanella* spp. is not well defined. Our objective was to define the incidence and determinants of *Shewanella* spp. bloodstream infections by using population-based surveillance in Queensland, Australia during 2000–2019. The incidence was 1.0 cases/1 million persons annually and was highest during summer and in the tropical Torres and Cape region. Older persons and male patients were at highest risk. At least 1 concurrent condition was documented in 75% of case-patients, and 30-day all cause case-fatality rate was 15%. Aging populations in warm climates might expect an increasing burden of these infections.

*Shewanella* spp., most commonly *S. algae* and *S. putrefaciens*, are infrequent but occasionally severe causes of human infection associated with exposure to warm marine environments (1, 2). Cases of otogenic and skin and soft tissue infections caused by *S. (Pseudomonas) putrefaciens* were described in the 1960s, and case series of bacteraemic infections were reported in subsequent decades (3, 4). Vignier et al. reported 16 cases of *Shewanella* spp. infection that occurred in Martinique during 1997–2012 and identified an additional 239 cases in review of the published literature during 1973–2011 (5). That study observed that otogenic, skin and soft tissue, abdominal/biliary tract, and respiratory tract foci of infection were most common, and that 71 (28%) cases were bacteraemic (5). *Shewanella* spp. are frequently coisolated with other organisms, most notably Enterobacterales and *Aeromonas* and *Vibrio* spp., and occasionally might develop major antimicrobial drug resistance (2, 5, 6).

As a result of their rarity, the epidemiology of *Shewanella* spp. bloodstream infections (BSIs) is poorly defined. The existing body of literature is limited to case reports and small series. In addition, as a result of developments in genomic and phenotypic testing, it has been recognized that before the current millennium many reports of *S. putrefaciens* infection might have been actually caused by *S. algae* (7). The objective of this study was to determine the contemporary incidence of and risk factors for development of *Shewanella* spp. BSIs in the population of Queensland, Australia.

**Methods**

The study population was all residents (2019 population ≈5 million) of Queensland, Australia. Queensland is a large state with diverse geography that includes subtropical and tropical coastal regions and inland dry desert areas. Approximately two thirds of the population is concentrated around the Greater Brisbane/Gold Coast/Sunshine Coast areas in the southeastern corner of the state, and the remainder is distributed predominantly along the eastern coastal areas. Healthcare within the publicly funded system is administered through 16 hospital and healthcare service regions (8). All Queensland residents with incident BSIs caused by *Shewanella* spp. identified by Pathology Queensland during January 1, 2000–December 31, 2019, were included in this study. Approval of the health research ethics committee at the Royal Brisbane and Women’s Hospital was granted with a waiver of individual consent (LNR/2020/QRBW/62494).

During the study, we used different methods to identify *Shewanella* spp.. Before 2008, VITEK 1, API20E, API20NE, (all from bioMérieux), and Microscan (Baxter Diagnostics Inc., https://www.baxter.com) were used to identify *Shewanella* spp. but these methods were not able to identify *S. algae*. From ≥2008 onward, the VITEK database for gram-negative identification was upgraded to improve differentiation of *S. algae*. After matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was introduced during 2012, differentiation of the 2 *Shewanella* spp. was possible. In some instances in which earlier methods could not reliably distinguish between *S. putrefaciens* and *S. algae*, growth in nutrient broth with and without 6% NaCl was used to confirm the species identification. In instances in which methods were inadequate to reliably differentiate species, isolates are reported as *Shewanella* spp. We performed antimicrobial drug susceptibility testing by using an automated method (i.e., VITEK AST Card, bioMérieux) and disc diffusion according to recognized standards (Clinical and Laboratory Standards Institute, https://clsi.org) at the time of testing.

All blood cultures that had growth of *Shewanella* spp. were retrospectively identified by the Clinical Information Systems Support Unit, Queensland Health, during the study period. We defined incident BSIs by the first isolation of a *Shewanella* spp. for a patient; all subsequent isolations of the same species from that patient within 30 days were deemed to represent the same episode. Polymicrobial infections were those from which a *Shewanella* spp. was coisolated with ≥1 other major pathogens within a 48-hour period. Two independent sets of cultures were required for common contaminants to define significance (9).

Once incident episodes were identified, we obtained additional clinical and outcome information by using linkages to statewide databases. We identified all healthcare encounters with private and public institutions within the 2 years before 1 year after index blood cultured within the Queensland Hospital.
Admitted Patient Data Collection. We used this collection to determine healthcare encounters and hospital admission and discharge dates, discharge survival status, as well as all diagnostic codes (International Classification of Diseases, 10th Revision, Australian modification). Multiple admission episodes occurring within a continuous time period (such as with interhospital transfers) were deemed to represent 1 hospital admission for purposes of length of stay. We queried the Registry of General Deaths (https://info.australia.gov.au) as of December 31, 2020, to identify all deaths.

We classified BSIs as hospital-onset if the index blood culture was obtained 2 calendar days after admission or within 2 calendar days of hospital discharge (10). BSIs infections diagnosed in the community or within the first 2 calendar days of stay in hospital were classified as community-onset. Healthcare-associated BSIs were those community-onset BSIs that fulfilled ≥1 criteria: nursing home resident, encounter at a healthcare institution within 30 days, or admission to a hospital for >2 days within 90 days before index blood culture (10). We classified community-onset BSIs that did not fulfill criteria for healthcare-associated infections as community-associated. We defined comorbid medical illnesses by using Charlson comorbidity index and established these illnesses by using validated coding dictionaries (11,12). We assigned a clinical focus on the basis of review of diagnosis-related group and primary hospital discharge codes.

We analyzed data by using Stata version 16.1 (StataCorp LLC, https://www.stata.com). The primary unit of analysis was incident BSI episodes, reported as crude incident rates per million persons annually. We excluded cases identified among persons who were not Queensland residents and obtained denominator data stratified by age, sex, and hospital and health service area from Queensland Health (13). We obtained the total annual number of sets of blood cultures performed by Pathology Queensland to calculate the overall culturing rate per population as described (14). We obtained average monthly mean peak and low temperatures and rainfall from Queensland weather stations from the Australian Bureau of Meteorology (15). Incidence rate differences were expressed as incidence rate ratios and reported with exact 95% CIs. p values <0.05 represented statistical significance.

Results

During >86 million person-years of surveillance, 86 *Shewanella* spp. BSIs occurred to give an incidence of 1.0 cases/1 million Queensland residents/year. We identified 4 additional episodes of *Shewanella* spp. BSIs for persons residing in other states in Australia, and we excluded them from analysis. No second episodes of incident *Shewanella* spp. BSIs occurred. Most (65, 76%) cases of BSIs were caused by *S. algae*, 4 (5%) were caused by *S. putrefaciens*, and 17 (20%) were not identified to the species level.

There was moderate year-to-year variability in the overall incidence of *Shewanella* spp. BSIs (Figure 1). Occurrence of cases of *Shewanella* spp. BSIs varied according to the month of the year; there was a peak in the warmer, wetter months and nadir in the drier, cooler months (Figure 2). Incidence of *Shewanella* spp. BSIs varied considerably among the regions of the state (Figure 3). No cases were observed within the western outback regions, and low rates were observed in the Greater Brisbane area. The highest rates occurred in the tropical coastal Torres and Cape area (Figure 3).

The median age of case-patients was 71.4 (interquartile range [IQR] 60.3–82.8) years, and 72 (84%) incident episodes were in male patients. There was an increased risk for development of *Shewanella* species BSIs with advancing age, particularly among Figure 1. Incidence (cases/1 million persons) of *Shewanella* species bloodstream infections and number of blood samples collected per 1 million persons, Queensland, Australia.

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SYNOPSIS

male patients (Figure 4). Male patients had an overall 5-fold increased risk compared with female patients (1.7 cases/1 million vs. 0.3 cases/1 million; incidence rate ratios for male patients 5.2 [95% CI 2.9–9.0]; p<0.0001).

Most BSIs were community-onset; 25 (29%) were classified as healthcare-associated, 54 (64%) as community-associated, and 6 (7%) as hospital-onset. Of the 25 healthcare-associated case-patients, 21 (84%) had hospital visits within 30 days, and 10 (40%) had hospital admissions within 90 days before the index episode. None were nursing home residents. The median Charlson comorbidity index was 2 (IQR 1–3), and only 21 (25%) patients had no underlying conditions documented. The most common focus of infection was soft tissue (35 cases, 41%) cases. Seven (8%) case-patients had an abdominal focus, 6 (7%) had a lower respiratory focus, 2 (2%) had an endovascular focus, and 1 case each had a bone/joint, head/neck, or genitourinary focus of infection. No focus was identified for 33 (38%) case-patients.

Most (73, 70%) infections were monomicrobial. Among the 13 polymicrobial infections, 4 patients had 3 isolates, and 2 patients had 4 isolates. The co-isolated organisms included Escherichia coli in 7 cases, and 1 each of Candida spp., Corynebacterium diphtheriae, Enterobacter cloacae, Klebsiella oxytoca, Proteus mirabilis, Sphingomonas paucimobilis, Pseudomonas oryzihabitans, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, group G streptococcus, Vibrio vulnificus, and 1 unidentified gram-negative bacillus. Four isolates (n = 65; 6%) were meropenem resistant and 1 (n = 70; 1%) was ceftazidime resistant. No resistance to gentamicin (n = 76) or ciprofloxacin (n = 74) was observed among Shewanella spp. isolates tested.

All but 1 patient was admitted to a hospital for management (median length of stay 8 [IQR 5–16] days). Four patients required admission to an intensive care unit. Twelve (14%) patients died during the index hospitalization, and 13 (15%) died within 30 days of BSI diagnosis. Among the 13 patients who died, 6 (46%) had no focus identified, 3 (23%) had an abdominal focus, and 2 (15%) had soft tissue and lower respiratory tract infections. Eleven (85%) patients who died had ≥1 Charlson comorbidity.
Discussion
We describe the epidemiology of *Shewanella* species BSIs in a large population in Australia. We confirm these infections as rare infections and provide novel incidence data. In addition, although usually considered community-associated pathogens, 29% of our cases were healthcare-associated and 7% hospital-onset. We also observed that the most patients had underlying medical illnesses and that the elderly were at highest risk. This finding is useful given that the Queensland population, like those in many other high-income countries, is aging and showing an increased prevalence of chronic illnesses. Therefore, we might expect that the burden of *Shewanella* species BSIs will increase in the coming years.

There are few previous contemporary studies for which to compare our results. Although case series have been published since the turn of the millennium, they have included small numbers of cases, of which only a minority have been associated with BSIs (2,5). Our observation of a temporal relationship between seasonal temperature and rainfall is much more pronounced than when similarly examined in a previous study conducted in Reunion Island (2). Climate factors probably play a major role in these infections given that they are usually identified in warm regions. Therefore, these infections probably involve a complex interaction between environmental and human activity–related factors.

Vignier et al. reported a case series from Martinique and summarized the world literature on *Shewanella* species infections during 2013 (5). Although that study provided useful results, similar to all summaries of case reports, these results must be interpreted with caution because unusual or atypical cases might be more likely to be submitted and accepted for publication. Therefore, summaries of such reports might not reflect the average or usual characteristics of such infections. Our study has the benefit of comprehensive and consistent identification of all cases identified by a statewide laboratory such that selection biases are minimized and secular changes over time might be observed.

Nearly all of the *Shewanella* species BSIs occurring in our population during years when we had adequate means of identification were caused by *S. algae*. It has been recognized that many *S. putrefaciens* cases previously reported were probably *S. algae* because the clinical characteristics of these infections might be confused (5,7). Unfortunately, we do not have all of our original isolates for retesting.

Our study benefited from surveillance of a large population in Queensland. Including BSI data from public hospital and community collection sites over a 20-year period resulted in >86 million person-years of observations. The relatively small number of BSIs attributed to *Shewanella* species (86) limited the statistical significance of further analysis of the data, such as differences between species, underlying conditions, and outcome. A strength of our study is that our laboratory surveillance was statewide in scope and included specimens sent from hospital and community collection sites.

However, our study was limited to the publicly funded system. Thus, cases identified within private hospitals and collection sites were not included. Although we speculate that these cases represent a small proportion of missed cases, our incidence rates should be interpreted as potential underestimates of the true population rate. A second limitation was that our study was retrospective; therefore, we were restricted in data variables for analysis. It would have been
informative to examine environmental exposures, such as risk factors and the effect of specific antimicrobial drug treatments on outcome (16). A third limitation was that as a result of small numbers, we did not age and sex standardize our incidence rates. The possibility exists that at least some of the increased incidence observed in recent years of the study could have been related to demographic changes. Finally, as is the case for any study examining BSIs, our ability to detect a case is dependent on whether clinicians obtain blood for culturing from patients who have suspected infections. In addition, only a portion of all disease attributable to Shewanella species is associated with positive blood cultures. Accordingly, the true rate of disease attributable to Shewanella species in our population is likely higher than we detected (14).

In conclusion, we present a novel study of Shewanella species BSIs that details the epidemiology of these infections in a large population in Australia. We observe that older persons are at highest risk and that their incidence is increased in association with higher environmental temperatures. Although Shewanella species BSIs are rare, there is a major potential for large increases in coming years as a result of aging populations and climate change. Ongoing surveillance is warranted.

Acknowledgments

We thank the team of the Statistical Services Branch, Queensland Health, for linking the data sets used for this study.

About the Author

Dr. Laupland is an infectious diseases specialist, intensivist, and director of research in the Department of Intensive Care Services, Royal Brisbane and Women’s Hospital, Queensland, Australia. His primary research interest is the epidemiology of severe infections in populations.

References


Address correspondence: Kevin B. Laupland, Intensive Care Services, Level 3 Ned Hanlon Bldg, Royal Brisbane and Women’s Hospital, Butterfield St, Brisbane, QLD 4029, Australia; email: kevin.laupland@qut.edu.au
The Aedes aegypti mosquito is the primary vector of arboviruses such as dengue (DENV), Zika (ZIKV), chikungunya (CHIKV), and yellow fever. This mosquito species is common in urbanized areas in the tropics because it is highly adapted to live in close association with humans, preferentially feeding on blood of human hosts and laying eggs in containers located around human dwellings (1–6). Estimates indicate that ≈3 billion persons live in areas with ongoing DENV transmission (7).

Traditional entomologic surveillance for Ae. aegypti mosquitoes is based on periodic inspections of larvae and pupae in domestic breeding sites, which provide measures of infestation known as the house index (HI), the percentage of houses in which >1 larva or pupae was collected, and Breteau index (BI), the number of containers positive for larvae or pupae divided by the number of inspected houses. By using available infestation data, public health managers intensify control strategies in the areas with higher indices. Of note, indices based on collection of immature mosquitoes face many criticisms because surveys are costly to perform with the frequency required for adequate surveillance; indices are highly dependent on the agent’s motivation to effectively search for larvae in myriad container types, including cryptic and hard-to-access containers; surveys do not consider container productivity (i.e., these surveys might only provide measures of presence or absence immature mosquitoes); and larval density has proven to be a poor indicator of adult mosquito density (8–12).

Traps capturing adult mosquitoes could be a promising alternative to larval surveys because they sample the vector life stage that is directly responsible for transmission and provide qualitative (percent positive traps) and quantitative (number of captured mosquitoes per trap) indices (8,13–17). Adult traps provide relative measurements of the vector population, expressed in units of mosquitoes by area, mosquitoes per person, or mosquitoes per trap (8,18,19). Therefore, adopting adult traps in an arbovirus-endemic setting likely would provide relevant information regarding the spatiotemporal dynamics of Ae. aegypti mosquitoes.
Effective arbovirus surveillance should be able to accurately predict when and where an outbreak will occur. Routine virologic surveillance in field-caught *Ae. aegypti* mosquitoes, (entomo-virologic surveillance) is one measure that could be adopted to enhance surveillance effectiveness (20). In DENV-endemic settings of developing countries, screening for natural infection in field-caught *Ae. aegypti* mosquitoes has been performed in various situations but rarely as a component of a long-term routine surveillance to direct control interventions to critical areas (21–23). By adding entomo-virologic surveillance to routine surveillance based on large-scale adult mosquito trapping across an entire city, health managers ideally would be able to identify hotspots of disease transmission and intensify vector control in those regions before human cases arose (19,24).

We report on a 4-year integrated citywide vector surveillance approach that involved extensive use of adult mosquito traps, molecular diagnostic testing for natural arbovirus infection in live collected mosquito specimens, construction of transmission risk maps, and performance of timely vector control intervention ≤48 h after mosquito collection. In this scheme, vector control was intensified in areas with higher risk for transmission instead of maintaining homogeneous vector control efforts over the landscape. In addition, we evaluated the correspondence of larval- and adult-based indices with the epidemiologic trend in the city of Foz do Iguaçu, Brazil, during 2017–2020.

**Methods**

**Study Site**

We implemented an entomo-virologic surveillance system in the city of Foz do Iguaçu (25°30′58″S, 54°35′07″W), Brazil, which is located on the triple border with Argentina and Paraguay. Foz do Iguaçu has ≈250,000 inhabitants and an intense daily population movement across the 3 countries’ border cities. Foz do Iguaçu is divided into 73 urban areas of ≈1,500 premises each (25), plus 3 rural areas that were not included in this study. We defined premises as a property occupied by a residence or a business at ground level. According to the Brazil Ministry of Health, apartment buildings are not included, and surveillance and vector control interventions take place only at the foyer. The climate in Foz do Iguaçu is classified as humid tropical, according to the Köppen-Geiger system, and is characterized by hot and humid summers (mean temperature >27°C) and cold to mild winters (mean temperature <15°C), with an annual rainfall >1,850 mm.

**Adult Mosquito Collection**

During January 2017–December 2020, a total of 3,476 Adultraps (Berdon, https://adultrap.com.br) were installed in the city, and 1 trap could be found in the peridomestic environment for every 25 premises. This system was originally designed to capture gravid *Ae. aegypti* female mosquitoes during oviposition because Adulttraps use water as the principal attractant. These traps have an opening on the top where females enter, then are trapped in an interior chamber (16,26). Water remains confined in a compartment at the bottom of the trap that the mosquitoes cannot access, thus deterring egg laying. Local health agents visit all Adulttraps every 2 months, within the first 4 days of the first week of odd months, when agents usually conduct larval surveys as part of traditional entomologic index. Therefore, during the study period, the 3,476 Adulttraps were inspected 24 times in the same premises, a total of 83,424 trap inspections.

**Entomologic Indices**

Besides the traditional HI and BI based on larval surveys, the Adultrap inspections produced 3 entomologic indices based on adult collections. The trap positivity index (TPI) is the number of positive traps among the total number of traps inspected multiplied by 100; the adult density index (ADI) is the total number of *Ae. aegypti* mosquitoes captured divided by the total number of inspected traps multiplied by 100; and the mosquitoes per inhabitant index (MII) is the total number of adult *Ae. aegypti* mosquitoes collected, divided by the number of persons in each house with an Adulttrap multiplied by 1,000. We calculated all entomologic indices every 2 months during 2017–2020, a total of 24 observations per index.

**Entomo-Virologic Screening**

Mosquitoes collected alive during each 2-month period were sent to the entomology laboratory for further taxonomic identification by using appropriate keys. Mosquitoes classified as *Ae. aegypti* were placed in cryogenic tubes for diagnosis of arbovirus infection by quantitative real-time PCR (qPCR). Depending on the number of mosquitoes captured in traps on the same city block, we pooled ≤10 mosquitoes per block, separating male from female mosquitoes. We calculated minimum infection rate (MIR) by dividing the number of positive pools by the total specimens tested, then multiplied by 1,000 (27). To estimate MIR, we used only data from DENV-positive pools because only a few pools were positive for ZIKV or CHIKV.
RNA Extraction and Real-Time qPCR
We extracted viral RNA from *Ae. aegypti* mosquitoes by using the MagMAX Viral/Pathogen Nucleic Acid Ultra Isolation KIT (Applied Biosystems/Thermo Fisher Scientific, https://www.thermofisher.com), according to the manufacturer’s instructions. We added single or pooled mosquitoes to electromagnetic mixing beads (MagMAX Viral/Pathogen Binding Beads; Applied Biosystems) and macerated using TissueLyser II (QIAGEN, https://www.qiagen.com). After RNA extraction, we separated an aliquot of 2 µL from each sample and used this to read the concentration of viral RNA recovered in a NanoDrop One Spectrophotometer (Thermo Fisher Scientific).

For arboviral genome amplification, we used the ZDC Biomol Kit (Instituto Biologia Molecular do Paraná [IBMP], https://www.ibmp.org.br) (28–31), which enables identification of ZIKV, CHIKV, and differentiation of DENV serotypes with an internal control (IC) of the reaction that uses probes specific to each molecular target. We used a 96-well QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) for PCR and analyzed results by using QuantStudio Design and Analysis Software versions 1.3.1 and 1.5.1 (Applied Biosystems). We considered samples positive when the amplification plot curve exceeded the specific threshold for each target ≤35 cycle threshold.

Epidemiologic Surveillance and Case Report
The health system in Foz do Iguaçu is composed of 30 basic health units, including 2 emergency care units, 3 private hospitals, and 1 public hospital. The Ministry of Health lists dengue, Zika, and chikungunya as diseases of compulsory notification that can be registered in any of the local health facilities. Epidemiologic surveillance for arboviruses is carried out passively after symptomatic persons seek care in the city health system. Zika and chikungunya cases were reported in the city before 2017, but no further large outbreaks were reported in Foz do Iguaçu (33). We assumed a Gaussian distribution for the continuous response variable and implemented the models in the lme4 package in R (R Foundation for Statistical Computing, https://www.r-project.org). We obtained significance values for fixed effects in the Imertest software package (R Foundation for Statistical Computing). We chose the best scenario by using Akaike information criteria (AIC) to rank models (ΔAICc), and calculated Akaike weights (wAICc) to evaluate the relative support of each model (34). We used ΔAICc to evaluate the differences in AIC score between the best model and the other models. We used Akaike weights to evaluate model selection uncertainty, which quantified the probability that the model was the best among those considered based on the data (34,35). We selected the best supported model based on rejection of GLMM null hypothesis (p<0.05), the lower AIC value, and an AIC weight >0.7 (70% confidence set) (34). We considered models with ΔAICc of <4.0 to have no differences (36). We implemented the ΔAICc and wAICc in the bbmle package (R Foundation for Statistical Computing).
Results

Entomologic Survey of Larvae

In each 2-month period, an average of ≈4,883 (range 4,781–5,021) premises were inspected, which correspond to ≈6.25% of houses in Foz do Iguaçu. During 2017–2020, a range of 3.5%–17.7% of inspected Adulttraps were positive for mosquitoes, and an average of 9.5% of traps had ≥1 Ae. aegypti mosquito. We used the number of positive houses to create HI and the number of containers to create BI and observed strong seasonal variation; values were 7 times higher during the wet summer (November–March) than in the dry winter (July–September) (Appendix 1, https://wwwnc.cdc.gov/EID/article/28/4/21-1547-App1.xlsx). The average HI of Foz do Iguaçu was 2.58% during the 24 observations of 2017–2020, and only twice was HI above the 4% alert level adopted by Ministry of Health, reaching 5.41% in March 2019 and 5.29% in May 2019.

We used the number of positive houses to estimate HI and number of positive breeding sites in each of larval survey to estimate traditional BI (Figure 1). Indices based on larval surveys showed an expected seasonal variation with higher values during the rainy summer (=November–March), but HI and BI fluctuations were only partially in accordance with the dengue notification curve (Figure 1).

Entomologic Survey for Adult Mosquitoes

Adult Ae. aegypti mosquitoes were collected on the same premises where larval surveys were performed. The average number of inspected traps was 2,468 (range 2,239–2,767). Therefore, a mean of 73% of adult traps were inspected bimonthly. A total of 11,962 adult Ae. aegypti mosquitoes were captured in the adult traps, showing a massive predominance of female mosquitoes, 95.4% of all captured insects (Appendix 1).

In contrast to the indices based on larval surveys, indices based on adult capturing corresponded more closely to the dengue notification curve (Figure 2). Ultimately, we observed high infestation levels based on adult indices in Foz do Iguaçu that aligned with dengue notification.

Entomo-Virologic Survey

Of the 11,962 adult Ae. aegypti mosquitoes trapped during 2017–2020, a total of 1,563 (13.1%) were captured alive. In addition, 1,459 (93.3%) were screened for arbovirus infection through real-time qPCR. Subsequently, mosquitoes were screened for infection in 20/24 months of thorough monitoring that summed up 221 pools (Table 1). From the 221 pools tested, 29 (13.1%) were positive for arboviruses, among which 22 (75.9%) pools were positive for DENV, 3 (10.3%) for ZIKV, and 4 (13.8%) for CHIKV. The average MIR for DENV was 42.6 (range 19.6–75.0), and MIR
peaked in March 2020. The entomo-virologic results of natural DENV, ZIKV, and CHIKV infection in field-caught mosquitoes was available ≤36 hours after Adultrap inspection.

**Dengue Prediction of Entomologic Indices**
All entomologic indices based on adult sampling (TPI, ADI, and MII) showed a statistically significant relationship with dengue incidence in Foz do Iguacu during 2017–2020 (Table 1). Indices based on larval surveys had limited statistically significant relationships with dengue incidence in the same week for both BI and HI and for BI after 2 weeks. Of note, indices based on adult trapping best predicted the incidence of dengue after 4 weeks, with emphasis on ADI and MII (Table 1). Adult indices showed a stronger prediction of future dengue incidence than traditional larval surveys indices on the basis of GLMM results for each index in the 5 scenarios (Appendix 2 Tables 1–5, https://wwwnc.cdc.gov/EID/article/28/4/21-1547-App2.pdf). Of note, coefficients for HI and BI were negative in most scenarios whereas positive coefficients were observed for TPI, ADI, and MII.
Choropleth Maps
We constructed maps for the 5 entomologic indices in each of the 24 months of collection during 2017–2020. We selected January 2019 to illustrate the differences between maps based on larval surveys (HI) from the one using adult trapping (TPI), and considered HI and TPI analogous (Figure 3). Of note, January 2019 marked the initial rise in dengue cases in the city, which peaked during March–May 2019, the moment in which a sensitive tool could foresee an increase in dengue transmission. In January 2019, HI classified 52 (71.2%) areas as being low and moderate risk for dengue transmission, whereas TPI estimated 25 (34.2%) areas under the same risk. The relative frequency of high-risk areas before the start of a dengue outbreak increased from 28.7% when measured by HI to 65.8% when measured by TPI (Figure 3).

Discussion
Epidemiologic surveillance is defined as the systematic collection, analysis, and interpretation of determinants of disease activity to support the planning and implementation of further actions to mitigate disease burden. Systematic literature reviews have stressed a general lack of evidence for the usefulness of arboviral surveillance for early outbreak detection and emphasized the lack of indicators and alert signals to trigger response (37,38). When most arbovirus-endemic countries rely on passive surveillance with clinical but few laboratory diagnostics to confirm infection, epidemiologic data frequently are not able to provide a sensitive alert signal before an outbreak takes place. We report on implementation of a citywide integrated surveillance system using entomologic, epidemiologic, and entomo-virologic data gathered during a 4-year period. The extensive fieldwork provided a large dataset and enabled robust analysis. The entomologic indices based on adult trapping provided a more reliable alert signal of dengue outbreaks than widespread traditional indices based on larval surveys.

Dengue entomologic surveillance using larval inspections is a time- and resource-consuming activity widely used in many tropical countries (8,10,39–42). Larval surveys can identify key containers but often fail to provide fast or localized measurements of mosquito abundance. In this context, adopting adult mosquito traps as a complementary approach can improve dengue vector surveillance by providing information previously unknown in larval surveys, such as adult female mosquito abundance (8,43,44). Traditional larval surveillance indices often fail to demonstrate a strong correlation with adult mosquito density and dengue transmission. Thus, developing other indices to serve as indicators of an imminent dengue outbreak should be encouraged (41). Of note, TPI, ADI, and MII were developed by using Adultrap and thus should not be seen as universal adult indices. Although specific for Adultrap, our results highlight that analogous approaches, such as extensive time series data, traditional entomologic data, and high cover in a city, should be pursued by using other traps (41).

Ultimately, even though 73 areas of the city were tested, the HI rarely reached values above the 4% alert threshold; the only exceptions were in March

Table. Akaike information criteria results ranking the most parsimonious a priori models (descending order) that predict the incidence of dengue cases based on surveys of Aedes aegypti mosquitoes, Foz do Iguaçu, Brazil*

<table>
<thead>
<tr>
<th>Scenarios</th>
<th>Index</th>
<th>ΔAICc</th>
<th>wAICc</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 4 weeks</td>
<td>Mosquitoes per inhabitant</td>
<td>0.0</td>
<td>0.6079</td>
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<tr>
<td></td>
<td>Adult density</td>
<td>0.9</td>
<td>0.3908</td>
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<tr>
<td></td>
<td>Trap positivity</td>
<td>12.3</td>
<td>0.0013</td>
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<tr>
<td>After 2 weeks</td>
<td>Adult density</td>
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</tr>
<tr>
<td></td>
<td>Trap positivity</td>
<td>407.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mosquitoes per inhabitant</td>
<td>407.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Adult density</td>
<td>426.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>After 6 weeks</td>
<td>Mosquitoes per inhabitant</td>
<td>1,151.2</td>
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</tr>
<tr>
<td></td>
<td>Adult density</td>
<td>1,156.3</td>
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</tr>
<tr>
<td></td>
<td>Trap positivity</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>During the same week</td>
<td>Adult density</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mosquitoes per inhabitant</td>
<td>2,148.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Trap positivity</td>
<td>2,151.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>House</td>
<td>2,159.7</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>Breteau</td>
<td>2,161.1</td>
<td>&lt;0.001</td>
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<tr>
<td>After 8 weeks</td>
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<td></td>
<td>Mosquitoes per inhabitant</td>
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<tr>
<td></td>
<td>Trap positivity</td>
<td>2,297.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Based on p<0.05 in a generalized linear mixed model. Each model was denoted as a specific scenario, from the same week to 8 weeks after entomologic surveys, and index. House and Breteau indices are based on larval surveys of Aedes aegypti mosquitoes; mosquito per inhabitant, trap positivity, and adult density indices are based on adult trapping. ΔAICc, difference in corrected Akaike information criteria; wAICc, weights of corrected Akaike information criteria.
Integrated *Aedes aegypti* Mosquito Surveillance

2019 (5.41%) and May 2019 (5.29%). Furthermore, the most intense dengue transmission peak in Foz do Iguaçu was recorded during January–May 2020. In this window, we had 3 HI estimates, 3.21 in January, 1.32 in March, and 0.7 in May. We observed the same pattern of poor correlation with local dengue transmission for BI, evincing criticisms directed to larval surveys as both entomologic and epidemiologic indicators. Instead, indices based on adult trapping showed low variation during the nonendemic years of 2017–2018 and peaked accordingly in the 2019 and 2020 dengue seasons.

Standard larval surveys were not sufficient to issue proper alerts in Foz do Iguaçu. By comparison, Adulttraps detected increased mosquito infestation during the dengue transmission seasons, indicating the system’s ability to detect mosquito density variation and thus the likelihood of generating indices that could be used as part of an early warning system to trigger vector control response. The greater sensitivity of traps to mosquito density variation is probably because they can cover >1 premises, whereas surveys of immature mosquitoes only encompass those houses included in the sample (8).

Comparing the predictive ability of traditional versus adult indices revealed that indices based on adult trapping consistently performed much better than indices based on larval surveys. In fact, we observed negative GLMM coefficients for HI and BI but saw positive estimates for indices based on adult trapping. In addition, MII, ADI, and TPI performed better as predictive indicators of dengue outbreaks 4 weeks after the trapping period. Therefore, local health managers would have ≈1 month after estimating the index values to promote and intensify vector control in areas with higher risk on a choropleth map. In addition, health managers could create additional criteria to prioritize areas for vector control in case the cost to cover all high-risk areas of a city becomes too expensive to be covered by health agencies.

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**Figure 3.** Choropleth maps comparing larval and adult *Aedes aegypti* mosquito infestation indices and dengue notifications in 73 urban areas of Foz do Iguaçu, Brazil, January–February 2019. A) Traditional house index (HI) calculated from larval surveys; B) trap positivity index (TPI) calculated from dengue virus positivity among captured adult *Ae. aegypti* mosquitoes. Dots represent dengue notification and numbers inside dots represent the total of dengue cases reported on that city block.
SYNOPSIS

One criterion that could be used to prioritize areas is the occurrence of Ae. aegypti female mosquitoes naturally infected with DENV, ZIKV, or CHIKV. In Foz do Iguaçu, inspection of the 3,476 Adulttraps took 4 days, and real-time qPCR results were available, on average, 36 h after all Adulttraps were inspected and live mosquitoes collected. Thus, within 5 days of starting trap inspection, additional entomologic information, such as geographic position of traps, the infestation index, and the choropleth maps, were made available for local health managers. In the early hours of the next business day, the local health manager could meet with field supervisors to decide which area to prioritize and which vector control activities to perform considering local contexts. Therefore, a week after the start of Adulttrap inspection, the dengue transmission risk among the 73 areas of Foz do Iguaçu would be known by the local health managers, triggering vector control interventions in prioritized areas.

In conclusion, traditional entomologic indices have shown a poor relationship with dengue transmission, if any. We conducted a 4-year citywide study to deepen the entomologic and epidemiologic features of dengue transmission in Foz do Iguaçu by focusing on developing indicators based on adult mosquito trapping. We demonstrated the process we used to develop the 3 adult trapping indices, all of which have a higher prediction behavior to foresee dengue outbreaks than the widely adopted traditional larval survey indices. Our proposed surveillance system can predict a dengue outbreak with high accuracy, and in districts based on adult trapping are able to predict a dengue outbreak 4 weeks after DENV detection in adult mosquitoes. In addition, adoption of easily accessible technological resources makes it possible for the model to be replicated to other localities.

Acknowledgments

We thank all Foz do Iguaçu health agents for their dedication and support in the implementation of this integrated surveillance approach.

We thank Fiocruz, Fundação Carlos Chagas Filho de Amparo a Pesquisa no Estado do Rio de Janeiro (FAPERJ), and the Coordination for the Improvement of Higher Education Personnel, Financial code 001 (CAPES) for their support.

About the Author

Dr. Leandro is a veterinarian in the Zoonoses Control Center at Foz do Iguaçu, Brazil. His primary research interests are zoonosis, arbovirus epidemiology, and global health.

References

SYNOPSIS


Address for correspondence: Rafael Maciel-de-Freitas; Avenida Brasil, 4365. Laboratório de Transmissores de Hematozoários, sala 414, Pavilhão Carlos Chagas, Instituto Oswaldo Cruz, IOC. Fiocruz. Manguinhos, Rio de Janeiro, CEP 21040-360, Brazil; email: freitas@ioc.fiocruz.br

EID Podcast: People with COVID-19 in and out of Hospitals, Atlanta, Georgia

For many people, coronavirus disease (COVID-19) causes mild respiratory symptoms. Yet others die of from complications caused by the infection, and still others have no symptoms at all. How is this possible? What are the risk factors, and what role do they play in the development of disease?

In the pursuit to control this deadly pandemic, CDC scientists are investigating these questions and more.

COVID-19 emerged less than 2 years ago. Yet in that short time, scientists have discovered a huge body of knowledge on COVID-19.

In this EID podcast, Dr. Kristen Pettrone, an Epidemic Intelligence Service officer at CDC, compares the characteristics of hospitalized and nonhospitalized patients with COVID-19 in Atlanta, Georgia.

Visit our website to listen: http://go.usa.gov/xHUME
Antimicrobial resistance (AMR) is a global crisis and one of the world’s most complex challenges, threatening a century of health progress. AMR affects human and animal health and poses a serious threat to reaching sustainable development goals and food security. Drug-resistant infections account for 700,000 deaths globally each year and could cumulate to 10 million by 2050 if no sustained efforts to contain AMR are implemented (1–3). Factors contributing to the emergence and spread of AMR in the EMR include the high burden of infectious diseases; weak health and surveillance systems; inadequate regulatory frameworks; poor infection prevention and control (IPC) in healthcare facilities; limited capacities of microbiology laboratories; lack of access to quality-assured antimicrobial drugs for humans and animals; poverty; inadequate access to water, sanitation, and hygiene; and limited antimicrobial stewardship (AMS) programs (6). Antimicrobial drugs are available over the counter, and self-medication is a common practice in most countries. Inappropriate prescription practices among physicians are widespread. Antimicrobial drugs are used to compensate for the lack of basic public health infrastructure (e.g., vaccination coverage and IPC) (7,8).

WHO identified surveillance as 1 of the 5 strategic priorities of the global and national AMR action plans (9,10). Because most countries did not have good quality AMR data, in 2015, WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS, https://www.who.int/glass). To measure the regional AMR burden and generate quality data, WHO supported countries to establish and enhance national AMR surveillance. We evaluated the burden of AMR for selected serious resistant bacterial infections reported to WHO through GLASS over 3 years (2017–2019), along with the regional capacities of AMS and IPC programs. We also explored the challenges faced by countries responding to AMR and propose priority actions to advance the AMR control agenda in the EMR.
Methods

GLASS Design
Each country assigned a variable number of sentinel surveillance sites on the basis of good quality microbiology laboratories. The AMR data are generated through processing of specimens (e.g., blood, urine, stool) collected for clinical purposes. Isolating and identifying bacteria through antimicrobial sensitivity testing (AST) formed the basis of reporting; countries used either Clinical Laboratory Standards Institute (https://clsi.org) or European Committee on Antimicrobial Sensitivity Testing (https://www.eucast.org) guidelines. Surveillance sites used WHONET software ([11]), which was adapted to improve data entry and reporting to GLASS through an aggregated format. Some countries reported demographic and epidemiologic variables such as age, sex, and origin of infection. However, these data were incomplete ([10]), and we did not use them for analysis.

AMR Data Collection, Case Definitions, and Data Analysis
For this article, we report only bloodstream infections (BSIs) caused by resistant organisms and used the data reported to GLASS by 11–14 countries during 2017–2019 (Appendix, https://wwwnc.cdc.gov/EID/article/28/5/21-1975-App1.xlsx). We prioritized BSIs because they are among the most serious and life-threatening infectious conditions and are used as the main sustainable development goal indicator for AMR. The presence of a pathogen in blood samples is used as a proxy for BSI in a patient. We report BSIs caused by carbapenem-resistant *Acinetobacter* spp. (CRAsp), third-generation cephalosporin (3GC)–resistant *Enterobacteriaceae* (Escherichia coli and Klebsiella pneumoniae), methicillin-resistant *Staphylococcus aureus* (MRSA), and carbapenem-resistant *Enterobacteriaceae* (CRE) (*E. coli* [CREC] and *K. pneumoniae* [CRKP]).

To calculate the proportion of patients with BSIs caused by resistant pathogens, we used as a numerator the number of patients with BSIs caused by one of the specific resistant pathogens and as the denominator the total number of patients with BSIs that were tested by AST (susceptible, intermediate, resistant) for the same pathogen. For example, we calculated the proportion of patients with BSIs caused by CRAsp by dividing the number of patients with BSIs caused by CRAsp by the number of patients with BSIs caused by *Acinetobacter* spp. with AST results (susceptible, intermediate, or resistant) for carbapenem. We used box-and-whisker plots to display the proportion of BSIs caused by specific resistant pathogens. We also described the distribution of resistance over time with a line graph and the geographic distribution with maps.

Antimicrobial Drug Prescriptions among Hospitalized Patients
Seven countries participated in a standardized regional point prevalence survey that measured antimicrobial drug use among hospitalized patients. Countries selected a nationally representative sample of healthcare facilities and assigned national teams to collect the data. WHO trained the data collection teams to ensure standardization and collection of good quality data. We calculated the prevalence of antimicrobial drug use by dividing the number of patients prescribed ≥1 antimicrobial drug at the time of the survey over the total number of inpatients surveyed.

IPC Program Capacity Assessment
To conduct a regional survey at the end of 2019 and assess the national IPC programs in countries, we used the WHO IPCAT2 tool ([12]). The main purpose of the IPCAT2 tool is to describe the status of the national IPC activities according to WHO guidelines ([13]) and identify strengths and weaknesses to plan for improvement. We collected data through personal interviews with representatives at national IPC focal points or their alternatives.

AMS Programs Capacity Assessment
To assess the preparedness of countries regarding their national AMS programs, we completed a regional survey in early 2020 by using the WHO AMS assessment tool ([14]). The tool assesses 4 core elements of AMS programs: 1) national plans and strategies; 2) regulations and guidelines; 3) awareness, training, and education; and 4) supporting technologies and data. We collected the data through virtual personal interviews with national AMR/AMS representatives in each country.

Results

AMR Surveillance

Reporting to GLASS
The number of countries and health facilities reporting to GLASS increased over time. For 2017, a total of 235 health facilities in 12 countries reported data on any type of infection, increasing to 373 health facilities in 15 countries in 2018 and to 527 health facilities in 18 countries in 2019. The number of countries...
that reported BSIs to GLASS also increased; 11 countries reported BSIs in 2017, 12 in 2018, and 14 in 2019. Also, the number of reported BSIs caused by priority pathogens increased from 6,957 BSIs in 2017 to 16,454 in 2018 and 23,104 in 2019 (15,16).

AMR Data
In 2019, the median proportion of patients with BSIs caused by CRAsp was highest at 70.3% (IQR 62.4%–81.3%), followed by K. pneumoniae resistant to 3GC (66.3%, IQR 54.0%–3.8%). The lowest median proportion was for CREC (4.6%, IQR 1.8%–18.2%). The proportion of BSIs caused by resistant pathogens varied widely across countries: 41.7%–88.2% for CRAsp, 28.2%–95.0% for 3GC-resistant K. pneumoniae; 32.6%–88.6% for 3GC-resistant E. coli; 17.4%–79.6% for MRSA, 6.8%–67.8% for CRKP, and 0.7%–28.1% for CREC (Figure 1). Although assessing trends with only 3 years of data is difficult, especially with the changes in number of reporting countries and surveillance sites, the proportion of resistance tended to increase over time, from 71.4% in 2017 to 74.5% in 2019 for CRAsp, from 55.3% in 2017 to 65.4% in 2019 for 3GC-resistant K. pneumoniae, from 36.6% in 2017 to 45.8% in 2019 for MRSA, and from 24.2% in 2017 to 37.5% in 2019 for CRKP. 3GC-resistant E. coli increased minimally from 58.4% in 2017 to 59.5% in 2019, and CREC increased from 6.1% in 2017 to 7.1% in 2019 (Figure 2). Egypt and Pakistan had the highest proportion of resistance for 5 of the 6 resistant pathogens; Qatar and United Arab Emirates had the lowest proportion of resistance for 5 (Qatar) and 3 (United Arab Emirates) of the 6 resistant pathogens (Figure 3).

Antimicrobial Drug Prescriptions among Hospitalized Patients
A total of 128 hospitals in 7 countries (Jordan, Sudan, Pakistan, United Arab Emirates, Tunisia, Lebanon, and Iraq) participated in the prevalence survey for antimicrobial drug use among hospitalized patients. Among the 16,551 patients hospitalized, 8,814 (53.3%) received ≥1 antimicrobial agent, ranging from 38.7% to 77.7% across countries. The most common indication for prescribing antimicrobial drugs in all countries was treatment of community-acquired infections. Respiratory infections accounted for the largest proportion of infections treated. The top 3 antimicrobial drugs prescribed were 3GC (22.6%), imidazoles (9%), and carbapenems (8.5%). According to the WHO Access, Watch or Reserve (AWaRe) classification of antibiotics (17,18), only 34% of the prescribed antimicrobial drugs were from the access group, 61% were from the watch group, and 5% were from the reserve group.

IPC Program Assessment
Among the 22 countries in the region, 13 (59%) had a national IPC program established within their ministries of health. However, for 8 (61.5%) of the 13 countries with an IPC program, the existing structures were not functioning and did not implement IPC policies and procedures in healthcare facilities. Nine (40%) of the 22 countries had developed national IPC guidelines within the past 5 years, 7 had active IPC education programs, and 6 had either multimodal strategies or national IPC monitoring plans (Table 1).

Figure 1. Proportion of patients with bloodstream infections caused by antimicrobial resistant pathogens in 14 World Health Organization Eastern Mediterranean Region countries. Data from the Global Antimicrobial Resistance Surveillance System (https://www.who.int/glass) for 2019. Each dot represents the percentage of patients with resistant organisms in a country. Horizontal lines within boxes indicate medians, box tops and bottoms indicate interquartile ranges (middle 50% of data), and error bars (upper and lower whiskers) represent scores outside the middle 50%. CRAsp, carbapenem-resistant Acinetobacter spp.; CREC, carbapenem-resistant Escherichia coli; CRKP, carbapenem-resistant K. pneumoniae; E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae; MRSA, methicillin-resistant Staphylococcus aureus; 3CG, third-generation cephalosporins.
AMS Assessment
We assessed national AMS core capacities for 20 of the 22 EMR countries. Only 1 (5%) country had dedicated funding for AMS, 4 (20%) had established national AMS technical working groups, and 1 (5%) had developed an AMS implementation plan. With regard to regulations and clinical guidelines, 13 (65%) countries reported having an Essential Medicines List, 2 of which adopted the AWARe classification; 5 (25%) had treatment guidelines, and 10 (50%) had a prescription-only sale policy for antibiotics, of which only 5 enforced this policy (Table 2).

Discussion
Of the 22 EMR countries, 20 developed their national AMR action plans in alignment with the global AMR action plans. Since 2017, several countries in the region started generating data on AMR and antimicrobial drug use. Hence, detection and surveillance capabilities increased in most countries along with awareness of the scope and complexity of AMR.

The median percentage of patients with BSIs caused by CRAsp was highest at 70.3%. This figure is extremely high compared with the percentage in the United States, where CRAsp among healthcare-associated infections is at 33.9% (19); in European Union countries, 32.6% of Acinetobacter spp. isolates identified in blood or cerebrospinal fluid were resistant to carbapenems (20). CRAsp is a high-threat pathogen; resistant clones are spreading in healthcare settings. Transmission is exacerbated by limited implementation of IPC (21). CRAsp and CREs have become resistant to nearly all available antimicrobial drugs, contributing to patient deaths and case-fatality rates >50% (22,23).

The data for antimicrobial drug prescriptions among hospitalized patients pointed to high use of 3GC and carbapenems, which may explain the high levels of resistance for these drugs (K. pneumoniae resistant to 3GC and CREs). The overall prevalence of antimicrobial drug use in the 7 (53.5%) countries that implemented the point-prevalence survey is lower than that in some countries in Africa (Ghana 70.7%, Nigeria 80%) (24,25), similar to the prevalence for Latin America (49.5%) (26) but higher than that for countries in Europe (27). The most common indication for antimicrobial drug prescription in the EMR as well as in several countries in Europe and Africa is treatment of community-acquired infections (25,27). However, in the EMR, 3GC are the most prescribed antimicrobial drugs, and in Europe, penicillins with β-lactamase inhibitors are most commonly prescribed (27).

AMS programs prevent further emergence of resistance. In the EMR, these programs are still in their infancy but are evolving with various progress among countries. Only 5 countries in the region are enforcing a prescription-only sale policy for antimicrobial drugs in pharmacies, and 2 countries are adopting AWARe classification in their national Essential Medicines List to increase the use of the Access group of antimicrobial drugs (first or second empiric choice) for common infections (17,18). Legislation to enforce this policy must be combined with adequate access to universal health coverage. Despite the high AMR burden, major barriers for AMS implementation
Increasing AMR in Eastern Mediterranean Region

exist in the EMR: limited numbers of infectious disease and clinical pharmacy experts in several countries, limitations in microbiological diagnostic capacities, lack of national AMR governance including AMS, knowledge gaps regarding optimum antimicrobial drug use across healthcare providers, insufficiently staffed and overcrowded healthcare systems in some countries, and absence of information technology (including electronic hospital records and clinical decision support systems) to monitor antimicrobial use.

After resistant organisms have emerged, IPC programs are essential for preventing spread. Unfortunately, unlike other preventive and curative interventions, IPC has never been an integrated

Table 1. Key elements of national IPC core components in 22 countries of the World Health Organization Eastern Mediterranean Region; 2019*

<table>
<thead>
<tr>
<th>National IPC core components</th>
<th>Countries, no. (%)</th>
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</thead>
<tbody>
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<td>IPC focal point/group</td>
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<tr>
<td>Evidence-based national IPC guidelines within past 5 y</td>
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</tr>
<tr>
<td>National IPC education and training program</td>
<td>7 (32)</td>
</tr>
<tr>
<td>National healthcare-associated infection surveillance program</td>
<td>5 (23)</td>
</tr>
<tr>
<td>National IPC multimodal improvement strategies</td>
<td>6 (27)</td>
</tr>
<tr>
<td>National monitoring/auditing of IPC practices and feedback</td>
<td>6 (27)</td>
</tr>
</tbody>
</table>

*IPC, infection prevention and control.
function of healthcare systems. IPC programs were developed mainly to respond to global or national infectious disease epidemics or pandemics (e.g., bloodborne pathogens, Middle East respiratory syndrome coronavirus, pandemic influenza virus, severe acute respiratory syndrome coronavirus 2). IPC has progressed in several high-income countries, whereas implementation in low- and middle-income countries remains limited and compliance with IPC measures is often low (28). Decision makers rarely recognize the role of IPC as a health system–strengthening element with cross-cutting value for AMR response and prevention or control of other infectious diseases. The coronavirus disease pandemic led to recognition of the value of IPC, but many countries in the EMR have yet to establish or enhance their IPC national programs. Although investing in IPC will need resources, the coronavirus disease pandemic demonstrated that such investment will be highly cost-effective for preventing spread of infection among patients and healthcare workers in addition to reducing infections caused by drug-resistant strains (29,30).

Among limitations of these AMR surveillance data, the increase in number and type of reporting healthcare facilities over time for some countries could affect the proportion of drug-resistant infections reported. First, use of routine clinical data has the potential to overestimate resistance because of the tendency to culture specimens of patients experiencing treatment failure. Second, most AMR data are driven by hospitals with limited understanding of AMR data at the community level. Third, although countries were encouraged to report demographic and clinical characteristics, the completeness of these data is limited for most reporting countries. Fourth, reporting only BSIs does not reflect the complete spectrum of AMR. Last, reporting aggregated data from countries prevents detailed epidemiologic analysis.

In conclusion, the prevalence of AMR in EMR countries is high, and the continued increase threatens health security in the region. AMS programs that prevent emergence of AMR and IPC programs that reduce spread are still developing with variable capacities among countries. This situation calls for political engagement and leadership. EMR countries need to accelerate implementation of the national AMR plans with effective national AMR governance systems, including highly specialized human resources, adequate funding, and empowerment of responsible staff at all levels. Countries within the EMR must continue to enhance and expand their national AMR surveillance programs with a focus on strengthening microbiology laboratories and to fully implement and strengthen AMS and IPC with a focus on safety and quality of services, especially in countries with weak health systems (1,3,6,14,31). Last, legislation to promote antimicrobial drug use and IPC in this region is urgently needed.

Acknowledgments
We acknowledge the significant contribution of various collaborators, WHO AMR and IPC focal points in WHO country offices, who provide the technical support to countries to enroll and share data with GLASS. Special thanks go to the national AMR, IPC, and GLASS focal points in countries in the EMR for their great work and contribution to push the AMR agenda forward. M.T. conceptualized and formulated overall goals and aims of the manuscript; coordinated the implementation of regional work on AMR surveillance, IPC and AMS; and wrote the manuscript. B.Z. provided technical support to countries for implementing national AMR surveillance and coordinated the regional assessments. S.T. supported all countries in the region.

Table 2. Implementation status of select core elements of national AMS programs in 20 countries in the World Health Organization Eastern Mediterranean Region, 2020*

<table>
<thead>
<tr>
<th>Activities</th>
<th>Countries, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>National core elements, national plans, and strategies</td>
<td></td>
</tr>
<tr>
<td>Dedicated funding for AMR and AMS activities</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>Establishment of AMS technical working group with defined terms of references</td>
<td>4/20 (20)</td>
</tr>
<tr>
<td>Development of AMS implementation plan with short-, medium-, and long-term goals</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>Regulations and guidelines</td>
<td></td>
</tr>
<tr>
<td>Presence of national Essential Medicines List</td>
<td>13/20 (65)</td>
</tr>
<tr>
<td>National EML adopts AWaRe classification</td>
<td>2/13 (15.5)</td>
</tr>
<tr>
<td>Development of updated treatment guidelines for common infections</td>
<td>5/20 (25)</td>
</tr>
<tr>
<td>Existing treatment guidelines integrate the AWaRe classification</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Presence of prescription only sale policy for antimicrobial drugs</td>
<td>10/20 (50)</td>
</tr>
<tr>
<td>Enforcement of prescription only sale policy for antimicrobial drugs</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>Awareness, training, and education</td>
<td></td>
</tr>
<tr>
<td>In-service training for AMS teams on AMS and antimicrobial drug prescribing</td>
<td>0/20</td>
</tr>
<tr>
<td>In-service training for healthcare professionals on AMS and antimicrobial drug prescribing</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td>AMS criteria for hospitals accreditation, set by ministry of health</td>
<td>4/20 (20)</td>
</tr>
</tbody>
</table>

*AMR, antimicrobial resistance; AMS, antimicrobial stewardship; AWaRe, Access, Watch, or Reserve.
Increasing AMR in Eastern Mediterranean Region

establish national AMR surveillance and analyzed the data. E.H. conducted the regional AMS assessment and participated in writing the interpretation of the AMS sections of the manuscript. M.G. supported countries with regard to reporting AMR data to GLASS. D.I. provided technical support to the national AMR laboratories in the region. Y.H. supervised the analysis and assisted with revision of the manuscript. R.H. critically reviewed the manuscript.

About the Author
Dr. Talaat is the regional advisor for AMR and IPC at the WHO EM regional office. Her research interests are public health and epidemiology of emerging infectious diseases, focusing on combating AMR and strengthening IPC programs.

Reference


Address for correspondence: Maha Talaat, World Health Organization, Eastern Mediterranean Region, Monazamet El Seha El Alamia St, Box 7608, Nasr City, Cairo, Egypt; email talaatm@who.int

Want to stay updated on the latest news in Emerging Infectious Diseases? Let us connect you to the world of global health. Discover groundbreaking research studies, pictures, podcasts, and more by following us on Twitter at @CDC_EIDJournal.
An HIV outbreak investigation during 2017–2018 in Unnao District, Uttar Pradesh, India, unearthed high prevalence of hepatitis C virus (HCV) antibodies among the study participants. We investigated these HCV infections by analyzing NS5B and core regions. We observed no correlation between HIV–HCV viral loads and clustering of HCV sequences, regardless of HIV serostatus. All HCV isolates belonged to genotype 3a. Monophyletic clustering of isolates in NS5B phylogeny indicates emergence of the outbreak from a single isolate or its closely related descendants. The nucleotide substitution rate for NS5B was $6 \times 10^{-3}$ and for core was $2 \times 10^{-3}$ substitutions/site/year. Estimated time to most recent common ancestor of these isolates was 2012, aligning with the timeline of this outbreak, which might be attributable to unsafe injection practices while seeking healthcare. HIV–HCV co-infection underlines the need for integrated testing, surveillance, strengthening of healthcare systems, community empowerment, and molecular analyses as pragmatic public health tools.

Hepatitis C virus (HCV) infection is a major public health concern worldwide and recognized as the leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC). India, Pakistan, Georgia, and many countries in the western world have recorded high HCV prevalence among persons who inject drugs (PWID) (1–4). In addition, unsafe injection practices in therapeutic settings have been responsible for spread of HCV infection in the general population (5,6). HCV shares the same routes of transmission as HIV, although heterosexual sex is a less efficient one. Owing to the shared routes of transmission, about one third of persons living with HIV have been estimated to be coinfected with HCV in the United States, Europe, and some developing countries (7,8).

Increased detection of HIV among the attendees of the Integrated Counselling and Testing Center, located in the district hospital of Unnao, in the northern state of Uttar Pradesh, India, was reported in July 2017. A case–control investigation was initiated while 2 patients from Premganj Township of Bangarmau Block, Unnao, raised concerns that a local doctor (frequented mostly by persons from lower socioeconomic status) had been using a single syringe and needle to inject different persons. That investigation indicated that unsafe injecting practices in therapeutic settings were associated with HIV transmission and identified clustering around a monophyletic HIV-1 clade C. Serologic analyses revealed very high prevalence of antibodies to HCV among HIV-infected persons (85%) and controls (56%), suggesting a concerning level of spread of HCV infection in the study community, which was a serendipitous finding (5). Detection and molecular analysis of community HCV outbreaks is important because they can inform targeted public health interventions to interrupt HCV transmission. These initiatives are even more imperative as India aims to achieve the Sustainable Development Goal 3.3, which seeks to end viral hepatitis by 2030, as articulated in the National Health Policy (9). In this article, we describe the findings of molecular and phylogenetic analysis of HCV infections discovered during the HIV outbreak investigation in Unnao, India.
Methods

Ethics Statement
We obtained approval for the investigation from the Ethics Committee of the Indian Council of Medical Research—National AIDS Research Institute (Pune, India). All study participants provided written informed consent.

Clinical Specimens and Data
We conducted molecular and phylogenetic analyses for 98 HCV seroreactive participants, including 70 with HCV and 28 with HIV–HCV co-infection. We enrolled these participants as part of our previous case–control investigation, which was undertaken to identify the factors associated with increased detection of HIV in Unnao District Hospital, Unnao, India, during September–December 2018, details of which are published elsewhere (5). In brief, case-patients (n = 33) were persons found to be HIV seroreactive during a 6-month period (November 2017–April 2018) from 3 locations (Premganj, Karimuddinpur, and Chakmeerapur) in the Bangarmau Block of Unnao District. Controls (n = 125) were persons who lived in the same geographic locale as case-patients and tested HIV seronegative either in health camps or at the Integrated Counselling and Testing Centers where the cases were detected. Eighty-five percent (28/33) of HIV-positive and 56% (70/125) of HIV-negative study participants were HCV seroreactive. We used the HCV seroreactive specimens stored at −80°C for the molecular analysis for HCV infection, along with corresponding metadata pertaining to sociodemographic and laboratory data.

Viral Load Estimation
We performed HCV viral load testing by using the Xpert HCV viral load assay (Cepheid, https://www.cepheid.com). In brief, we placed 1 mL serum into the Xpert cartridge, scanned it, and loaded it into the GeneXpert IV instrument. We recorded the results according to the manufacturer’s instructions.

We estimated HIV-1 viral load by using the Abbott Real Time HIV-1 assay (Abbot https://www.abbott.com), which uses reverse transcription PCR (RT-PCR) with homogenous detection of real-time fluorescence. We carried out automated sample preparation on the m2000sp instrument for RNA isolation by using magnetic microparticle technology and dispensed it to a PCR tray along with the amplification reagents. We then transferred the PCR tray to the Abbott m2000rt instrument for amplification and real-time detection according to the manufacturer’s instructions.

HCV RNA Extraction, NS5B and Core Gene Amplification, and Sequencing
We extracted viral RNA by using the NucliSens EasyMag total nucleic acid extraction system (bioMérieux, https://www.biomerieux.com). We amplified the extracted RNA for the core and NS5B region of HCV. We amplified the core region with outer sense primer 5′-ACTGCTCTGAGGTGCTTGC-3′; outer antisense primer 5′-ATGTACCCCAT-GAGGTCGGC-3′; inner sense primer 5′-AGGTCTCTGAGACCCTGCA-3′; and inner antisense primer 5′-CATGTCAGGTGATCGATGAC-3′ (10). We amplified the NS5B region with outer sense primer 5′-CNTAYGGITTCCARTACTCC-3′, antisense primer 5′-GAGGARCAIGATGTTATIARCCT-3′, inner sense primer 5′-TATGAYACCCGCTYTTT-GACTC-3′ and inner antisense primer 5′-GCNGAR-TAYCTVGTATAGCCTC-3′ (11). We used the Onestep RT-PCR kit (QIAGEN, https://www.qiagen.com) for cDNA synthesis. We carried out the outer PCR in 25 µL reaction containing 4 µL 5× PCR buffer, 1 µL 2.0 mmol/L dNTP, 4.5 µL RNase-free water, 2 µL each sense and antisense primers, 1 µL RT-PCR enzyme mix, 0.5 µL RNase out (Invitrogen, https://www.thermoscientific.com), and 10 µL extracted RNA (treated with heat at 65°C for 30 s for core and 42°C for 5 min for NS5B) with PCR conditions: 50°C for 35 min; 95°C for 15 min; 95°C for 20 s, 55°C for 45 s, 72°C for 2 min, 35 cycles; and 72°C for 10 min. We carried out the inner PCR in 25 µL reaction containing 2.5 µL 10× PCR buffer, 1 µL 2.5 mmol/L dNTP, 11 µL water, 1 µL each sense and antisense primers, 0.5 µL Taq enzyme, and 5 µL template cDNA with PCR conditions: 95°C for 1 min, 95°C for 15 s, 56°C for 45 sec, 72°C for 1 min, 30 cycles; and 72°C for 5 min. We verified the PCR products by gel electrophoresis. We sequenced all amplified products on the Applied Biosystems 3130XL genetic analyzer (Applied Biosystems/ThermoFisher, https://www.thermoscientific.com). We then performed sequence assembly and base-calling with the FASTA files generated for further analysis. We submitted nucleotide sequences to GenBank (accession nos. MW675966–MW676030 for the core gene and MW675899–MW675965 for the NS5B gene).

HCV Genotyping and Phylogenetic Analysis
We genotyped partial sequences of core and NS5B genes of HCV samples from persons with HIV–HCV coinfection (labeled as i) and HCV monoinfection (labeled as ic) by using GenomeDetective (https://www.genomedetective.com/app/typingtool/hcv)
and HCV-Blast (12). We performed multiple sequence alignments by using the prototype sequence of H77 isolate (GenBank accession no. NC_038882) belonging to genotype 1, using MAFFT (13). We determined the exactly identical sequences by using the CD-HIT web server (14). We then extracted core and NS5B genes belonging to India and global HCV isolates from GenBank (15) by using blastn (16). We carried out recombination detection by using RDP4 (17) and then reconstructed phylogenetic trees for core and NS5B sequences by using the maximum-likelihood method as implemented in IQTREE (18). We used partial sequences of core and NS5B genes belonging to other India and global isolates, which clustered with Unnao samples for evolutionary analysis. We used BEAST version 1.10.4 to determine time to most recent common ancestor (tMRCA) (19).

We used the generalized time reversible model as a nucleotide substitution model with relaxed clock and log-normal distribution and coalescent constant growth as demographic model for tMRCA calculation. We carried out Markov Chain Monte Carlo simulations for 100 million steps and sampled every 10,000 steps. We used Tracer 1.6 (https://bioweb.pasteur.fr/packages/pack@Tracer@v1.6) for assessing convergence and iTOL (https://itol.embl.de) and FigTree (https://github.com/rambaut/figtree) for visualization of phylogenetic trees. To generate information on drug-resistant mutation substitutions observed in NS5B protein sequences, we mapped to sofosbuvir and ribavirin drug-resistant variants (http://hcv.geno2pheno.org).

### Statistical Analysis

We compared the means between 2 samples by using an independent t test. We used Pearson’s correlation analysis to determine whether the values of 2 variables were associated. We considered a probability level lower than the conventional 5% (p<0.05) as statistically significant. We assessed the association between dependent variable and cofactors by using Pearson’s χ² or Fisher exact tests as appropriate.

### Results

#### Participant Profile

We profiled persons with HCV monoinfection (n = 70) and those with HIV–HCV co-infection (n = 28) (Table 1); sex, area of residence, occupation, and sexual risk distribution were not significantly different.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV–HCV co-infection</th>
<th>HCV monoinfection</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. persons</td>
<td>28 (100)</td>
<td>70 (100)</td>
<td></td>
</tr>
<tr>
<td>Mean age, y</td>
<td>50</td>
<td>38</td>
<td>0.044</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>09 (32.1)</td>
<td>33 (47.1)</td>
<td>0.175</td>
</tr>
<tr>
<td>F</td>
<td>19 (67.9)</td>
<td>37 (52.9)</td>
<td></td>
</tr>
<tr>
<td>Area of residence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chakmeerapur</td>
<td>12 (42.9)</td>
<td>32 (45.7)</td>
<td>0.957</td>
</tr>
<tr>
<td>Kirvidyapur</td>
<td>1 (3.6)</td>
<td>2 (2.9)</td>
<td></td>
</tr>
<tr>
<td>Premganj</td>
<td>15 (53.6)</td>
<td>36 (51.4)</td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>17 (60.7)</td>
<td>29 (41.4)</td>
<td>0.147</td>
</tr>
<tr>
<td>Farmer</td>
<td>5 (17.9)</td>
<td>12 (17.1)</td>
<td></td>
</tr>
<tr>
<td>Nonagricultural</td>
<td>6 (21.4)</td>
<td>29 (41.4)</td>
<td></td>
</tr>
<tr>
<td>Ever had sex with female casual partner (as reported by male participants)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (14.3)</td>
<td>2 (6.1)</td>
<td>0.453</td>
</tr>
<tr>
<td>No</td>
<td>6 (85.7)</td>
<td>31 (93.9)</td>
<td></td>
</tr>
<tr>
<td>Ever had sex with male casual partner (as reported by female participants)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (5.26)</td>
<td>0</td>
<td>0.345</td>
</tr>
<tr>
<td>No</td>
<td>18 (94.74)</td>
<td>36 (100)</td>
<td></td>
</tr>
<tr>
<td>Condom use during last sex†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (12.5)</td>
<td>11 (17.2)</td>
<td>0.750</td>
</tr>
<tr>
<td>No</td>
<td>21 (87.5)</td>
<td>53 (82.8)</td>
<td></td>
</tr>
<tr>
<td>Intravenous injection in therapeutic setting in past 5 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27 (96.4)</td>
<td>47 (67.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>No</td>
<td>1 (3.6)</td>
<td>23 (32.86)</td>
<td></td>
</tr>
<tr>
<td>Intramuscular injection in therapeutic setting in past 5 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27 (96.4)</td>
<td>45 (64.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>1 (3.6)</td>
<td>25 (35.7)</td>
<td></td>
</tr>
<tr>
<td>Way syringe and needle used while receiving intramuscular injection in past 5 years†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injected by used syringe and needle</td>
<td>9 (32.1)</td>
<td>3 (4.3)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Injected by new syringe and needle</td>
<td>13 (46.4)</td>
<td>60 (85.7)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are no. (%) except as indicated. HCV, hepatitis C virus.
†Because of nonresponse from some participants, numbers may not sum to total.
between the 2 groups. We observed statistically significant differences between the 2 groups with regards to age and receipt of injection during treatment-seeking within the last 5 years.

We compared the characteristics of HCV reactive (n = 98) and nonreactive (n = 60) persons and observed that a statistically significant higher proportion of HCV reactive persons reported being exposed to used syringes and needles while seeking treatment and were HIV seropositive compared with HCV non-reactive persons. We did not observe significant differences with regards to age, sex, area of residence, occupation, and sexual risk distribution between the 2 groups (Appendix Table, https://wwwnc.cdc.gov/EID/article/28/4/18-1845-App1.pdf). None of the study participants reported ever injecting drugs for nonmedicinal or recreational purposes.

**Association between HCV and HIV Viral Load**

We tested all HCV antibody-positive specimens for HCV viral load. Samples collected from 2 participants with HIV–HCV co-infection (identified by presence of antibody) and 3 with HCV monoinfection (seroreactive) had insufficient volume. Thus, we used specimens from 26 participants with HIV–HCV co-infection and 67 participants with HCV monoinfection for viral load testing. Eighty-eight percent (23/26) of specimens from HIV–HCV co-infected persons and 71% (49/67) specimens from HCV monoinfected participants had detectable HCV RNA (chronic infection). HCV RNA load in participants with HIV–HCV co-infection was (mean ± SD log IU/mL) 5.93 ± 0.91 and HCV monoinfection was 5.46 ± 1.09; the difference was not statistically significant (p = 0.07). HCV RNA load did not differ significantly in HIV-positive persons with detectable (6.06 ± 1.15) and undetectable (5.85 ± 0.76) viral load (p = 0.65) (Table 2). We did not observe any correlation between HIV and HCV viral load (r = 0.0567; p = 0.88).

**Genetic Variability in the HCV Core and NS5B Genes and HCV genotypes**

We obtained a total of 67 (23 with HIV–HCV coinfection and 44 with HCV monoinfection) and 65 (22 with HIV–HCV coinfection and 43 with HCV monoinfection) sequences belonging to core and NS5B regions. The partial core sequence mapped to 394–722 (329 bases) and NS5B gene mapped to 8,196–8,647 (452 bases) of H77 prototype. We observed the extent of sequence similarities in the core HIV–HCV coinfection was 98.15%, in HCV monoinfection was 96.9%, and in combined datasets of the sequence similarity was 96.6%. Similarly, for the NS5B sequence similarities in HIV–HCV coinfection was 98.9%, HCV monoinfection was 98.03%, and in combined datasets of the sequence similarity was 97.36%. All the isolates from Unnao belonged to genotype 3a regardless of HIV co-infection status. No recombination was observed in the regions of NS5B and core genes sequenced.

With reference to the H77 prototype, we observed a total of 71 nucleotide and 12 amino acid substitutions in the core protein, whereas the NS5B protein showed 70 nucleotide and 43 amino acid substitutions in Unnao isolates. Of the 12 amino acid substitutions in the core protein, 6 (N16I, L36V, R70Q, P71S, T75S, and T110N) were part of highly variable sites in other India isolates. Of the 43 NS5B substitutions, 6 were associated with resistance to the known drugs ribavirin and sofosbuvir. The substitutions Q309R (observed in all Unnao isolates) and R345S (observed in 4 isolates in the HCV monoinfection group) are known to be associated with resistance to ribavirin. We also observed the substitutions A207M, S218F, C289F, and A333R (associated with sofosbuvir resistance) in all isolates.

**HCV Phylogenetic and Evolutionary Analysis**

The phylogenetic trees derived using partial sequences of NS5B and core genes of the Unnao isolates showed clustering of isolates with HIV–HCV co-infection and HCV monoinfection, indicating lack of distinct clusters pertaining to HIV status (Figures 1, 2). In the phylogenetic tree derived for NS5B gene using global sequences that include India HCV isolates from other studies as well as the Unnao isolates, Unnao isolates were observed to form a monophyletic cluster (Appendix Figure 1). The closest branch joining the Unnao isolates includes 1 India isolate (GenBank accession no. GQ275355, isolated during "EID/article/28/4/21-1845-App1.pdf"). None of the other India isolates showed sequence similarity was 97.36%. Similarly, for the NS5B sequence similarities in HIV–HCV coinfection was 98.9%, HCV monoinfection was 98.03%, and in combined datasets of the sequence similarity was 97.36%. All the isolates from Unnao belonged to genotype 3a regardless of HIV co-infection status. No recombination was observed in the regions of NS5B and core genes sequenced.

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**Table 2.** Viral load among HCV antibody–positive persons identified during HIV outbreak investigation, by HIV serostatus, Unnao, India*

<table>
<thead>
<tr>
<th>Category</th>
<th>Viral load, log IU/mL, ± SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persons with HCV monoinfection</td>
<td>5.46 ± 1.09</td>
<td>0.07†</td>
</tr>
<tr>
<td>Persons with HIV–HCV co-infection</td>
<td>5.93 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>Persons with undetectable HIV-1 viral load</td>
<td>5.85 ± 0.76</td>
<td>0.65‡</td>
</tr>
<tr>
<td>Persons with detectable HIV-1 viral load</td>
<td>6.06 ± 1.15</td>
<td></td>
</tr>
</tbody>
</table>

*HCV, hepatitis C virus.
†For comparison between HCV monoinfection and HIV–HCV co-infection.
‡For comparison between undetectable and detectable HIV-1 viral load.
Spread of Hepatitis C Virus, Unnao, India

2003), 3 isolates from the United States (GenBank accession nos. DQ430819–20, isolated during 2000, and AY956467, isolated in 2002), and 2 isolates from the United Kingdom (GenBank accession nos. GQ356207 and GQ356217, isolated during 2006). In the phylogenetic analysis using the core gene, Unnao isolates were part of 3 clusters. Most Unnao isolates (56 of the total 65) formed a distinct cluster. The second cluster included 1 Unnao isolate ic31460 and 5 HCV isolates (GenBank accession nos. MT953835 and MT953776, isolated during 2019 from India, and GenBank accession nos. KY620638, KY620807, and KY620806, isolated during 2014 [country of isolation is not available]). The third cluster included Unnao isolates i10711, i30501, i10911, i30071, i20851, i10811, ic31220, and ic31370 and other India isolates (GenBank accession nos. MN697780, isolated during 2016, and MN697854 and MN697827, isolated during 2017) from a previous study (20) (Appendix Figure 2).

We estimated tMRCA for Unnao isolates by using the partial sequences of NS5B genes of HIV–HCV co-infection and HCV monoinfection, together with the India and global isolates that are observed in close proximity in the phylogenetic tree. tMRCA for Unnao was observed to be 2012 (Figure 3). The nucleotide substitution rate of partial sequences of the core gene belonging to Unnao and global isolates is $2 \times 10^{-3}$ substitutions/site/year (95% highest posterior density interval 1.16–3.54 $\times 10^{-3}$) and for the NS5B gene (that clustered with Unnao isolates) is $6 \times 10^{-3}$ substitutions/site/year (95% highest posterior density interval 1.28–8.46 $\times 10^{-3}$).
Discussion
We describe the investigation of a HIV–HCV dual outbreak in a general community setting from India by means of sequencing of partial regions of 2 HCV genes; namely, NS5B (nonstructural) and core (structural). The NS5B is relatively conserved and used as a marker for genotyping (21). The isolates sequenced from participants with HCV mono-infection and co-infection (HIV–HCV) were characterized by using phylogenetic analysis to understand the circulating genotypes, time of introduction of HCV in the community, and the functional significance of substitutions, if any.

Phylogenetic analysis revealed the presence of genotype 3a in all samples. This genotype is the most commonly reported from the state of Uttar Pradesh and the neighboring regions (2,22,23). All HCV sequences from Unnao shared high sequence similarity (>96%) regardless of HIV infection status. The clustering pattern observed in the phylogenetic tree derived from NS5B suggests monophyly and thereby indicates that the Unnao isolates are descendants of 1 (or many) closely related 3a isolates that are circulating in the community. Similarly, the estimated nucleotide substitution rates reported in our study are not only in agreement with previous reports (24,25) but also corroborate with the timeline of the HIV outbreak reported. Thus, the phylogenetic clustering supports the observation pertaining to the use of unsterile injection equipment as a potential route of entry of HCV in the study setting (5). The phylogenetic tree of core sequences from Unnao showed that 1 of the 3 observed clusters consisting of 8 sequences (6 i and 2 ic) group with isolates reported in a previous study describing HCV diversity among HIV-infected persons (20). However, clustering proximity of Unnao isolates with isolates from recreational drug users (based on core region) is insufficient (21) and therefore the use of recreational drug abuse can be ruled out in the Unnao outbreak (Appendix Figure 2). None of the study participants reported injecting drugs for nonmedical or recreational purposes. Thus, the exceptionally high rate of HCV infection in Unnao might be explained by exposure of the local community (mostly agrarian and belonging to lower socioeconomic status) to unsafe injection practices during healthcare seeking; an assertion supported by the observation of 2 patients, which prompted the case-control study described previously and earlier reports documenting reuse of injection equipment in nearby rural and urban settings (5,26).

HIV is reported to accelerate the progression of liver disease in HCV-infected persons because HCV replication increases in the presence of HIV, resulting in elevated HCV RNA levels (7,25). We did not observe correlation between HIV and HCV RNA load, nor was any specific clustering of HCV sequences based on HIV serostatus noted. Thus, it appears that
Figure 3. Time to most recent common ancestor estimated using hepatitis C virus (HCV) NS5B gene sequences of isolates from HCV antibody–positive persons identified during HIV outbreak investigation, Unnao, India. Red indicates samples with HIV–HCV coinfection; blue indicates samples with HCV monoinfection. Mean estimated time to most recent common ancestor of HCV isolates with the respective 95% highest posterior density interval is 2012 (2008–2014), with posterior probability value of 1. GenBank accession numbers are provided for reference sequences. Scale bar indicates branch lengths. I, HIV–HCV co-infection; IC, HCV monoinfection.
the influence of duration of HIV infection was not sufficiently strong and probably succeeded (rather than preceded) the highly evolved or adapted HCV that circulated in defined clusters.

We observed certain mutations associated with ribavirin and sofosbuvir resistance; these drugs form the main component of HCV treatment regimens in India (9). The low detection rate of resistance mutations in the isolates corroborates with the HCV treatment–naïve status of the community in concern. Knowledge of baseline mutations is important for predicting potential response to antiviral therapy and will help to determine local policies for HCV treatment.

The first limitation of our study is that it was not primarily designed as an HCV outbreak investigation and hence was restricted in terms of the samples tested for HCV phylogeny. The nature of the investigation did not enable us to link HCV infection to any specific source. HCV RNA testing was performed after the detection of the HIV outbreak in Unnao. Hence, we could not define the time between infection and the onset of viremia. A similar outbreak investigation in the Roka rural commune in Cambodia showed that concurrent spread of HIV and HCV was linked with unsafe injecting practices in therapeutic settings (6).

The ongoing HCV epidemic we uncovered in our investigation highlights the need for HCV surveillance in the area and in neighboring districts. Furthermore, after our investigation, an antiretroviral treatment center was established at the Bangarmau Community health center, the coordination site for the case–control investigation described previously, to ensure access of local residents to testing, treatment, and follow-up services.

In conclusion, characterization of NS5B and core sequences with HCV monoinfection and HIV–HCV co-infection using phylogenetic analysis not only substantiates our previous results (5) but also indicates a potential HCV outbreak in the community from a single isolate or its closely related descendants in Unnao, India. The HIV–HCV dual epidemic in a general community setting highlights the need for systematic surveillance and integrated approach combining hepatitis testing along with HIV for early detection and timely interventions to arrest transmission. We reiterate the need for adhering to good infection prevention and control practices, deploying single-use injection equipment (preferably autodisabled ones), strengthening healthcare delivery systems, and empowering the community to reduce the further spread of HIV and HCV. Molecular analyses of all HCV outbreaks is warranted to decipher the circulating genotypes, genetic diversity, and transmission dynamics. These findings in turn will provide valuable inputs to the National Viral Hepatitis Control Program in planning targeted interventions for mitigating the spread of HCV in India.

This work was supported by the Indian Council of Medical Research, New Delhi under Short Term New Scheme (HIV/50/183/2/2018-ECD II).

About the Author

Dr. Arati Mane is a scientist with the Division of Microbiology at the Indian Council of Medical Research–National AIDS Research Institute in Pune, India. Her research interests include molecular epidemiology of sexually transmitted infections, evaluation of rapid and low cost diagnostics, and antimicrobial resistance.

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Address for correspondence: Dr. Samiran Panda, Division of Epidemiology and Communicable Diseases, Indian Council of Medical Research, New Delhi 110 029, India; email: pandasamiran@gmail.com
Coronavirus disease (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in China in 2019 and spread around the world in 2020. By August 2021, >200 million persons were infected by SARS-CoV-2 and >4 million had died (1). A large proportion of infected persons remain asymptomatic or have only mild symptoms (2,3). The role of all infected persons should be considered in the maintenance of disease transmission, especially because asymptomatic or mildly symptomatic persons often are not tested or reported to public health authorities (4). In addition, because of economic, political, and social difficulties, molecular tests for detecting SARS-CoV-2 often are limited, particularly in developing countries (5,6). In this context, alternative measures must be explored to generate reliable data that enable government decisions to contain viral spread.

Several studies report a rapid immune response that culminates in the production of SARS-CoV-2 antibodies in the first weeks after infection (5–8). Assessment of serologic SARS-CoV-2 IgG can be an essential tool to measure the dynamics of virus transmission.

Some authors hypothesized that serosurveillance in blood donors can help monitor the evolution of SARS-CoV-2 infections (9–11). We conducted a large longitudinal study using the records of reported COVID-19 cases and SARS-CoV-2 serology results from blood donors as inputs and delivers estimates of hidden variables, such as daily values of SARS-CoV-2 transmission rates and cumulative incidence rate of reported and unreported SARS-CoV-2 cases. We concluded that the information about cumulative incidence of a disease in a city’s population can be obtained by testing serum samples collected from blood donors. Our proposed method also can be extended to surveillance of other infectious diseases.

Our study also provides evidence supporting the possibility of using serology from blood samples collected in blood donation centers to estimate the accumulated incidence of disease. Our results cover blood samples collected in 7 blood donation centers. Our proposed method provides a consistent picture of the evolution
of COVID-19 in the 7 representative cities and describes the cumulative incidence, daily transmission rate, and proportion of reported and unreported cases.

Materials and Methods

Study Population
We enrolled blood donors at 7 blood donation centers (Fundação HEMOMINAS) in Minas Gerais, Brazil, during March 1–December 31, 2020. These 7 blood donation centers account for ≈60% of the blood collections performed by HEMOMINAS and are in the cities of Belo Horizonte, Governador Valadares, Juiz de Fora, Montes Claros, Pouso Alegre, Uberaba, and Uberlândia. We used Epitools (Ausvet, https://epitools.ausvet.com.au) to calculate the number of samples to include monthly from each selected blood center based on the prevalence of COVID-19 cases reported by the municipal health secretaries in each city. However, we analyzed more samples than the Epitools-defined quantity in all centers during all periods. The study was approved by the Ethics Committee of Fundação HEMOMINAS (approval no. CAAE 31087720.2.0000.5118). Data from all donors were collected from the records of each blood donation center.

Sample Collection and Testing
We randomly selected samples collected for serologic screening (n = 7,854) in the 7 blood donation centers, aliquoted, and froze at −80°C until SARS-CoV-2 IgG testing was performed. We used the SARS-CoV-2 IgG Kit (Abbott, https://www.abbott.com), following the manufacturer’s instructions, to determine the IgG of the nucleocapsid protein of samples that tested positive or negative for SARS-CoV-2. Among the samples tested, we excluded 17 (0.2%) from the study because they corresponded to different donations from the same donors that were IgG positive for SARS-CoV-2. Testing results in different donations from the same donor (n = 17) always showed the same result. We only kept the first donation from each SARS-CoV-2 IgG-positive repeat donor in the analysis.

Epidemic Model
Studies using dynamic models of COVID-19 epidemics usually use compartmental models with SEIR structures (13). We propose a SEIR model that uses the same compartments defined by R. Li et al. (12), in which persons are susceptible, exposed (those in the latent period of infection), reported as infected (those that can propagate the virus and are reported in public health statistics), unreported infected, and removed (persons who have either recovered and become immune, at least temporarily, or have died). In addition to the number of persons in each compartment, our model also defines the transmission rate, β, as a variable that changes with time, and differential equations describe the time evolution of variables (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-1961-App1.pdf). Our version of the model does not yet consider vaccination or the possibility of reinfection. Vaccination could be included in our model by moving vaccinated persons to the removed compartment, but further studies on the loss of immunity in both recovered and vaccinated persons are needed to elucidate usefulness of the model for longer timeframes. Other issues that could be studied include the response of serology tests in detecting vaccine antibodies and the effect of IgG waning in test results.

Our model has 2 parameters that are mainly biologically determined: the average time a person stays in the compartment of exposed before changing to infected and the average duration of infection. Other parameters depend on both biologic and social factors.

Most studies related to the dynamic modeling of COVID-19 epidemics consider either a constant or a piecewise constant, β, that changes as governments enact or remove social distancing and other containment measures (14). However, the actual dynamics of COVID-19 epidemics vary faster due to the shifting response of populations to virus containment measures. In this study, we assumed that β has a daily value, which we estimated by minimizing the error between the number of reported infected persons delivered by the model and the number of infected persons reported by public health services. This assumption creates an implicit feedback loop that forces the model’s internal variables to adapt to mirror the corresponding real hidden variables of the epidemic process. A model capable of delivering estimates of hidden internal variables of a system is called a state observer (13,15).

After β is estimated, the remaining model parameter to be found is the fraction (α) of infected persons detected by testing and becoming reported cases. This value is determined by comparing the accumulated number of reported cases with the accumulated incidence indicated by seroprevalence in blood donors.

We assessed our proposed method by using data of apparent COVID-19 lethality (deaths divided by reported cases). We were able to determine apparent lethality because the testing policy used in the state of Minas Gerais defined patients’ COVID-19 testing eligibility on the basis of severity of symptoms. These criteria were very restrictive at first and were relaxed after the testing infrastructure was expanded in July 2020. Therefore, α changed from one fixed value to
another fixed value, leading to a change in the apparent lethality by the same factor of the change in \( \alpha \). Because the data relative to deaths, reported cases, and incidence in blood donor samples are mutually independent, our proposed model could be assessed by checking its simultaneous compatibility with these data in all cities. For this purpose, we used the apparent lethality to infer \( \alpha \) instead of using the proportion between the accumulated number of reported cases and incidence in blood samples.

**Statistical Analysis**

We calculated the number of occurrences of each outcome and the frequencies for categorical variables. We made comparisons by using the Fisher exact test. We calculated the median and interquartile range (IQR) for continuous variables and performed comparisons by using 2-sided Mann-Whitney tests. We calculated all tests and confidence intervals at 95%. We estimated the proportion of positive IgG tests for each blood donation center by aggregating the number of tests and positive results each month after removing repeat donors who had positive tests recorded in previous visits. We used Epitool (Ausvet) to calculate the unadjusted and test-adjusted seroprevalence for sensitivity (90%) and specificity (99%) (16,17), using Wilson’s CI for apparent prevalence and Blaker’s CI for true prevalence.

**Results**

**SARS-CoV-2 IgG Seroprevalence among Blood Donors**

Our study included data from 7,837 donors who gave blood at 7 donation centers in Brazil during March 1–December 31, 2020. The total number of samples included in the study represents 6.4% of all blood donations in the selected centers during the study period. Serologic testing to identify SARS-CoV-2 IgG revealed 441 (5.63%) positive blood donors during the study period. When adjusted for sensitivity and specificity of the test, the overall rate of positivity was 5.20% (95% CI 4.65%–5.80%). Male donors had 1.35 (95% CI 1.12–1.63) times the odds of being seropositive than did female donors. The type of donor (first-time vs. repeat donor) did not represent a statistically significant difference between groups who were positive or negative for SARS-CoV-2 IgG. Age also was not a statistically significant difference, either across age groups after adjusting for multiple hypotheses tests using the Holm correction or when regressing the rate of positivity on age by using simple linear regression (Table). We calculated the evolving seroprevalence over all months of 2020 in each blood center and its geographic location in Minas Gerais (Figure 1). In most blood centers, the increase in seroprevalence rates was slower in the first months, accelerated in August, but became faster during October (Appendix Table).

**Modeling SARS-CoV-2 Infection among the General Population**

We used the seroprevalence rates of SARS-CoV-2 IgG among blood donors to infer the proportion of persons in the general population infected in the 7 cities’ blood centers, according to the statistical model established. We chose the \( \alpha \) parameter so that the accumulated incidence rate delivered by the model, including the reported and unreported cases, fits the prevalence of COVID-19 in the blood donors in each blood center for each month.

**Model Assessment**

The evolution of apparent lethality of COVID-19 (deaths divided by reported cases) in the cities that

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total samples</th>
<th>Positive samples</th>
<th>% (95% CI)</th>
<th>Test-adjusted seroprevalence, % (95% CI)*</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>4,284</td>
<td>273</td>
<td>6.4 (5.7–7.1)</td>
<td>6.0 (5.3–6.9)</td>
<td>1.4 (1.1–1.6)</td>
</tr>
<tr>
<td>F</td>
<td>3,553</td>
<td>168</td>
<td>4.7 (4.1–5.5)</td>
<td>4.2 (3.5–5.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Age range, y</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16–30</td>
<td>2,895</td>
<td>153</td>
<td>5.3 (4.5–6.2)</td>
<td>4.8 (4.0–5.8)</td>
<td></td>
</tr>
<tr>
<td>31–40</td>
<td>2,330</td>
<td>135</td>
<td>5.8 (4.9–6.8)</td>
<td>5.4 (4.4–6.5)</td>
<td></td>
</tr>
<tr>
<td>41–50</td>
<td>1,701</td>
<td>115</td>
<td>6.8 (5.7–8.1)</td>
<td>6.5 (5.3–7.9)</td>
<td></td>
</tr>
<tr>
<td>51–60</td>
<td>829</td>
<td>32</td>
<td>3.9 (2.8–5.4)</td>
<td>3.2 (2.0–4.9)</td>
<td></td>
</tr>
<tr>
<td>61–70</td>
<td>82</td>
<td>6</td>
<td>7.3 (3.4–15.1)</td>
<td>7.1 (2.7–15.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Donor type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First-time donor</td>
<td>1,483</td>
<td>72</td>
<td>4.9 (3.9–6.1)</td>
<td>4.3 (3.2–5.7)</td>
<td>0.8 (0.7–1.1)</td>
</tr>
<tr>
<td>Repeat donor</td>
<td>6,353</td>
<td>369</td>
<td>5.8 (5.3–6.4)</td>
<td>5.4 (4.8–6.1)</td>
<td></td>
</tr>
</tbody>
</table>

*Considering sensitivity of 90% and specificity of 99%. Analysis performed by Epitool by using Wilson’s confidence interval for apparent rate of positivity and Blaker’s CI for true rate of positivity.
host blood centers (except Uberlândia) suggests that the proportion of infected persons tested changed around day 122, July 16, and increased by nearly 70% (Figure 2). That date coincides with the time when additional laboratories were integrated into the testing infrastructure for SARS-CoV-2 provided by the state government. In the case of Uberlândia, the municipality provided a relevant testing infrastructure in addition to the infrastructure provided by the state government in all other cities. Those data provide independent information about the relative changes in the value of \( \alpha \), which can be used for assessing the model consistency.

We performed this assessment by running the model with \( \alpha \) taking the same value in all cities except Uberlândia, assuming a fixed value that we increased by 70% at day 122 (July 16; \( t = 122 \) days) and that remained fixed on this new value from July 16–December 31. We determined the initial value of \( \alpha \) that enabled the best fitting of the observed IgG rates of positivity in the blood centers was 0.18 for the proportion of reported cases up to \( t = 122 \) days and was 0.31 for \( t > 122 \) days in Belo Horizonte, Governador Valadares, Juiz de Fora, Montes Claros, Pouso Alegre, and Uberaba. For Uberlândia, we found the values were 0.37 for \( t \leq 122 \) and 0.41 for \( t > 122 \).

**Figure 1.** Temporal evolving cumulative severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) seroprevalence in HEMOMINAS Foundation blood donation centers in 7 cities of Minas Gerais, Brazil, March–December 2020. A) March; B) June; C) September; D) December. Data represent SARS-CoV-2 IgG seropositivity among persons eligible to donate blood. Scale bar represents cumulative proportion of SARS-CoV-2 IgG seropositivity per 100,000 population. Gov., Governador.
We superimposed the seroprevalences in the blood centers each month onto the estimated curves of accumulated number of cases (reported and unreported) as predicted by the model in the respective cities (Figure 3). In 5 cities (Belo Horizonte, Juiz de Fora, Montes Claros, Uberlândia, and Uberaba), these parameter values resulted in reasonable matches with almost all data points. In 2 cities, Governador Valadares and Pouso Alegre, the seroprevalence data in the last period (October–December) were not well-adjusted to the model, suggesting that the pattern of variation of α in those cities could be different from the variations in other cities. However, because Governador Valadares and Pouso Alegre are small cities, a separate analysis of the change in the apparent lethality is not possible, which prevents the possibility of applying the same methodology for refining the estimates when comparing cities of varying populations.

Discussion
In this study, we evaluated the rate of blood donors who tested positive for SARS-CoV-2 IgG and donated blood in 7 cities in Minas Gerais, Brazil, during March–December 2020. We used the data to estimate the rate of infection in the general population, then used the infection rate in a dynamic model with a SEIR structure.

The higher rate of IgG positivity found in male donors (6.4% vs. 4.7% for female donors) in our study did not agree with the reported COVID-19 cases in Minas Gerais during the same period (49.2% for male vs. 50.8% for female persons). The higher proportion of positive tests among male donors suggests that the epidemiologic profile of infection might change when more persons with asymptomatic or mild COVID-19 are tested, such as expected for blood donors. The rate of positivity associated with sex has been previously observed (18), but different works did not identify this association in blood donors (19–21) or in the general population (22).

Concerning differences of positivity between age groups, we found no statistically significant difference in this study. Differences in positivity between age groups is a controversial issue; some studies report higher seroprevalence in younger persons (19,23), but other studies indicate greater seroprevalence in older persons (24) or do not find statistically significant associations between seroprevalence and age (25). A study conducted in 133 cities in Brazil found that persons 20–59 years of age, an age group that corresponds to most blood donors included in this study, were more likely to be infected (26). The differences between studies might be partly explained by cultural and population issues, making it difficult to consolidate a general conclusion. Loss of statistical power resulting from corrections for testing multiple hypotheses might also play a role in the observed differences not achieving statistical significance, particularly if the effect size is moderate.

Figure 2. Apparent lethality of coronavirus disease (COVID-19) in 6 cities (Belo Horizonte, Pouso Alegre, Montes Claros, Juiz de Fora, Governador Valadares, and Uberaba), Minas Gerais State, Brazil, April–December 2020. Gray shading and gray data line indicate the beginning of the COVID-19 epidemic in Minas Gerais, days 1–60, in which few cases were reported and the testing infrastructure was still being organized. Red data line indicates days 61–290 of the epidemic. Our model predicted the apparent lethality during days 60–120 to be nearly 5.2% and to fall during days 121–290 to nearly 3.0% (gray horizontal lines). This change corresponds to a nearly 70% increase in the value of α in our model (proportion of infected persons that are reported), assuming that the actual lethality has not changed. We did not include data from the city of Uberlândia in this estimation due to local legislation regulating COVID-19 testing, which resulted in a much larger proportion of people being tested in that municipality than in the other cities. Markings for each month represent the first day of the month.
Seroprevalence in the blood donation centers showed the proportion of positive donors increased slowly in the first 6 months, and higher proportions of positivity were recorded from August onward, with regional variations. In Minas Gerais, COVID-19 cases increased in June, peaked in August, decreased slowly until October, and then reached the highest numbers in December 2020. Our results agree with this scenario, suggesting that seroprevalence rates in blood donors correlated with reported COVID-19 case rates. A crucial feature of the rate of positivity indicated by serologic testing in the blood centers is that seroprevalence is much greater than prevalence that would be obtained by the accumulated number of reported COVID-19 cases. However, we expected this difference because of underreporting. Notwithstanding, public communication about COVID-19 epidemics is commonly articulated on the basis of reported cases in the community, which strongly underestimates the actual spread of the disease. This difference

Figure 3. Proportion of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) IgG–positive results among blood donors in blood donation centers in 7 cities in Minas Gerais, Brazil, March–December 2020. A) Belo Horizonte; B) Governador Valadares; C) Juiz de Fora; D) Montes Claros; E) Pouso Alegre; F) Uberaba; G) Uberlândia. Blue dots indicate proportion of SARS-CoV-2–positive donors at the end each month; vertical blue lines indicate 95% CIs. Black squares indicate the official cumulative prevalence of reported coronavirus disease cases for each city. Red lines represent model estimates of the number of infected persons, including reported and unreported cases, in each city, as a proportion of the city’s population. Vertical dashed lines indicate national holidays.
underscores the convenience of using a model-based approach, as we propose, because it enables the use of measured data for estimating hidden variables, such as the total number of infected persons.

Although all the cities we evaluated had increased COVID-19 positivity rates in December 2020, Governador Valadares had the highest rates. This finding is in consonance with the fact that Governor Valadares had a higher accumulated COVID-19 incidence, 4,227.8 cases/100,000 population, than was seen in Minas Gerais (2,270.1 cases/100,000 population) and Brazil overall (3,383.6 cases/100,000 population). Governor Valadares also had a higher COVID-19 mortality rate, 143 deaths/100,000 population, than Minas Gerais (51.3 deaths/100,000 population) or Brazil (88 deaths/100,000 population).

Several countries are implementing serial SARS-CoV-2 serologic surveillance studies by using blood donors (19,23,27). These studies provide relevant results to complement population seroprevalence data (19) and valuable information for decision-making in countries where such data are not available. However, some issues should be considered, including the appropriate test to assess seroprevalence and the threshold for identifying positive and negative samples. Of note, the automated serologic tests that are available were validated by using samples from symptomatic COVID-19 patients with a confirmed diagnosis by reverse transcription PCR (E.W. Eyre et al., unpub. data, https://doi.org/10.1101/2020.07.21.20159038). Results obtained in other studies using the same chemiluminescence test indicated a lower sensitivity to detect SARS-CoV-2 IgG in newly infected persons (28), which might affect the extrapolation of seroprevalence data to the population when using blood donors’ samples (11,29). These data reinforce the need for choosing serologic assays with high sensitivity, specificity, and durable antibody detection, even months after infection (30; M. Stone et al., unpub. data, https://doi.org/10.1101/2021.09.04.21262414).

Blood donor–based estimates of SARS-CoV-2 seroprevalence might deviate from the seroprevalence in the general population for several reasons, including the exclusion of populations who cannot donate blood, such as persons <16 and >70 years of age and residents of nursing homes and prisons. The proportion of different groups (e.g., male and female donors, or different age groups) represented in the samples might differ from their respective proportions in population. In addition, recruitment and eligibility criteria for blood donations recommended by the Brazil Ministry of Health during the COVID-19 pandemic excluded asymptomatic candidates who had contact with infected persons <30 days before going to a donation center; those donors had to wait 14 days from the date they were first seen at the blood center before they could donate. The recommendation also excluded potential blood donors who had a COVID-19 diagnosis until ≥30 days after their symptoms disappeared (31). Such guidelines might result in decreased SARS-CoV-2 IgG seropositivity rates among blood donors.

The results of our study comparing prevalence estimates obtained using the SEIR model with actual health system notification data suggest that blood donor serosurveillance data can provide valuable information for monitoring the epidemic and evaluating the effectiveness of measures to fight the virus spread in the cities that have blood donation centers. Our study also showed that the evolution of the epidemic can be considerably different from city to city, even considering cities within the same state in Brazil, suggesting that the application of the proposed SEIR model in other cities would require some strategy of periodic collection of blood samples for serologic analysis from a sufficient number of persons spread across the population.

Some aspects of the proposed modeling approach should be highlighted. First, the procedure for estimating the time-varying transmission rate β for the SEIR model enables a reasonable automatic estimation of that parameter, thus circumventing a major difficulty in COVID-19 modeling (12,14). As a byproduct, this procedure also eliminates the difficulty usually encountered in determining adequate initial conditions. In fact, the SEIR model, endowed with the estimation procedure for β, becomes equivalent to a state observer model (13,15), producing estimates of the model’s hidden variables that will approximate the real unmeasured variables, regardless of initial conditions, provided that the model parameters are reasonable approximations of the actual parameters.

The estimated hidden variables might be quite useful in practice. For instance, β(t) provides information that is not contained in the reproduction number, R0, because β(t) does not vary with the number of recovered persons, representing a better descriptor of social isolation intensity. Perhaps counterintuitively, the cumulative incidence estimate provided by the model can be considered more reliable than the monthly point estimates derived from raw data of serologic analysis in blood centers because the model performs a filtering of the random variation in data that results from sampling.

Concerning assessment of the proposed model, users could choose different values for the α
parameter for each city and for each month, according to the outcomes of serologic tests in the respective blood donation centers. Thus, the accumulated incidence of cases estimated by the model would be forced to follow the trajectory of serology results, which would not confirm the model validity. The procedure of model assessment we adopted used the same trajectories for $\alpha$ in 6 cities and got the changes in $\alpha$ from an independent source. The consistency of the model outcomes with the serology results in most of data points, considering cities with rather different trajectories of the epidemics, provides corroborated of our proposed model.

The first limitation of our study is that the stratification of the blood donors by sex or by age would enable the correction of the seroprevalence estimates according to the demographic composition of the general population, leading to more precise results. As we observed (Figure 3), the prevalence in some cities showed a systematic tendency to remain below the values predicted by the model in the last 3 months, which also suggests that a relevant process of seroconversion might exist as IgG wanes. Modeling of such a decay process might help provide the correct interpretation of data in the last months of our experiment. Finally, some of the blood donation centers considered in this study are relatively small (Pouso Alegre and Uberaba), which increases the uncertainty associated with the data collected in those centers, not only by reducing the sample size, but also by reducing the robustness to skewed data.

In conclusion, the results of our study suggest that blood donation centers could be incorporated into COVID-19 surveillance systems with the role of regularly providing quantitative estimates of SARS-CoV-2 seroprevalence in the population. For this purpose, public health agencies should use an epidemic model with a state observer property, which performs a track of some measured variable and produces outputs that converge to the system’s hidden variables. Thus, we propose a specific SEIR epidemic model that performs the adjustment of the transmission rate $\beta$ such that the model tracks the measured number of reported COVID-19 cases. Our model used seroprevalence data collected in blood centers to adjust the proportion of reported cases considered. This model provided consistent estimates of relevant variables that otherwise would not be accessible, thus supporting a well-informed decision-making process. The methods we propose can be adapted for surveillance of other infectious diseases by using other kinds of input information from sentinel surveillance systems combined with serosurveillance data gathered in blood donation centers.

Acknowledgments
We thank all the professionals who contributed to the enrollment of blood donors in the study.

Secretaria Estadual de Saúde de Minas Gerais (SES/MG) and Fundação Hemominas provided financial support. E.F.B.-S., R.H.C.T., and I.R.O. are fellows from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

About the Author
Dr. Chaves is head of the Research Service at Fundação Hemominas. His research interests include hematology and infectious diseases.

References


Address for correspondence: Daniel Gonçalves Chaves, Fundação Centro de Hematologia e Hemoterapia de Minas Gerais (Fundação HEMOMINAS), Alameda Ezequiel Dias 321, Belo Horizonte, Minas Gerais 30.130-110, Brazil; email: daniel.chaves@hemominas.mg.gov.br
Persistence of vaccination-induced cellular and humoral immune responses is crucial to prevent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection or at least provide protection against severe coronavirus disease (COVID-19) that requires hospitalization. As in many other countries, the SARS-CoV-2 vaccination strategy in Germany was based on prioritization by occupation, underlying medical conditions, or advanced age (1). Although those priority groups have been vaccinated, a debate has emerged as to whether a third booster dose may be necessary to maintain or raise levels of protection within some of these groups. Decisions on whether to recommend a third dose needed to be made within a short timeframe, because SARS-CoV-2 infection case numbers were expected to increase again in the upcoming cold season, as previously observed in late 2020 (2).

To date, however, data are lacking regarding the longevity of vaccination responses, and most published studies only provide follow-up data until 3 months after the second dose (3). Only 2 studies report data on extended time frames of 6 months after a completed 2-dose scheme (4, 5), and, to our knowledge, no studies have considered follow-ups in patients receiving chronic hemodialysis. Data on the actual effect of a third dose are equally scarce and, so far, limited to organ transplant recipients, where a third dose substantially increased antibody responses.

Patients undergoing chronic hemodialysis were among the first to receive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccinations because of their increased risk for severe coronavirus disease and high case-fatality rates. By using a previously reported cohort from Germany of at-risk hemodialysis patients and healthy donors, where antibody responses were examined 3 weeks after the second vaccination, we assessed systemic cellular and humoral immune responses in serum and saliva 4 months after vaccination with the Pfizer-BioNTech BNT162b2 vaccine using an interferon-γ release assay and multiplex-based IgG measurements. We further compared neutralization capacity of vaccination-induced IgG against 4 SARS-CoV-2 variants of concern (Alpha, Beta, Gamma, and Delta) by angiotensin-converting enzyme 2 receptor-binding domain competition assay. Sixteen weeks after second vaccination, compared with 3 weeks after, cellular and humoral responses against the original SARS-CoV-2 isolate and variants of concern were substantially reduced. Some dialysis patients even had no detectable B- or T-cell responses.

Persistence of vaccination-induced cellular and humoral immune responses is crucial to prevent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection or at least
to first-generation vaccines is reduced for SARS-CoV-2 variants of concern (VOCs) (7), which now account for most infections worldwide (8), making the decision of whether a third dose is advisable even more critical for those with underlying conditions, immunodeficiencies, or an increased exposure risk (e.g., healthcare workers).

One particular risk group for SARS-CoV-2 infection and severe COVID-19 disease is hemodialysis patients; currently, ≈80,000 persons requiring regular renal replacement therapy in Germany (9). Their various underlying medical conditions and dialysis therapy often lead to a state of generalized immunosuppression (10). At the same time, these patients bear a continuous exposure risk because of the regular need for in-center hemodialysis therapy, which prevents them from self-isolating or reducing contacts to avoid infection. We and others have identified impaired cellular and humoral responses towards several viral vaccinations (e.g., SARS-CoV-2, influenza A, or hepatitis B) (10–13); however, there is a lack of longitudinal vaccination response studies against SARS-CoV-2 within this population. To guide future vaccination strategies as to whether additional booster vaccinations for at-risk groups to prevent severe COVID-19 are required, we provide follow-up data for a previously reported cohort of 76 persons receiving hemodialysis and 23 healthcare workers with no underlying conditions (13) for systemic and mucosal B- and T-cell responses 16 weeks after full BNT162b2 vaccination and the neutralizing potency of vaccination-induced antibodies. Because of the emergence of VOCs, and because all currently licensed vaccines are formulated against the original wild-type isolate (B.1), we also examined antibody binding and neutralization toward the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.3) and Delta (B.1.617.2) VOCs.

**Methods**

**Study Design and Sample Collection**

We collected blood samples by using vascular access before the start of dialysis or by venipuncture for the control population 16 weeks after the standard 2-dose vaccination with a 21-day interval of BNT162b2 (Pfizer-BioNTech, https://www.pfizer.com) was completed (T2). An analysis of samples from this population that were collected 3 weeks after the second dose of BNT162b2 (T1) has been published previously (13). A total of 76 patients on maintenance hemodialysis and 23 healthcare workers from the same dialysis center participated in the longitudinal follow-up (13). Demographic characteristics (e.g., age and sex), body mass index, time on dialysis, use of immunosuppressive medications, and anti-S1 domain IgG levels at T1 of persons who did not provide a sample at T2 were not substantially different compared with persons included in this analysis (Table; Appendix Table 1, 2, https://wwwnc.cdc.gov/EID/article/28/4/21-1907-App1.pdf). We obtained plasma by using an S-Monovette lithium heparin blood collection kit (Sarstedt, https://www.sarstedt.com). We used whole-blood samples immediately for an interferon-γ (IFN-γ) release assay (IGRA). To inactivate potential pathogens, we treated collected saliva samples with Tri-n-butyl phosphate for a final concentration of 0.3% and Triton X-100 for a final concentration of 1%.

**Ethics Considerations**

The study was approved by the Internal Review Board of Hannover Medical School (approval number

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**Table.** Characteristics of participants in a study of immune response against variants of concern in dialysis patients 4 months after SARS-CoV-2 mRNA vaccination*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nondialysis control group</th>
<th>Hemodialysis group</th>
<th>p value for difference between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) patients</td>
<td>23 (100)</td>
<td>76 (100)</td>
<td>NA</td>
</tr>
<tr>
<td>Median age, y (IQR)</td>
<td>55 (14)</td>
<td>70.5 (18.25)</td>
<td>2.78 × 10⁻⁴</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>1.01 × 10⁻⁵†</td>
</tr>
<tr>
<td>M</td>
<td>6 (26.09)</td>
<td>43 (56.58)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>17 (73.91)</td>
<td>33 (43.42)</td>
<td></td>
</tr>
<tr>
<td>Median days since start of hemodialysis (IQR)</td>
<td>NA</td>
<td>1,337 (1,686.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Using immunosuppressive medication</td>
<td>0</td>
<td>10 (13.16)</td>
<td>6.77 × 10⁻²</td>
</tr>
<tr>
<td>Underlying condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesity, BMI &gt;30‡</td>
<td>4 (17.39)</td>
<td>16 (21.05)</td>
<td>8.68 × 10⁻¹</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>19 (25)</td>
<td>7.30 × 10⁻³</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>0</td>
<td>35 (46.05)</td>
<td>2.93 × 10⁻⁵</td>
</tr>
</tbody>
</table>

*Values are no. (%) except as indicated. Percentages are for total group. BMI, body mass index; IQR, interquartile range; NA, not applicable; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

†p value reflects difference in male-to-female ratios between the two groups, not differences explicitly for either male or female persons.

‡BMI for 1 person was not known.
Immune Responses after SARS-CoV-2 Vaccination

8973_BO-K_2020). We obtained written informed consent from all participants before the start of the study.

Bead Coupling
We coupled antigens to spectrally distinct MagPlex beads (Luminex, https://www.luminexcorp.com) by using EDC/s-NHS coupling for all standard (MULTICOV-AB) antigens (14). We coupled receptor-binding domains (RBDs) from VOCs by using Anteo coupling (AnteoTech, https://www.anteotech.com) according to the manufacturer’s instructions (15).

MULTICOV-AB
We analyzed IgG and IgA binding and levels by using MULTICOV-AB, a multiplex coronavirus immunoassay, as previously described (14). For our study, we used a panel of recombinant proteins as antigens (Appendix Table 3). In brief, we immobilized antigens on spectrally distinct populations of MagPlex beads either by EDC/s-NHS coupling (14) or by Anteo coupling according to the manufacturer instructions (15). We then incubated the combined MagPlex beads with samples. After conducting a wash step to remove unbound antibodies, we detected IgG or IgA with either R-phycoerythrin labeled goat anti-human IgG (Jackson ImmunoResearch, https://www.jacksonimmuno.com) or IgA (Jackson ImmunoResearch) as secondary antibodies. After conducting another wash step and bead resuspension, we measured samples once on a FLEXMAP 3D instrument (Luminex) by using the following settings: timeout, 80 s; gate, 7,500–15,000; reporter gain, standard photomultiplier tube; 40 events. Raw median fluorescence intensity (MFI) values or normalized values (MFI/MFI of quality control [QC] samples) (15) are reported. Three QC samples were measured per individual plate to monitor MULTICOV-AB performance. We measured all samples once.

Angiotensin-Converting Enzyme 2 Receptor Binding Domain Competition Assay
We carried out an angiotensin-converting enzyme 2 receptor-binding domain (ACE2-RBD) competition assay as previously described (15; D. Junker et al., unpub. data, https://doi.org/10.1101/2021.08.20.21262328) to determine IgG neutralization capacity against SARS-CoV-2 wild-type and the VOCs. For this assay, we combined biotinylated ACE2 with individual samples (and as a control, ACE2 alone) and incubated with the previously described MULTICOV-AB bead mix. Before and after ACE2 detection with Streptavidin-PE (Moss, Fisher Scientific, https://www.fishersci.com), we conducted washes. We measured samples once on a FLEXMAP 3D instrument with the same settings as MULTICOV-AB and analyzed them by using normalization of MFI values against the control. We considered samples with a neutralization ratio <0.2 as nonneutralizing. This cutoff is based on comparison to a classic virus neutralization test (D. Junker et al., unpub. data).

Euroimmun ELISA QuantiVac
As a control for the MULTICOV-AB results, we also analyzed plasma samples by using the Anti-SARS-CoV-2 QuantiVac ELISA IgG (Euroimmun, https://www.euroimmun.com). Samples were measured as previously described (13). We measured all samples once.

IGRA
We analyzed SARS-CoV-2–specific T-cell responses from whole blood by measuring IFN-γ production after stimulation with a peptide pool from the SARS-CoV-2 spike S1 with the SARS-CoV-2 Interferon Gamma Release Assay (Euroimmun) and the IFN-γ ELISA (Euroimmun), as previously described (13). We subtracted background signals from negative controls and calculated final results in milli-IU (mIU) per milliliter by using standard curves. Results from positive and negative controls were not statistically significantly different between timepoints T1 and T2. We considered IFN-γ concentrations >200 mIU/mL as reactive. We defined this arbitrary cutoff by using average background IFN-γ activity without antigen-stimulation in all samples of T1 multiplied with 10 for the threshold for IGRA positive. Using this cutoff, we found negative IGRA results in all of the 15 control samples (prepandemic persons) (16). The upper limit of reactivity was 2,000 mIU/mL.

Data Analysis and Statistics
We matched sample metadata and collected results from different assay platforms in Microsoft Excel 2016 (https://www.microsoft.com). We used GraphPad Prism 8.4.3 (https://www.graphpad.com) for statistical analysis. We generated figures in RStudio 1.2.5001 running R 3.6.1 (https://www.rstudio.com). We used the beeswarm add-on package to visualize data as strip charts with overlaying boxplots and to create nonoverlaying datapoints and used the RcolorBrewer add-on to generate specific colors for plots. We then edited the figures by using Inkscape 0.92.4 (https://inkscape.org).
Results

Substantial Decrease in Antibody Titers from 3 Weeks to 4 Months Postvaccination

Because antibody levels are considered a proxy for protection, we initially examined the seroreversion rate by using MULTICOV-AB (14), a previously published bead-based multiplex immunoassay that simultaneously analyses >20 different SARS-CoV-2 antigens, including the RBDs of VOCs and the endemic human coronaviruses. Similar to findings from our previous report (13), RBD IgG responses within the dialysis group (median normalized MFI 4.26 among 76 patients) toward SARS-CoV-2 wild-type RBD were significantly reduced compared with those for the control group (median normalized MFI 13.6 among 23 persons; p<0.001) (Figure 1, panel A) 16 weeks after complete vaccination (T2). Compared with titer levels at 3 weeks after the second dose (T1), at 16 weeks after (T2), antibody titers had significantly decreased, by 61% in the control group and 75% in the dialysis group (p<0.001) (Figure 1, panel A). RBD IgG levels measured by MULTICOV-AB were additionally verified with a commercial quantitative in vitro diagnostic antibody test (Spearman rank 0.956) (Appendix Figure 1). Although none of the samples of the control group were classified as seronegative (titer below the cutoff) (Appendix Figure 2), 19.7% (15/76) of dialysis samples were defined as such 16 weeks after the second dose (T2), which constitutes a substantial increase from 3 weeks after second vaccination (T1),

Figure 1. Significant decrease in humoral and cellular responses induced by Pfizer-BioNTech vaccine BNT162b2 (https://www.pfizer.com) against SARS-CoV-2 from 3 weeks to 16 weeks after second vaccination, observed in a study of immune response against variants in dialysis patients 4 months after SARS-CoV-2 mRNA vaccination. A) IgG response in plasma; B) IgG response in saliva; C) neutralizing capacity toward SARS-CoV-2 wild type B.1; D) T-cell response measured by IFN-γ release assay. Blue circles indicate dialysis patients (n = 76) and red circles controls (n = 23). Samples were taken 3 weeks (T1) and 16 weeks (T2) after vaccination. Saliva (panel B) has reduced sample numbers in both groups because of issues in sample collection (T1 control, n = 22; T1 dialysis, n = 69; T2 control, n = 23; T2 dialysis, n = 71). T1 timepoint data has been published previously (13) and is reproduced here for clarity. Horizontal lines within boxes indicate medians; box tops and bottoms indicate the 25th and 75th percentiles; whiskers show the largest and smallest nonoutlier values. Outliers were determined by 1.5 times interquartile range. Statistical significance was calculated by Wilcoxon matched-pairs signed rank test when comparing T1 and T2, and 2-sided Mann–Whitney–U test when comparing control and dialysis groups. ACE2, angiotensin-converting enzyme 2; IFN-γ, interferon γ; MFI, median fluorescence intensity; NS, not significant; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; T1, timepoint 1; T2, timepoint 2.
Immune Responses after SARS-CoV-2 Vaccination

When examining plasma titers against nucleocapsid, we did not observe any dialysis patients, other than one who had a PCR-confirmed infection before the first dose, having a value above the cutoff that would indicate infection.

To evaluate whether this reduction in plasma RBD IgG was also present at the mucosal site, we profiled the local antibody response in saliva by using MULTI-COV-AB. As observed in plasma, a significant reduction occurred in saliva RBD IgG titers in the dialysis (median 143 among 71 patients) compared with the control group (median 313.5 among 23 persons) (p = 0.02) (Figure 1, panel B). When comparing saliva RBD IgG levels at T1 to those at T2, we observed a statistically significant decline in both groups (p<0.001) (Figure 1, panel B), suggesting that the antibodies have potentially lost competence to prevent transmission if infected. When examining RBD IgA, we observed a significant difference in titers between persons in the control and dialysis groups (p = 0.003) (Appendix Figure 3, panel A); 47.8% of controls and 75% of dialysis patient samples were classified as seronegative. This more pronounced reduction in IgA versus IgG levels most likely represents the shorter IgA half-life. Saliva RBD IgA tended to be higher in the dialysis group, although not significantly (p = 0.051) (Appendix Figure 3, panel B).

Reduced T-cell Response after Vaccination in Dialysis Patients and Decrease Over Time

Because some persons might be able to control and clear SARS-CoV-2 infections with a strong T-cell response alone, we examined spike-specific SARS-CoV-2 T-cell responses by using a commercially available IGRA. Although absolute mean IFN-γ responses in the dialysis group compared with the control group tended to be lower (median 370 vs. 651 mIU/mL), this difference was not significant (p = 0.13) (Figure 1, panel D). In the control group, IFN-γ release after restimulation declined significantly from the first timepoint (median 1,505; p<0.001) (Figure 1, panel D), whereas for dialysis patients, this decline was not significant (median 580; p = 0.13) (Figure 1, panel D). This difference is probably attributable to most control samples being at the assay’s upper limit.
of detection at the first timepoint, when the dialysis samples already showed reduced IFN-γ release. Overall, the number of nonresponders was higher in the hemodialysis group (40.8% [31/76]) than the control group (21.7% [5/23]) (Appendix Figure 2). A lack of serologic response appears to be more driven by T-cell immunity than B-cell immunity; 2.6% (2/76) of the dialysis group having a T-cell response but no B-cell response, compared with 23.6% (18/76) who had a B-cell response but no T-cell response. In total, 17.1% (13/76) of the dialysis group were classified as complete nonresponders because of the absence of detectable SARS-CoV-2 wild-type B- and T-cell responses, compared with none in the control group.

**Significantly Reduced Antibody Binding and Neutralization Capacity against VOCs**

Having characterized response against wild-type SARS-CoV-2, we then assessed humoral response against the VOCs Alpha, Beta, Gamma, and Delta. As shown with classical cell-culture based virus neutralization assays (7), neutralization responses were also reduced for all VOCs compared with wild-type when we used the previously described ACE2-RBD competition assay. Compared levels at with the initial timepoint, neutralization decreased significantly for the Alpha and Beta VOCs (p<0.001 for both) (Figure 2, panel A, B). We were unable to determine these changes for Gamma and Delta because these variants were not measured in the initial analysis. In a comparison between the dialysis and the control cohort, dialysis patients had significantly reduced neutralization against Alpha (p<0.001) (Figure 2, panel A), Gamma (p = 0.014) (Figure 2, panel C), and Delta (p = 0.002) (Figure 2, panel C) but not for Beta (p = 0.08) (Figure 2, panel B). The number of nonresponders was variable between the different strains although consistently high; 87.0% of the control group and 93.4% of the dialysis group were considered nonresponders against Alpha, 95.7% of the control group and 100% of the dialysis group against Beta and Gamma, and 95.7% of the control group and 96.1% of dialysis group against Delta.

**Discussion**

After our initial study (13), which focused on humoral and cellular responses 3 weeks after administering the second BNT162b2 vaccination, we provide longitudinal data for 4 months after the second dose. In comparison with other vaccine studies, which have mostly examined peak humoral response within 1 month or alternative prime-boost vaccination schedules with BNT162b2 (12), our data reveal a substantial decrease in the subsequent months in hemodialysis patients and healthy controls. Overall, the decline in neutralizing anti-spike RBD antibodies was comparable in both groups, and the difference between groups was mostly driven by differences in the magnitude of the initial humoral response. Although this decrease is expected and can be attributed to the memory phase, the extent of the reduction was unpredicted because it resulted in a substantial proportion of persons being classified as seronegative. The reduction of salivary antibodies is particularly important because their presence has been linked to reduced transmission potential (15). This pattern of reduced antibody binding with increasing time post-vaccination was also reflected in diminishing neutralization potential.

Most persons tested were classified below our defined neutralization threshold for wild-type RBD with an almost complete nonresponder rate against Delta, which was the dominant strain in many parts of the world at the time of our analysis (8). Although this finding does not automatically translate to a failure of vaccine efficacy, given that any active challenge of the immune system should result in expansion of memory B- and T-cell populations along with increased (neutralizing) antibody titers, it does suggest nevertheless that active protection against infection may be reduced. Although a recent study by Pfizer (4) indicated that BNT162b2 vaccine efficacy did only slightly decrease 6 months postvaccination in the study cohort (from 95% to 91%) in fully immunocompetent persons, data from vaccinations in Israel identified a reduction in efficacy to 40% (19). In combination with our data, where 17.1% of the dialysis cohort were classified as having no evidence for vaccine-elicited T- and B-cell immunity after 4 months, the Pfizer study findings suggest that vaccine efficacy may be even further reduced within this patient group. For dialysis patients, this finding is particularly concerning because they often have underlying conditions that put them at additional risk for severe COVID-19 (10). The lack of a considerable SARS-CoV-2 specific T-cell response in dialysis patients may result from chronic inflammatory conditions, leading to T-cell exhaustion and suppression of IFN-γ levels (20). Differences in anti-SARS-CoV-2 T-cell kinetics between groups presumably reflect difference in the magnitude of T-cell responses after boost and during the contraction phase. To what extent T-cell immunity contributes to protection from COVID-19 and whether our IGRA results below a cutoff provide evidence for the lack of effective adaptive T-cell immunity, requires further investigation. However, we should state that
although we see reductions in titer, neutralizing activity, and T-cell responses, we did not see any new infections by T2 within our cohort.

Our study is limited by the relatively small sample size of persons, who were not matched by age or sex. However, the sample number and compromised matching is consistent with similar studies on dialysis vaccine responses (12). Although studies have indicated that differences exist in protection and antibody responses (21) after different COVID-19 vaccination schedules, our study of Pfizer’s BNT162b2 represents a real-world situation for most dialysis patients. Because of reduced anti-spike responses 4 weeks postvaccination in patients with other chronic conditions (6), these groups should undergo careful monitoring to determine whether their responses also decrease substantially over time.

Taken together, our results strongly argue that all persons undergoing chronic hemodialysis should be preferably administered a third dose of the BNT162b2 vaccine. Recent studies on administering a third dose to dialysis patients and transplant recipients has identified strong increases in humoral responses after vaccination, and a reduced percentage of recipients are considered nonresponders (22–25). However, longitudinal follow-up studies will be needed in early 2022 to monitor the rate of antibody decay after administration of a third dose in these and other vulnerable groups.

Acknowledgments
We thank staff and participants at the Dialysis Centre Eickenhof for their continued support to make this study possible.

This work was financially supported by the Initiative and Networking Fund of the Helmholtz Association of German Research Centers (grant no. SO-96), the EU Horizon 2020 Research and Innovation Program (grant agreement no. 101003480–CORESMA), the State Ministry of Baden–Württemberg for Economic Affairs, Labor and Tourism (grant nos. FKZ 3–4332.62-NMI-67 and FKZ 3–4332.62-NMI-68), and the European Regional Development Fund (Defeat Corona, grant no. ZW7–8515131). The funders had no role in study design, data collection, data analysis, interpretation, writing, or submission of the manuscript. All authors had complete access to the data and hold responsibility for the decision to submit for publication.

About the Author
Dr. Dulovic is a scientist in the Multiplex Immunoassay Group at the Natural and Medical Sciences Institute at the University of Tübingen. He has a background in molecular biology and genetics of parasitic nematodes and currently works on serologic assay development for a range of pathogens, including SARS-CoV-2, hepatitis virus, and influenza virus.

References


Address for correspondence: Gérard Krause, Helmholtz Center for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany; email: gerard.krause@helmholtz-hzi.de; Nicole Schneiderhan-Marra, Natural and Medical Sciences Institute at the University of Tübingen, Markwiesenstrasse 55, 72770 Reutlingen, Germany; email: nicole.schneiderhan@nmi.de; Georg M.N. Behrens, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany; email: behrens.georg@mh-hannover.de
Global genomic surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been repeatedly observed globally; shifts in human mobility drastically alter the source of virus importations, and only a small proportion of importations lead to sustained community transmission (2–6). Virus importations can sometimes be sufficiently intense to have a major effect on epidemic dynamics (e.g., super-seeding of the Alpha variant in the United Kingdom) (7). Genomic surveillance has been fundamental in tracking these dynamics, including detecting the emergence and spread of novel variants that alter risk to public health (8,9).

Gujarat is the fifth largest state in India and the ninth most populated of its 28 states (>60 million inhabitants). Most of the population lives in rural areas (57.4%), although the proportion living in cities has been increasing in past decades because of urbanization (from 37.4% in 2001 to 42.6% in 2011) (10). Gujarat has an international border with Pakistan, as well as state borders with Rajasthan and Madhya Pradesh to the northeast and east and with Maharashtra and the Union Territories of Daman, Diu, and Nagar Haveli to the south. The region has 2 international airports, in Ahmedabad and Surat (Figure 1, panel A). The airport in Ahmedabad served ≈11 million passengers and the airport in Surat served ≈1.5 million passengers (11) during April 2019–March 2020.

Although coronavirus disease (COVID-19) cases in India were first reported on January 30, 2020, and linked to travel from Wuhan, China, cases in Gujarat were initially identified on March 19, 2020 and were associated with travel from Saudi Arabia and the United Kingdom. A few days later, on March 24, 2020, a nationwide lockdown across India was announced. In Gujarat, special transportation (trains and buses) was arranged during March–May 2020 to enable ≈1.8 million stranded migrant workers to return to their home states (i.e., outside Gujarat) or districts within Gujarat. Most specially organized trains
originated from Gujarat (26%), which has one of the largest populations of migrant workers in India (12).

In this study, we investigated the introduction and transmission of SARS-CoV-2 in Gujarat during the first wave of the COVID-19 epidemic in India by using a combination of epidemiologic and genomic data. Through our analyses, we characterized the epidemiologic and lineage dynamics of SARS-CoV-2, evaluated key drivers of virus importation and transmission, and assessed whether major changes in human movement (i.e., because of lockdown and transport of migrant workers) shaped virus transmission dynamics in Gujarat. Our study demonstrates the limitations of current datasets and highlights the need for greater investment in virus genomic surveillance and collection of human mobility data to support comprehensive investigations into the origins and dissemination of future viral outbreaks.

Methods

Sample Collection, Library Preparation, Sequencing, and Data Analysis
During April–July 2020, we generated 434 SARS-CoV-2 genome sequences for investigation. We collected nasopharyngeal/oropharyngeal swab specimens from COVID-19–positive persons after obtaining informed consent and ethics approval. We transported samples and processed them for sequencing as described (13). In brief, we used the Ion AmpliSeq SARS-CoV-2 Research Panel and the Ion AmpliSeq Library Kit Plus (both from Thermo Fisher Scientific) for the library preparation. We performed sequencing on the Ion Torrent S5Plus system (Thermo Fisher Scientific) by using a 530 chip with 400-bp chemistry. We used a reference-based genome assembly, as described by Joshi et al. (13), to obtain whole-genome sequences. In brief, we used PRINSEQ-lite version 0.20.4 (14) for trimming and quality filtering. We mapped high-quality reads against a SARS-CoV-2 reference genome (GenBank accession no. NC_045512) by using CLC Genomics Workbench V 12.0 (QIAGEN, https://www.qiagen.com) to obtain consensus genomes.

Epidemiologic Analysis

We obtained COVID-19 case data from a crowdsourced initiative in India (https://www.covid19india.org and https://api.covid19india.org). These data were curated by volunteers from different data sources, such as state press bulletins and the Ministry of Health and Family Welfare of the Government of Gujarat. The source code is available in the GitHub repository (https://github.com/covid19india/api).

We estimated the instantaneous effective reproduction number at time $t$, $R_t$ (median and 95% equal-tailed Bayesian credible intervals), by using the Epidemic Filter method (15). This approach applies optimal recursive smoothing techniques to minimize the mean squared error in inferring $R_t$ from the incidence of cases under a renewal transmission model. For all analyses, we assumed that the SARS-CoV-2 generation time distribution is well approximated by the serial interval distribution from (16), and we applied a weekly averaging filter to daily incidence data to
reduce weekend effects and inconsistent reporting, which probably corrupts the incidence time series.

**Identifying Transmission Lineages**

On the basis of lineages detected in the Gujarat SARS-CoV-2 dataset, we collated a representative global dataset comprising 10,000 genome sequences sampled evenly by week and country. To maximize our power to detect within-India dissemination, we added all sequences from India on GISAID (https://www.gisaid.org) as of November 5, 2020, to this dataset, for a final dataset size of 12,180 sequences (including samples from Gujarat). We followed a similar pipeline to that outlined in du Plessis et al. (I) and used Thorney-Beast (https://beast.community/thorney_beast) to estimate the posterior molecular clock tree. In brief, we estimated a maximum-likelihood tree by using iqtree (https://www.iqtree.org), and a Juke-Cantor substitution model with Wuhan/WH04/2020 as an outgroup. Branch lengths in this tree were scaled and rounded to represent the expected number of mutations. We then estimated the posterior tree distribution under a Skygrid coalescent prior (17) and a strict molecular clock model with a fixed rate (0.00075 substitutions/site/year). We executed 10 chains of 400 million steps, logging every 7.2 million steps, removing the first 40 million steps as burn-in.

We used a 3-state asymmetric discrete trait analysis (DTA) model implemented in BEAST version 1.10 (18). We used this analysis to infer the ancestral node locations (Gujarat, India, or global) by using an empirical tree distribution comprising 500 time-calibrated trees sampled from the posterior tree distributions estimated previously from ThorneyBeast.

To identify transmission lineages, we followed the method described by du Plessis et al. (I). In brief, starting from a randomly drawn Gujarat node in the maximum clade credibility (MCC) tree, we initiated a depth-first search, continuing until a non-Gujarat node was encountered or there were no additional nodes left to explore. The subtree visited during the search represents a transmission lineage or a singleton (if only 1 node was visited). We repeated this approach iteratively until all Gujarat nodes in the MCC tree had been visited. Using this approach, we identified 7 transmission lineages with >10 sequences, which we selected for further analysis.

**Drivers of Virus Transmission**

To investigate the drivers of virus transmission, we focused on the 7 largest transmission lineages identified in the global dataset. Each dataset ranged from 11 to 75 sequences. We collated data on key predictors (population size and density, number of cases, geographic distance) at the district level. For each transmission lineage dataset, we used a separate exponential coalescent prior, while sharing a SRD06 substitutional model (19), a strict molecular clock model with a fixed rate (0.001 substitutions/site/year), and a generalized linear phylogeographic model (20). We executed 8 chains of 50 million steps (logged every 5,000 steps), then combined and thinned the chains by a factor of 10 after discarding the first 10% as burn-in.

**Results**

Analysis of the instantaneous reproduction number over time (R) of the epidemic indicated that the 3 most populous districts (Ahmedabad, Surat, and Vadodara) (Figure 1, panel A) showed rapid epidemic growth from late March through mid-April (Figure 1, panel C). However, after this initial period of growth, we observed a sharp decrease in R, for all 3 major cities (Figure 1, panel C). The initial growth period in March and April occurred during the period of national lockdown. This finding might be a consequence of a time lag between the number of reported cases and the epidemiologic effect of the lockdown (which began on March 24, 2020) or might reflect considerable population movements during the early phase of the lockdown (e.g., resulting from persons returning to their home states or districts).

After the lockdown ended on June 1, 2020 (Figure 1), we observed slower epidemic growth in Ahmedabad, indicating that restriction in human mobility had a notable effect on disrupting chains of transmission. In contrast, in other districts, including Surat and Vadodara, from first week in June, the number of cases started to increase rapidly. Although Ahmedabad, Surat, and Vadodara recorded their first cases on March 19–20, 2020, Ahmedabad accounted for most confirmed cases during the first wave (Figure 1, panel B). This pattern is consistent with faster epidemic progression in Ahmedabad, possibly driven by higher frequency of virus importation compared with other districts, which have comparatively weaker international links because of lack of airports or greatly decreased passenger flow, and differences in population density and population connectedness.

To determine the source of virus importations in Gujarat, we characterized the lineage dynamics of SARS-CoV-2 in the region by using phylogenetic analyses. Specifically, we combined the 434 genome sequences generated from Gujarat with a representative subset of global genetic diversity of SARS-CoV-2 sampled over a similar period. We detected 39 (95% highest posterior density [HPD] 33–44) distinct
Gujarat transmission lineages (defined as comprising ≥2 Gujarat genomes that belong to an ancestral lineage that originates from outside Gujarat), comprising 360 sequences, and 74 (95% HPD 63–83) singletons (genomes that could not be allocated to a Gujarat transmission lineage) across 2,000 posterior trees (Figure 2, panel A).

We identified 113 virus importations into Gujarat during the first wave. Consistent with previous analyses (1), larger transmission lineages were associated with longer duration times (the time between the oldest and most recently sampled genome within a transmission lineage), and smaller transmission lineages were associated with shorter duration times (Figure 2, panel A). This observation is further corroborated by the strong negative exponential relationship between transmission lineage size and the time to most recent common ancestor (tMRCA) (Figure 2, panel B). The mean tMRCA of transmission lineages was on April 17, 2020 (SD 35.8 days), and 75% of transmission lineages had a tMRCA from March 28 through May 7, 2020, which coincides with the period when the epidemic was increasing exponentially.

To determine likely sources of virus importation into Gujarat, we performed a 3-location discrete trait phylogeographic analysis (with location states of Gujarat, India [excluding Gujarat], and global [excluding India]). Despite having a border with other states with a high burden of disease (e.g., Maharashtra), our results suggest that virus importations into Gujarat had been driven primarily by international travel. However, the relatively low frequency of virus genome sampling across India is likely to mask importations from other states in India. Over the study period 3,092 SARS-CoV-2 genomes available from India were suitable for phylogenetic analysis (high-coverage and with complete temporal information), and only 930 were from neighboring states, predominantly from Maharashtra.

Next, to evaluate the spatial dynamics of SARS-CoV-2 within Gujarat, we undertook a more detailed phylogeographic analysis to evaluate predictors of virus lineage dissemination by using a generalized linear model. To ensure the results reflect the specific dynamics of SARS-CoV-2 in Gujarat, we included only those sequences from the 7 largest Gujarat transmission lineages (Figure 3, panel B), which contained 11–75 sequences. All 7 lineages were associated with virus importation from outside India and had a mean tMRCA that occurred close to the start of or before the national lockdown. Four lineages were composed mainly of genome sequences sampled from Ahmedabad, 2 were associated with Vadodara or Surat, and 1 was associated with the Aravalli District. The main predictors we tested

**Figure 2.** Size, duration, and importation of severe acute respiratory syndrome coronavirus 2 transmission lineages, Gujarat, India. A) Tree map summarizing the 113 detected transmission lineages by size. Colors indicate the duration of persistence of the lineage, and areas indicate the size of the transmission lineages. Lineage duration corresponds to time between the lineage’s oldest and most recently sampled genomes. B) Strong log–linear relationship between size and mean tMRCA of each transmission lineages. Gray shading indicates time of testing; dashed line indicates slope. C) Breakdown of virus importations into Gujarat from other states in India or other countries. The number of location state transitions were estimated by using a robust counting approach (21) and a 3-location discrete trait phylogeographic analysis. tMRCA, time to most recent common ancestor.
were case counts, population size, and population density at both origin and destination locations and the road distance between districts (Figure 3, panel A).

Overall, our findings indicate that viral lineages moved more intensely between districts that were geographically closer (Bayes factor >100) (Figure 3, panel C) and predominantly from districts that had higher population density than districts that had lower population density (Bayes factor 40) (Figure 3, panel C). Together with the epidemiologic data (Figure 1, panel C), these results suggest that viral lineage movement was concentrated in exports from urban centers, which had higher caseloads and population densities, to nearby districts, initially from Ahmedabad but also at later stages from Surat and Vadodara. These movements contributed to the spread of SARS-CoV-2 within Gujarat.

Although the global phylogeographic analysis suggested that importations of transmission lineages to Gujarat were associated mainly with international travel, phylogenetic analysis of the 2 largest Gujarat transmission lineages (Figure 4) provided evidence that these lineages were subsequently exported globally and to other states in India. Clusters (≥2) of sequences from Canada, Oman, Karnataka, and Odisha were detected nested within DTA_MCC_4 (Figure 4, panel A), suggesting that this transmission lineage might have seeded outbreaks in these locations. Furthermore, in DTA_MCC_5, we detected clusters of sequences from Bangladesh and Telangana, as well as singletons from the United States, the United Arab Emirates, Hong Kong, and France. Several of these non-Gujarat clusters and singletons are on long branches, which indicates that we cannot exclude the

**Figure 3.** Determinants of SARS-CoV-2 lineage spread, Gujarat, India. A) Choropleth maps of key predictors (left, no. cases; middle, population size; right, population density, persons per square kilometer) that were evaluated in the phylogeographic generalized linear model analysis along with geographic distance. B) tMRCA and sample distribution of the 7 largest transmission lineages. For each lineage, circles correspond to the estimated lineage tMRCA, and horizontal bars indicate the 95% highest posterior density interval of the tMRCA. Box indicates date range of the samples from Gujarat for each lineage. Total number of samples and duration of each lineage are shown on the right. C) Predictors of SARS-CoV-2 lineage movement in Gujarat on the basis of 20 sampled districts. The contribution of each predictor is indicated by the mean coefficient value (points) and 95% highest posterior density interval (horizontal bars). tMRCA, time to most recent common ancestor. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
possibility of intermediate locations in viral exportation. The mean TMRCAs of the 2 transmission lineages suggested probable importation before the first case in Gujarat was reported on March 19, 2020. However, given that the 95% HPD intervals overlapped with the start of the national lockdown (Figure 3, panel B) and low genomic sampling, these observations could reflect either multiple importations of closely related viruses from outside Gujarat (either another state in India or another country in which COVID-19 was circulating in early February or March) or a single importation into Gujarat before March 19, 2020, followed by cryptic community transmission. It is difficult to ascertain which scenario is more probable without additional data for human mobility and air travel, which is not available for the region.

Discussion
Using epidemiologic data and virus whole-genome sequences, we investigated the spread, importation, and lineage dynamics of the first wave of SARS-CoV-2 in Gujarat, India. Initially, the epidemic was concentrated mainly in the most populous district, Ahmedabad, which accounted for more than half of reported cases. Epidemiologic analysis showed that $R_t$ decreased across all districts after the national lockdown was announced. However, despite the slowdown in epidemic growth, the number of cases continued to increase across Gujarat after restrictions were gradually lifted on May 31, 2020, particularly in other populous districts, such as Surat and Vadodara. Phylogenetic analyses suggest that virus importations into Gujarat were comparatively higher than virus exportation from Gujarat and that virus importations were predominantly associated with international travel.

Virus lineage importations into Gujarat were associated mostly with global travel and visibly higher than either virus lineage importations from within India or viral exportations from Gujarat to other states in India, suggesting that mass transportation of migrant workers within and from Gujarat was not a major driver of virus transmission in Gujarat. However, the comparatively low genomic surveillance across India indicates that our study probably underestimated the number and rate of within-country virus movements. Nevertheless, our phylogeographic analysis identified geographic proximity between locations as a key driver of virus transmission in Gujarat, which was similarly observed for the Alpha variant in the United Kingdom (7). This finding suggests that disease control strategies should consider spatial context of SARS-CoV-2 spread (e.g., interventions should not only focus on populations with high disease prevalence but also be expanded to include geographically proximate populations to limit onward transmission). Particularly, more

Figure 4. Timing of exportation of severe acute respiratory syndrome coronavirus 2 from Gujarat, India. Maximum clade credibility trees are for the 2 largest transmission lineages identified in this study: A) DTA_MCC_4; B) DTA_MCC_5. UAE, United Arab Emirates.
coordinated, responsive approaches at the local level
could prevent traveling waves of infections without
the need for national lockdowns (22). This strategy
would probably require an improved understanding
of multiscale mobility patterns across India and else-
where to better respond to such epidemics (23).

As noted for the United Kingdom, virus importa-
tion is expected to occur earlier than the estimated tMRCA
of a transmission lineage (0–10 days) (1). How-
ever, because of a lack of detailed data on population
movements into Gujarat, we were not able to estimate
importation dates for the Gujarat transmission lineages.
Nonetheless, the tMRCA of transmission lineages serve
as upper bounds on the importation events (i.e., impor-
tation must occur before the tMRCA). The earliest trans-
mission lineages (based on tMRCA) were associated
with importation from outside India, although because
of the relatively small number of genomes from Gujarat,
we could not evaluate changes in the dynamics of virus
importation before and after the lockdown. However,
similar to findings from previous studies (1), our results
showed transmission lineages with earlier tMRCA tended
to be larger and have longer duration times.

Our analyses indicated that the Gujarat epidemic
during the first wave was associated with >100 virus
introductions. Given that this estimate was obtained
from a relatively small number of sampled genomes,
the true number is likely to be much larger. The faster
progression of the epidemic in Ahmedabad compared
with that in other districts strongly suggests this dis-
tric was the epicenter of the first wave, probably en-
abled by a combination of higher inflow of internation-
al travelers, population density, and connectedness
to the rest of Gujarat. Ahmedabad hosts the busiest
airport in the state and is a major destination for in-
testate movement in Gujarat. Because incidence kept
increasing after the gradual lifting of restrictions, sug-
gesting that some transmission chains persisted dur-
ing the lockdown and resumed growth once it ended,
the focus of transmission started to shift to other dis-
tricts, such as Surat and Vadodara, perhaps because of
regional differences in human mobility and behavior
(e.g., less movement or greater caution in Ahmedabad
compared with other districts) (12). However, because
of the limited temporal range of the sampled genomes,
we could not test this hypothesis phylogenetically.
Nevertheless, rapid advancement of epidemics in ur-
banized regions, followed by later movement into
less populous regions, has been commonly observed
elsewhere (24–26), including during the influenza
A(H1N1) virus pandemic in 2009 (27). This finding
strongly suggests that to reduce disease transmission,
interventions should be implemented rapidly and
robustly in major urban centers (e.g., as demonstrated

Although our study period preceded the detection,
emergence, and international spread of the Delta
variant (Pango lineage B.1.617.2) in 2021, the findings
about SARS-CoV-2 transmission dynamics from our
study offer insight into how the Delta variant arose
and spread within India and subsequently worldwide.
We show the role of international connectedness and
intraregional demographics in shaping virus lineage
dynamics in India, and we highlight the limitations of
investigating virus movement and origins because of
comparatively low genomic surveillance in this region.
All of these factors will need to be taken into consider-
ation as part of evaluating the emergence and epidemi-
ology of SARS-CoV-2 variants.

All data used in this study were collated from public
databases and are freely available. Regional case data,
SARS-CoV-2 genome sequences, and analysis files used
in this study are available (https://github.com/jnarag/
covid-gujarat).

This study was supported by the Wellcome Trust (grant
220414/Z/20/Z). S.C.H. was supported by the Wellcome
Trust (grant 220414/Z/20/Z). Sequencing work at the
Gujarat Biotechnology Research Centre was supported by
Department of Science and Technology, Government of
supported by the UK Research and Innovations Global
Challenges Research Fund UK Research and Innovation
One Health Poultry Hub (grant BB/S011269/1), 1 of 12
interdisciplinary research hubs supported by the UK
Government Grand Challenge Research Fund I
nterdisciplinary Research Hub Initiative.

J.R., S.C.H., G.F., M.J., and C.J. designed the study; J.R.,
L.d.P., J.T.M., K.V.P., J.T., D.K., A.P., and R.P. performed
the analysis; J.R. and L.d.P. visualized the study; J.R.,
L.d.P., and J.T.M. designed the methods; J.R. wrote
the original draft of the manuscript; and M.J., C.J, G.F., and
O.G.P. acquired funding. All coauthors reviewed and
edited the manuscript.

About the Author
Dr. Raghwani is a computational virologist at the
University of Oxford, Oxford, UK. Her primary research
interests are the evolutionary and ecologic dynamics of
emerging RNA viruses in natural populations.

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Reassessing Reported Deaths and Estimated Infection Attack Rate during the First 6 Months of the COVID-19 Epidemic, Delhi, India

Margarita Pons-Salort, Jacob John, Oliver J. Watson, Nicholas F. Brazeau, Robert Verity, Gagandeep Kang, Nicholas C. Grassly

India reported >10 million coronavirus disease (COVID-19) cases and 149,000 deaths in 2020. To reassess reported deaths and estimate incidence rates during the first 6 months of the epidemic, we used a severe acute respiratory syndrome coronavirus 2 transmission model fit to data from 3 serosurveys in Delhi and time-series documentation of reported deaths. We estimated 48.7% (95% credible interval 22.1%–76.8%) cumulative infection in the population through the end of September 2020. Using an age-adjusted overall infection fatality ratio based on age-specific estimates from mostly high-income countries, we estimated that just 15.0% (95% credible interval 9.3%–34.0%) of COVID-19 deaths had been reported, indicating either substantial underreporting or lower age-specific infection-fatality ratios in India than in high-income countries. Despite the estimated high attack rate, additional epidemic waves occurred in late 2020 and April–May 2021. Future dynamics will depend on the duration of natural and vaccine-induced immunity and their effectiveness against new variants.

India had just under 150,000 reported coronavirus disease (COVID-19) deaths in 2020, fewer per 1 million persons than other countries, such as Spain, France, the United Kingdom, and the United States (https://www.ourworldindata.org). This discrepancy could in part be because of a younger population but also because of incomplete documentation of overall deaths and of deaths with COVID-19 as a cause (1,2). Assessing the extent of underreporting of COVID-19 cases and deaths is essential for estimating actual disease burden and likely future trends in transmission.

Multiple severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) seroprevalence surveys conducted during 2020 in Delhi, one of India’s largest metropolitan areas (20 million residents), offered us an opportunity to assess the completeness of reported COVID-19 deaths and estimate the actual infection attack rate. SARS-CoV-2 transmission in Delhi has led to several waves of infection and death (Figure 1). At the beginning of the epidemic, all SARS-CoV-2 testing relied on reverse transcription PCR (RT-PCR), but after mid-June 2020, use of antigen-based rapid diagnostic tests (Ag-RDTs), which have lower sensitivity, quickly exceeded use of RT-PCR tests (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/4/21-0879-App1.pdf). Three serosurveys conducted in Delhi during 2020 that sampled participants >4 years of age found age- and sex-adjusted seropositivity rates (uncorrected for test sensitivity and specificity) of 22.8% in July, 28.7% in August, and 25.1% in September (Appendix Table 1) (3). The July survey found a difference in seropositivity between residents living inside or outside of slum areas (25.3% vs. 19.2%; p<0.001), but the August survey did not (28.9% vs. 28.8%; p = 0.94), and the September survey did not report this information.

Materials and Methods

Study Overview
We developed a SARS-CoV-2 transmission model to estimate the incidence of infection and changes in the reproduction number (R) after the start of non-pharmaceutical interventions, including lockdowns

Author affiliations: Imperial College London School of Public Health, London, UK (M. Pons-Salort, O.J. Watson, N.F. Brazeau, R. Verity, N.C. Grassly); Christian Medical College, Vellore, India (J. John, G. Kang)

DOI: https://doi.org/10.3201/eid2804.210879
We used Bayesian Markov chain Monte Carlo to fit the model to the 3 seroprevalence surveys and the time-series of reported deaths. We estimated the proportion of COVID-19 deaths reported by comparing reported deaths to the number expected based on the age-adjusted infection-fatality ratio (IFR) we used in the model. We used age-specific IFR estimates based on data from 7 countries in Europe; New York, USA; and Brazil (4) to estimate a median age-adjusted 0.39% IFR (95% prediction interval 0.21%-0.85%) for Delhi; median age-adjusted IFR in high-income countries with older populations, such as the United Kingdom, was ≈1%, based on data through June 2020 (5,6). The age-adjusted IFR for Delhi that we used was very similar to the 0.39% obtained using early data from China (6) and 0.40% from a meta-analysis based on data from advanced economies (as defined by membership in the Organization for Economic Cooperation and Development [https://www.oecd.org]) (7).

Epidemiologic and Demographic Data
We obtained data on the number of confirmed SARS-CoV-2 cases and deaths reported daily in Delhi beginning March 14, 2020, from COVID19India (8), a volunteer-driven, crowdsourced initiative that collates data from several sources, including the Ministry of Health and Family Welfare. Cases and deaths that occurred before March 14 were reported as cumulative numbers. Because we did not know specifically when these pre–March 14 cases and deaths occurred, we did not use these data for parameter inference. For our model, we used data from the 3 serosurveys conducted in Delhi (3) on dates of sample collection, number of samples tested, seropositivity rate found, and reported estimates of sensitivity and specificity of the assay used in each of the three serosurveys (Appendix Table 1). We used projections of the 2021 population in Delhi from the National Commission on Population (9) and stratified the population by 10-year age groups.

Transmission Model
To model SARS-CoV-2 transmission, we used a susceptible-exposed-infected-recovered (SEIR) deterministic transmission model (Appendix Figure 4). We did not stratify the population by age for the transmission parameters, assuming random mixing by
age, meaning that epidemic growth was equivalent in all age groups in the model. We did not account for births or deaths from causes other than COVID-19 because of the model’s short timeframe.

Because epidemic growth rate is determined by the reproductive number and the generation time, \( T_c \) (i.e., time interval between infection times of an infector-infectee pair) \( (10) \), we fixed the generation time to \( T_c = 6.5 \text{ days} \) on the basis of previous observations \( (11) \) and estimated the reproduction number. We split the generation time into the mean durations of the preinfectious \( (d_e = 1/\omega) \) and infectious \( (d_i = 1/\gamma) \) periods, so that \( T_c = d_e + d_i \) \( (10) \); we fixed \( d_e \) and \( d_i \) using information on the duration of the incubation period (i.e., time between infection and onset of symptoms) and the fact that infectiousness starts \( \approx 1 \text{ day before symptoms start} \) \( (12-14) \). Given an \( \approx 5.5\text{-day} \) incubation (i.e., presymptomatic) period \( (15,16) \), to give the correct generation time, we assumed a mean duration of the preinfectious period of \( d_e = 5.5 - 1.0 = 4.5 \text{ days} \) and a mean duration of the infectious period of \( d_i = 6.5 - 4.5 = 2 \text{ days} \).

**Disease Progression and Death Model**

We modeled disease progression and death after infection independent of the transmission process (Appendix Figure 4). Because the model has been used for other purposes, it also included transitions to hospitalizations, but these were not relevant for our work and did not affect the results. We used the 5.5-day mean incubation period and a peaked distribution modeled with an Erlang distribution with shape parameter 6 \( (15) \). We assumed that one third of infections were asymptomatic, although there is high variability in the observed proportion of asymptomatic infections across studies \( (17-19) \).

We separately tracked the proportion of total infections leading to hospitalization and those leading to death; those hospitalized who eventually died were represented in both groups. We age-adjusted the proportion of infections leading to hospitalization with versus without critical care using demographics from Delhi and age-stratified estimates from China \( (6) \). That is, we computed a weighted average of the age-stratified estimates, assigning weights by the share of the corresponding age classes. We based the proportion of infections leading to death on estimates of age-stratified IFR \( (4) \) applied to the population of Delhi.

We set average time from symptom onset to hospitalization as 5.8 days, consistent with observations in China \( (20) \). For hospitalization without critical care, we assumed a mean 9.8-day stay; if critical care was required, we assumed 9.8 days in critical care, followed by 3.3 days recovery outside of critical care, based on early estimates from the United Kingdom \( (21) \). The average time from symptom onset to death was \( \approx 16 \text{ days} \) \( (6) \). Using these estimates, we assumed a 10-day mean for time between hospitalization and death. These values might differ for India, but no domestic data were available at that time.

**Parameter Inference**

We fitted the transmission model to both the seroprevalence data and reported daily COVID-19 deaths (Appendix). We allowed the reproduction number to change at 5 different time points corresponding to changes in interventions (Appendix Table 2). Denoting the basic reproduction number during the first infection period (i.e., before any changes) as \( R_0 \), the reproduction number after \( i \) number of changes as \( R_i \) \( (i \text{ in periods 1-5}) \), we conducted parameterization of \( R_i \) as \( R_i = R_0 \times (1 + r_1) \times ... \times (1 + r_5) \), where \( r_1, ..., r_5 \) measured the relative change in the reproduction number from one period to the previous one.

We estimated \( R_0 \) and the subsequent changes at each time point, \( r_1, ..., r_5 \), the initial number of infected \( E(0) + I(0) \), the reporting, \( \theta \), and overdispersion of deaths, \( k \). We assumed February 19, 2020 \( (28 \text{ days before the first 10 cases were reported}) \), as the starting time \( (t_0) \) for the simulations and estimated the number of infected persons at that time point, \( [E(0) + I(0)] \). To prevent parameter estimates being biased by the earliest phase of the epidemic, when underreporting of deaths might have been greatest, we computed likelihood using data collected from March 29, 2020, when the first COVID-19 death was reported, through September 30, 2020, the end of the 6-month study period.

We could not estimate a change in transmission at the first time point \( (r_1) \), corresponding to the start of the lockdown on March 25, because no deaths were reported during March 15–28; we therefore assumed \( r_1 = 0 \). We assumed May 4, when the first lockdown relaxations were introduced, as the next time point for change in the reproduction number \( (r_2) \). Therefore, estimates of the reproduction number during February 19–May 4, 2020, from the beginning of the simulations through \( r_2 \) implicitly accounted for any effects of the lockdown during that time. Because \( R_0 \) was highly correlated with the initial number of infected, we estimated the total number of infections just before \( r_2 \) and back-calculated the initial number of infected persons using a simple exponential growth model to define the relationship between \( R_0 \) and the epidemic growth rate for a SEIR model \( (9,22) \).
We performed 100,000 iterations using Markov chain Monte Carlo in the lazymcmc software package (23) and uniform prior distributions to estimate model parameters; we ran 4 chains with different starting values to check convergence. We performed all analyses using R version 4.0.2 (24).

**Results**

Our model fit the data well for both the death time-series (Figure 2, panel A) and seroprevalence survey data (Figure 2, panel B), except for the last serosurvey, in which we estimated an increase in seropositivity from the previous survey, instead of a slight decrease. This difference might have been because the observation model did not account for waning antibodies and the possibility of seroreversion. However, the third serosurvey used a different testing kit, which might also have contributed to this difference. We estimated that the first peak in infection incidence was reached on May 31, at a median of 294,930 (95% credible interval [CrI] 143,271–440,702) new infections per day (Appendix Figure 5). Incidence at the second peak, reached on September 17, was lower, at a median of 79,032 (95% CrI 40,484–109,140) new infections per day. Assuming that changes in transmission occurred beginning at the times of each change in interventions and accounting for the reduction in susceptible persons, we estimated that the effective reproduction number, $R_{eff}$, increased with the first relaxation of the lockdown introduced May 4 (beginning of phase 3); in June and July, during the first 2 reopening phases, $R_{eff}$ was <1; in August, $R_{eff}$ then increased again to >1 (Figure 3, panel A), resulting in a median infection attack rate of 48.7% (95% CrI 22.1%–76.8%) by the end of September. After that, Delhi experienced a large third wave of cases and deaths (Figure 1), suggesting that even with approximately half the population having been infected, the herd immunity threshold had not yet been reached at that time. Of interest, a serosurvey conducted in January 2021 found a sex- and age-adjusted seroprevalence of 56.1%, probably indicating a steep increase in the cumulative number of infections, reflecting the effects of this third wave of transmission.

Using a 0.39% age-adjusted IFR, we estimated reported deaths to be 15.0% (95% CrI 9.3%–34.0%) of actual deaths (Figure 4; Appendix Figure 6). Repeating the analysis using an age-adjusted IFR of 0.21%, corresponding to the lower bound of the 95% prediction interval for IFR based mostly on age-specific high-income country (HIC) data (4), increased the proportion of reported deaths to 28% (95% CrI 18–59%) of actual deaths (Figure 4).

On the basis of infection incidence determined using our model, we also estimated the probability of detecting COVID-19 cases over time by comparing the number of reported cases to the estimated incidence of symptomatic infections (Figure 5, panel A). The probability of detecting infection quickly increased over the last weeks of March, fluctuated until mid-June, then remained relatively consistent through the end of September; a median of 7.1% of all symptomatic infections was detected during July 1–September 30, 2020 (Figure 5, panel B).

**Discussion**

The low proportion of reported deaths relative to actual deaths we found is consistent with findings from other cities in India, where seroprevalence surveys suggested substantially greater exposure to infection than predicted on the basis of reported COVID-19 deaths. For example, comparing seroprevalence during the first half of July 2020 in Mumbai (25) with cumulative deaths at that time suggested that only 21% of deaths were reported (Appendix Table 3). Similarly, a large-scale prospective, active-surveillance study conducted in the district of Madurai, Tamil Nadu, India, during the first wave of COVID-19 in summer...
2020 found that only 11.0% of deaths were reported, compared with expected deaths based on IFR estimates from other settings (26). This high level of underreporting might reflect incomplete or delayed reporting of deaths and a failure to report COVID-19 as a suspected or confirmed cause of death, particularly in the absence of a SARS-CoV-2 test result.

The extent of underreporting might also reflect our use of an age-specific IFR for India derived from mostly high-income countries (HICs). Age-specific IFR may be lower in India for several reasons. First, the prevalence of underlying medical conditions that increase the risk for severe COVID-19 after infection is somewhat lower in India than in the countries that informed the age-specific IFR estimates for our model (Appendix Figure 7) (27). However, correcting the Delhi IFR to account for the lower prevalence of underlying conditions only marginally reduced the age-adjusted IFR (≤0.02%). Second, a recent study that analyzed COVID-19 deaths from Mumbai and Karnataka by age found that IFR rose less steeply with age than in HICs (R. Cai et al., unpub. data, https://doi.org/10.1101/2021.01.05.21249264). Third, differences in immunity reflecting exposure to a greater number of pathogens, including related coronaviruses, or simply lower frailty among those surviving to older ages in India compared with HICs could theoretically reduce the IFR in older groups, although data supporting these hypotheses are lacking (28; B. Chatterjee et al., unpub. data, https://doi.org/10.1101/2020.07.31.20165696).

If the IFR in India was actually higher than in HICs, the proportion of deaths reported would be even lower. For example, using a 0.85% age-adjusted IFR, corresponding to the upper bound of the 95% prediction interval for IFR based on age-specific HIC data (4), would decrease the reported deaths to only 7% (95% CrI 4%–21%) of actual deaths (Figure 4).

The first limitation of our study is that we did not structure the transmission model by age, and therefore, did not account for differences in attack rates between age groups. However, age-structured models have predicted relatively homogeneous infection attack rates across age for India (29), consistent with age-stratified seroprevalence estimates (3), suggesting that any bias in our results from age-specific patterns of mixing and potentially lower attack rates in more susceptible older age groups is likely to be limited. Second, we assumed that the proportion of deaths

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**Figure 4.** Estimated reporting of coronavirus disease deaths, Delhi, India, March 15–September 30, 2020. Violin plots show the posterior distribution of the estimate of death reporting for 3 different values for the assumed age-adjusted IFR, using age-stratified estimates of IFR based on data from mostly high-income countries; 0.21% corresponds to lower bound, 0.39% to the median, and 0.85% to the upper bounds of the IFR based on data documented elsewhere (4). Horizontal black lines indicate the median values of the posterior distributions. IFR, infection-fatality ratio.

---

**Figure 5.** Reported cases of symptomatic severe acute respiratory syndrome coronavirus 2 infections and estimated actual number of cases, Delhi, India, March 15–September 30, 2020. A) Daily number of newly reported cases (black dots) and 50% (dark orange shading) and 95% (light orange shading) credible intervals (CrIs) for the estimated actual incidence of symptomatic infections, assuming that 2/3 infections are symptomatic. B) Estimated detection probability per symptomatic infection per day: 50% (dark orange) and 95% (light orange) CrIs.
reported was constant over the study period, but it might have changed over time. Therefore, our estimate of reported deaths represents an average over the study period. Finally, we used an age-specific IFR based on estimates mostly from HICs and explored sensitivity based on this assumption, including using data on underlying conditions in India. Further analyses using data from cohort studies or demographic surveillance specific to India will help to refine these estimates of IFR and the exact degree of underreporting of death.

The total number of new COVID-19 cases declined in India between mid-September 2020 and mid-February 2021 but started increasing again after that, and in April–May 2021, India experienced a devastating nationwide second epidemic wave bigger than the first one. How much of the country’s population had already been infected before the second nationwide wave and whether the herd immunity threshold had been reached were unclear (30). Seroprevalence surveys conducted in major cities, such as Mumbai, reported seroprevalence rates >50% in slum areas for the first half of July 2020 (25), suggesting that infection spread very quickly over the first few months of the epidemic in certain pockets. However, seroprevalence rates <20% in nonslum areas showed that the epidemic was spatially highly heterogeneous. Understanding what brought the number of cases down after the first wave in different parts of India and how to interpret the serosurvey results related to building population immunity are key to understanding and predicting the dynamics of subsequent waves of COVID-19.

The SARS-CoV-2 Delta variant emerged in Maharashtra in late 2020 and spread across India during the first few months of 2021, replacing other variants. In vitro data characterizing the Delta variant found that it was less sensitive to serum neutralizing antibodies from persons previously infected with other variants and that it also had higher replication efficiency (31). These findings suggest that the predominance of the Delta variant in the upsurge of SARS-CoV-2 cases seen in India during April and May 2021 resulted from either immune escape in previously infected persons, increased transmissibility, or both. These mechanisms, together with possible waning of population immunity over time, likely explain the increase in SARS-CoV-2 cases in Delhi, despite the high attack rate that we estimated in September 2020 and the high reported seroprevalence (≥56% for both) in the round 5 (January 2021) and 6 (April 2021) cross-sectional serosurveys. Analysis of epidemiologic data is needed to disentangle how these mechanisms contributed to the second nationwide epidemic wave.

In conclusion, our analysis found reported COVID-19 deaths in Delhi during the first 6 months of the pandemic were well below the number of actual deaths. Our estimate of underreporting of deaths might reflect incomplete or delayed documentation or failure to report COVID-19 as a cause of death but may also reflect our use of an age-specific IFR for India, derived from mostly HIC data.

Acknowledgments

We thank Nimalan Arinaminpathy for insightful comments on the manuscript, Marc Baguelin for helpful discussions on parameter inference, and James A. Hay for help using the lazymcmc R package.

M.P.-S. is a Sir Henry Dale fellow, a program jointly funded by the Wellcome Trust and the Royal Society (grant number 216427/Z/19/Z). M.P.-S., O.J.W., N.F.B., R.V., and N.C.G. acknowledge funding from the MRC Centre for Global Infectious Disease Analysis (MR/R015600/1), which is jointly funded by the U.K. Medical Research Council (MRC) and U.K. Foreign, Commonwealth & Development Office (FCDO), under the MRC/FCDO Concordat agreement, and is also part of the EDCTP2 programme supported by the European Union. M.P.-S., O.J.W., N.F.B., R.V., and N.C.G. also acknowledge funding from Community Jameel.

About the Author

Dr. Pons-Salort is a Sir Henry Dale fellow at Imperial College London. She uses statistical and mathematical models to study the dynamics of infectious diseases. Her recent work has focused on the epidemiology of COVID-19 and enteroviruses.

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Address for correspondence. Margarita Pons-Salort, Imperial College London, Norfolk Place, W2 1PG London, UK; email: m.pons-salort@imperial.ac.uk
Unique Clinical, Immune, and Genetic Signature in Patients with Borrelial Meningoradiculoneuritis


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Release date: March 22, 2022; Expiration date: March 22, 2023

Learning Objectives

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

• Assess the clinical features of specific clinical presentations of Lyme neuroborreliosis, based on a clinical and genetic study
• Evaluate the immune and genetic features, including the TLR1-1805GG single nucleotide polymorphism, of specific clinical presentations of Lyme neuroborreliosis, based on a clinical and genetic study
• Determine the clinical implications of the role of innate and adaptive immune responses and the TLR1-1805GG single nucleotide polymorphism in specific clinical presentations of Lyme neuroborreliosis, based on a clinical and genetic study

CME Editor

Dana C. Dolan, BS, Technical Writer/Editor, Emerging Infectious Diseases. Disclosure: Dana C. Dolan, BS, has disclosed no relevant financial relationships.

CME Author

Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. Disclosure: Laurie Barclay, MD, has disclosed the following relevant financial relationships: owned stock, stock options, or bonds for AbbVie (former).

Authors

Katarina Ogrinc, MD, PhD; Sergio A. Hernández, MS; Miša Korva, PhD; Petra Bogovič, MD, PhD; Tereza Rojko, MD, PhD; Lara Lusa, PhD; Geena Chiumento, MS; Franc Strle, MD, PhD; and Klemen Strle, PhD.

Author affiliations: University Medical Center Ljubljana, Ljubljana, Slovenia (K. Ogrinc, P. Bogovič, T. Rojko, F. Strle); New York State Department of Health, Albany, New York, USA (S.A. Hernández, K. Strle); University of Ljubljana, Ljubljana (M. Korva, L. Lusa); Massachusetts Department of Public Health, Boston, Massachusetts, USA (G. Chiumento)

DOI: https://doi.org/10.3201/eid2804.211831

1Part of the research reported here was presented at the 31st European Congress of Clinical Microbiology & Infectious Diseases (online), July 9–12, 2021.
2These first authors contributed equally to this article.
3These senior authors contributed equally to this article.
Lyme neuroborreliosis (LNB) in Europe may manifest with painful meningo-radiculoneuritis (also known as Banwarth syndrome) or lymphocytic meningitis with or without cranial neuritis (peripheral facial palsy). We assessed host immune responses and the prevalence of TLR1 (toll-like receptor 1)-1805GG polymorphism to gain insights into the pathophysiology of these conditions. Regardless of LNB manifestation, most mediators associated with innate and adaptive immune responses were concentrated in cerebrospinal fluid; serum levels were unremarkable. When stratified by specific clinical manifestation, patients with meningo-radiculoneuritis had higher levels of B-cell chemottractants CXC motif chemokine ligand (CXCL) 12 and CXCL13 and T-cell–associated mediators CXCL9, CXCL10, and interleukin 17, compared with those without radicular pain. Moreover, these patients had a higher frequency of TLR1–1805GG polymorphism and more constitutional symptoms. These findings demonstrate that meningo-radiculoneuritis is a distinct clinical entity with unique immune and genetic pathophysiology, providing new considerations for the study of LNB and borreial meningoradiculitis.

Lyme borreliosis, which is caused by several species of the *Borrelia burgdorferi* sensu lato complex, is the most common vectorborne disease in the Northern Hemisphere. The first sign of infection is usually an expanding skin lesion, erythema migrans (EM), that appears within days or weeks at the site of the tick bite. If untreated, the infection may disseminate to involve other organ systems, including the central nervous system (CNS), heart, or joints (1,2).

Lyme neuroborreliosis (LNB) is the most common extracutaneous manifestation of Lyme borreliosis in Europe and the second most common such manifestation in North America (1,2). In Europe, LNB in adult patients typically begins as painful meningoradiculoneuritis (also known as Garin-Bujadoux-Bannwarth syndrome or Banwarth syndrome), which is rare in the United States, or with cranial neuritis characterized by peripheral facial palsy (PFP) or lymphocytic meningitis (1–4). In addition, a subset of patients experience EM and symptoms indicative of LNB (e.g., headache, vertigo, concentration disturbance), but do not fulfill the European diagnostic criteria for LNB, namely cerebrospinal fluid (CSF) pleocytosis and intrathecal borrelial antibody production (5,6). This subset poses a particular diagnostic and clinical challenge because the etiology of illness is often unclear.

Pathogenic mechanisms that account for different clinical manifestations of LNB are not well understood. Clinical heterogeneity in Lyme borreliosis has been largely attributed to differences in the infecting *B. burgdorferi* s.l. species (1,2). In Europe, LNB is caused predominantly by *B. garinii* (and closely related *B. bavariensis*) but only rarely by *B. afzelii*, which is associated with skin manifestations, or *B. burgdorferi* sensu stricto, which is highly arthritogenic, implying a strong species-specific imprinting of clinically evidenced disease (1,2,7,8). However, although *B. garinii* has particular proclivity for the central nervous system (CNS) and undoubtedly serves as the initial trigger of LNB, Lyme borreliae do not express any known toxins that cause disease. Rather, the host immune response, which is shaped by various *Borrelia* species (9–13), is thought to be a key determinant of the clinical signs and symptoms of Lyme borreliosis, including LNB.

In addition to infection with a particular *Borrelia* species or strain, the immune responses are further augmented by alterations in the human genome. We have previously demonstrated that a single-nucleotide polymorphism (SNP; 1805GG) in the host toll-like receptor 1 (TLR1), a key pathogen sensing receptor for *borrelia*, is associated with more symptomatic early Lyme borreliosis and a greater frequency of postantibiotic, chronic inflammatory Lyme arthritis (12,14). This TLR1–1805GG SNP is associated with severe disease presumably because it enables maladaptive immune responses; EM patients with the TLR1–1805GG SNP had substantially higher levels of pro-inflammatory cytokines and chemokines in blood compared with those without the SNP. Moreover, in patients with Lyme arthritis, this SNP was associated with exceptionally high levels of the interferon (IFN) γ and IFN-γ–associated mediators CXC motif chemokine ligand (CXCL) 9 and CXCL10 in synovial fluid, supporting the predominant cellular and inflammatory T-helper 1 (Th1) responses in joints in these patients. Thus, host immune response, which is shaped by both microbial and host genetics, is a key driver of the clinical heterogeneity in Lyme borreliosis in general and probably in LNB, although this has not been tested directly.

Several studies in Europe and the United States have characterized host immune responses in patients with LNB in an effort to gain insights into pathogenesis and to identify biomarkers to aid in diagnosis (15–18). These studies demonstrated the activation of a broad-ranging innate and adaptive immune response in patients with LNB. In particular, LNB patients have marked CSF levels of CXCL13, a B-cell chemoattractant, compared with patients with other neurologic conditions (15–19), prompting the use of CXCL13 as an adjunct diagnostic biomarker of LNB (19–26). However, the role of host immunity in the pathogenesis of clinically distinct LNB manifestations and the possibility that these outcomes are associated with specific host genetic predisposition has not been explored.
We investigated the role of innate and adaptive immune responses and the TLR1–1805GG SNP in specific clinical manifestations of LNB. Our findings demonstrate that borrelial meningoradiculoneuritis is a distinct clinical entity with unique immune and genetic pathophysiology and offer new insights into the pathogenesis and diagnosis of this condition.

Methods

Patient Selection
This study is based on 61 adult patients who met the modified European diagnostic criteria for LNB (5), defined by the presence of symptoms suggestive of neurologic involvement; CSF lymphocytic pleocytosis; demonstration of borrelial infection with intrathecal synthesis of borrelia antibodies (IT-Bb-Abs), isolation of borreliae from CSF; or presence of EM. For comparison, we included 59 patients with signs and symptoms indicative of LNB but without pleocytosis and termed them suspected LNB. All patients were seen at the Lyme borreliosis outpatient clinic by the same clinical team in the Department of Infectious Diseases, University Medical Center Ljubljana, Slovenia, during 2006–2013. The Medical Ethics Committee of the Ministry of Health, Republic of Slovenia (no. 35/08/06), and the Institutional Review Board of the New York Department of Health (IRB protocol no. 20-013) approved the study.

Clinical Evaluation
We collected clinical and demographic information using a structured questionnaire as described previously (3). In addition to their general medical history, patients were asked about tick bites, EM, and new onset or worsening of symptoms. We also assessed signs indicative of neurologic involvement (meningeal signs, PFP, other cranial nerve palsies, sensory or motor deficits, tremor) and the presence of EM (27). We subsequently grouped patients into 3 categories according to clinical and diagnostic criteria: 1) painful meningoradiculoneuritis accompanied by CSF pleocytosis; 2) LNB without radicular pain, consisting of PFP with CSF pleocytosis or lymphocytic meningitis without cranial neuritis (PFP/meningitis); and 3) suspected LNB with EM and neurologic symptoms suggestive of LNB, but normal CSF cell counts (3,6). Serum and CSF samples were obtained at first visit, usually before antimicrobial therapy, and stored at −80°C for subsequent analyses.

Laboratory Evaluation
We defined CSF pleocytosis as a leukocyte count >5 × 10⁶ cells/L. We determined IgM and IgG levels against B. burgdorferi s.l. in serum and CSF with indirect chemiluminescence immunoassay using recombinant OspC and VlsE antigens (LIAISON; Diasorin, https://www.diasorin.com). We assessed intrathecal synthesis of Borrelia IgG as described previously (28); antibody index values >1.4 were indicative of IT-Abs against B. burgdorferi s.l. We cultivated Borrelia in CSF as reported previously (29–31).

Cytokine and Chemokine Determinations
We assessed the levels of 17 mediators associated with innate (CC motif chemokine ligand [CCL] 2, CCL3, CCL4, interleukin [IL] 8, IL-10, tumor necrosis factor, IFN-α), and adaptive T-cell (T₃,1, IFN-γ, CXCL9, CXCL10; Tₜ,17, IL-17A, IL-21, IL-23; CCL19, CCL21) or B-cell (CXCL12, CXCL13) immune responses. We tested for these levels in matched patient serum and CSF samples using bead-based multiplex assays (EMD Millipore, https://www.emdmillipore.com) coupled with the Luminex-200 Analyzer (Luminex, https://www.luminexcorp.com). We quantified each inflammatory mediator in all samples in one complete experiment to minimize sample freeze-thaw and assay variation. We then stratified cytokine and chemokine levels according to clinical and laboratory findings in the same patients.

TLR1–1805 Genotyping
We determined TLR1–1805 genotypes (TT, TG, GG; G denotes the SNP) using PCR amplification followed by restriction fragment length polymorphism (RFLP) as described previously (12). In brief, we extracted total DNA from patients’ blood (QIAamp DNA Blood Mini Kit; QIAGEN, https://www.qiagen.com) and amplified it using specific primers: forward: 5’-CCAGGGGACAATCCATTCCAA-3’; reverse: 5’CCCCAGAAAGAATCGTGCCCA-3’ (IDT). Each reaction contained 0.4 µM forward/reverse primers, 2 µL DNA (≥50 ng), and 2XDreamTaq PCR Master Mix (ThermoFisher Scientific, https://www.thermofisher.com). PCR amplicons were digested with PsI1 enzyme (New England BioLabs, https://www.neb.com) and visualized by electrophoresis (1.5% agarose gel, GelRed nucleic acid stain). We determined TLR1–1805 genotype in 65 patients with confirmed LNB (meningoradiculoneuritis, n = 40; LNB without radicularneuritis [termed PFP/meningitis], n = 25) and for comparison in 71 EM patients without neurologic involvement. Because of the requirement for consent for genetic studies, these 65 LNB patients represent a different cohort from the 61 patients assessed in the rest of the study. In addition, we ascertained TLR1–1805 genotype in the general European population.
(n = 503) by datamining the Ensembl genome browser 104 (https://useast.ensembl.org/index.html).

Statistical Analyses
We evaluated categorical variables (presence/absence of symptoms or TLR1-SNP) using Fisher exact test. We assessed quantitative variables (number of symptoms, cytokine levels) using Mann-Whitney nonparametric rank-sum test in Prism version 9.1.1 (https://www.graphpad.com). We applied Spearman correlation for cytokine associations. We adjusted for multiple comparisons using the Benjamini-Hochberg test; we set the false discovery rate (FDR) to 0.05 and considered p<0.05 statistically significant.

Results

Clinical Characteristics According to LNB Presentation
Of the 120 patients in this study, 19 had meningoradiculoneuritis, 42 had LNB without radiculoneuritis (PFP/meningitis; 35 PFP, 7 lymphocytic meningitis), and 59 were classified as suspected LNB because they had clinical signs and symptoms indicative of LNB (EM with neurologic symptoms) but lacked CSF pleocytosis (5) (Table 1). Seventy-two participants were female (60%) and 48 male (40%); median age was 51 years. When stratified by specific clinical presentation, patients with meningoradiculoneuritis were more likely to have had an EM skin lesion during the course of disease than LNB patients without radicular pain (68% vs. 24%; p = 0.004). Meningoradiculoneuritis patients had more symptoms than LNB patients without radiculitis (median 6 vs. 3 symptoms; p<0.001) and their symptoms lasted longer (30 days vs. 10 days; p<0.001). Meningoradiculoneuritis patients had more symptoms (median 6 vs. 4 symptoms; p = 0.01) and symptoms of longer duration (30 vs. 16 days; p<0.03) than patients with suspected LNB. Of the 13 constitutional symptoms we assessed, patients with meningoradiculoneuritis had a higher frequency of 8 specific symptoms, 4 of which reached statistical significance (Figure 1). The most common symptoms were sleep disturbance, headache, and fatigue.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MR, n = 19</th>
<th>PFP/meningitis, n = 42</th>
<th>Suspected LNB, n = 59</th>
<th>p value†</th>
<th>MR vs. PFP‡</th>
<th>MR vs. sLNB</th>
<th>PFP† vs. sLNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>51 (21–73)</td>
<td>52 (15–81)</td>
<td>51 (25–83)</td>
<td>0.4</td>
<td>0.8</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>9 (47)</td>
<td>17 (40)</td>
<td>45 (76)</td>
<td>0.8</td>
<td>0.02</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>10 (53)</td>
<td>25 (60)</td>
<td>14 (24)</td>
<td>0.9</td>
<td>0.7</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>No. tick bites/year</td>
<td>1 (0–17)</td>
<td>2 (0–20)</td>
<td>2 (0–30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or recent EM</td>
<td>13 (68)</td>
<td>10 (24)</td>
<td>59 (100)§</td>
<td>0.004</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Solitary EM</td>
<td>12 (63)</td>
<td>6 (14)</td>
<td>53 (90)</td>
<td>0.0007</td>
<td>0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Multiple EM</td>
<td>1 (5)</td>
<td>4 (10)</td>
<td>6 (10)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>EM duration, d</td>
<td>35 (6–128)</td>
<td>11 (3–60)</td>
<td>21 (1–240)</td>
<td>0.2</td>
<td>0.9</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>No. symptoms/patient</td>
<td>6 (1–12)</td>
<td>3 (1–10)</td>
<td>4 (1–13)</td>
<td>&lt;0.0001</td>
<td>0.01</td>
<td>0.001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Duration of symptoms, d</td>
<td>30 (7–75)</td>
<td>10 (3–365)</td>
<td>16 (2–270)</td>
<td>0.001</td>
<td>0.2</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Radicular pain</td>
<td>19 (100%)</td>
<td>0 (0%)</td>
<td>9 (15%)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>Peripheral facial palsy</td>
<td>9 (47%)</td>
<td>35 (83%)</td>
<td>0 (0%)</td>
<td>0.01</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>CSF findings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleocytosis</td>
<td>19 (100)</td>
<td>42 (100)</td>
<td>0</td>
<td>1.0</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Leukocyte count, × 10⁶ cells/L</td>
<td>160 (15–886)</td>
<td>56 (6–1579)</td>
<td>1 (0–4)</td>
<td>0.005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte count no. × 10⁶ cells/L</td>
<td>144 (15–811)</td>
<td>47 (4–1477)</td>
<td>1 (0–4)</td>
<td>0.005</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Immune Responses in Serum and CSF
In 61 patients with confirmed LNB (meningoradiculoneuritis or PFP/meningitis), most cytokines and chemokines associated with adaptive T-cell and B cell immune responses were more highly concentrated in CSF, the site of disease, than in serum (Table 2). This finding was particularly apparent for IFN-γ-inducible chemokines CXCL9 (median 347 vs. 170 pg/mL; p = 0.02) and CXCL10 (median 4,139 vs. 143 pg/mL; p<0.001) which recruit CD4+ T cells, as well as B-cell chemoattractants CXCL12 (median 1,541 vs. 820 pg/mL; p<0.001) and

Table 1. Clinical characteristics of patients with confirmed or suspected Lyme neuroborreliosis, Ljubljana, Slovenia, 2006–2013

†Values are no. (%) or median (range). CSF, cerebrospinal fluid; EM, erythema migrans; LNB, Lyme neuroborreliosis; MR, meningoradiculoneuritis; PFP, peripheral facial palsy; sLNB, suspected Lyme neuroborreliosis.
‡PFP group includes 42 LNB patients without radicular pain (35 patients with PFP and 7 with lymphocytic meningitis).
§Presence of EM was a criterion for suspected LNB.
¶Borrelia burgdorferi sensu lato positive culture result in CSF.

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CXCL13 (median 352 vs. 9 pg/mL; \( p < 0.001 \)) which recruit B cells to site of infection. In contrast, mediators associated with innate and Th17 immune responses were present at lower overall concentrations and often at similar levels in serum and CSF (Table 2).

### Immune Responses According to LNB Presentation

To determine if patients with different clinical presentations of LNB carry distinct immune signatures, we stratified cytokine and chemokine levels in CSF into 3 groups: meningoradiculoneuritis (n = 19), PFP/meningitis (n = 42), and suspected LNB (n = 59). Heat map analyses of global immune responses revealed that patients with meningoradiculoneuritis have the highest levels of most mediators tested; those with PFP/meningitis have intermediate levels, and those with suspected LNB have the lowest levels (Figure 2, panel A). We obtained similar results using unguided hierarchical clustering (Figure 2, panel B). Patients with meningoradiculoneuritis clustered primarily with the highest cytokine and chemokine levels and largest CSF leukocyte counts, whereas those with suspected LNB clustered with the lowest cytokine and chemokine levels and CSF leukocyte counts. Patients with PFP/meningitis were more interspersed. We observed these trends for cytokines and chemokines associated with both innate and adaptive immune responses.

We identified through statistical analyses 8 cytokines and chemokines with significant differences between groups (Figure 3), including mediators associated with innate (IL-8, IL-10, CCL3), and adaptive T-cell (CXCL9, CXCL10, IL-17) and B-cell (CXCL12, CXCL13) immune responses. We observed the highest levels and most pronounced differences

### Table 2. Immune mediators in cerebrospinal fluid and serum in 61 confirmed patients with Lyme neuroborreliosis, Ljubljana, Slovenia, 2006–2013*

<table>
<thead>
<tr>
<th>Cytokine or chemokine</th>
<th>CSF, pg/mL</th>
<th>Serum, pg/mL</th>
<th>( p ) value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Innate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>39 (8–289)</td>
<td>8 (0–473)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.3 (0–54)</td>
<td>0.3 (0–28)</td>
<td>0.0001</td>
</tr>
<tr>
<td>TNF</td>
<td>5 (1–54)</td>
<td>7 (0–170)</td>
<td>0.3</td>
</tr>
<tr>
<td>IFN-α</td>
<td>88 (0–406)</td>
<td>22 (0–205)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCL2</td>
<td>343 (71–10,000)</td>
<td>213 (7–516)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCL3</td>
<td>5 (0.3–16)</td>
<td>1 (0.3–227)</td>
<td>0.07</td>
</tr>
<tr>
<td>CCL4</td>
<td>5 (0.3–139)</td>
<td>20 (0.3–221)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>T-cell adaptive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>2 (0–77)</td>
<td>1 (0–25)</td>
<td>0.3</td>
</tr>
<tr>
<td>CXCL9</td>
<td>347 (4–6833)</td>
<td>170 (2–3,508)</td>
<td>0.02</td>
</tr>
<tr>
<td>CXCL10</td>
<td>4,139 (156–46,025)</td>
<td>B (3–932)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-17</td>
<td>3 (0–8)</td>
<td>2 (0–25)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-23</td>
<td>2 (2–33)</td>
<td>2 (2–4,949)</td>
<td>0.01</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5 (0–22)</td>
<td>3 (0–22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCL19</td>
<td>B (19–768)</td>
<td>28 (2–143)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCL21</td>
<td>17 (17–241)</td>
<td>17 (17–541)</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>B-cell adaptive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL12</td>
<td>1,541 (89–5,518)</td>
<td>820 (89–4184)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CXCL13</td>
<td>352 (1–76,869)</td>
<td>9 (1–86)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Values are median (range). CCL, CC motif chemokine ligand; CSF, cerebrospinal fluid; CXCL, CXC motif chemokine ligand; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.
†Statistical analyses were performed using Mann-Whitney nonparametric rank sum test. Bold type indicates statistically significant \( p \) values after Benjamini-Hochberg correction for multiple comparisons (false discovery rate FDR = 0.05).
between groups for B-cell chemoattractants CXCL12 (median values for meningoradiculoneuritis groups, 2,568 pg/mL; for PFP/meningitis, 1,170 pg/mL; for suspected LNB, 819 pg/mL) and CXCL13 (meningo-radicular neuritis, 1,000 pg/mL; PFP/meningitis, 53 pg/mL; suspected LNB, 1 pg/mL) and for T-cell chemoattractants CXCL9 (meningo-radicular neuritis, 680 pg/mL; PFP/meningitis, 252 pg/mL; suspected LNB 15 pg/mL) and CXCL10 (meningo-radicular neuritis, 5,159 pg/mL; PFP/meningitis, 4,087 pg/mL; suspected LNB, 236 pg/mL). These mediators correlated strongly with leukocyte counts in CSF (p<0.001) (Figure 4), implying the predominance of CSF-localized T- and B-cell responses in meningoradiculoneuritis.

**Frequency of TLR1–1805GG Polymorphism in LNB**

We previously demonstrated that the TLR1–1805GG SNP is associated with excessive immune responses,

![Figure 2](https://example.com/image2.png)

**Figure 2.** Heat map analysis of inflammatory mediators in CSF stratified by clinical presentation of LNB among patients with Lyme neuroborreliosis treated in Ljubljana, Slovenia, during 2006–2013. A) Levels of inflammatory mediators stratified by LNB manifestation. B) Hierarchical clustering analysis (Euclidian distance) of inflammatory mediators and CSF leukocyte counts. We assessed the levels of 17 cytokines and chemokines associated with innate and adaptive (T- and B-cell) responses in CSF of patients with meningoradiculoneuritis, PFP/meningitis, or suspected LNB. Each column represents an individual patient, with the corresponding mediators along the different rows. Heat map was constructed using Morpheus software (https://software.broadinstitute.org/morpheus). In the case of IFN-α, 3 values were exceptionally low (at lower limit of detection), resulting in disproportionately intense red coloring of other values. CSF, cerebrospinal fluid; CXCL, CXC motif chemokine ligand; GM-CSF, granulocyte macrophage colony–stimulating factor; IL, interleukin; IFN, interferon; LNB, Lyme neuroborreliosis; MIP, macrophage inflammatory protein; PFP, peripheral facial palsy; TNF, tumor necrosis factor.
more symptomatic early infection, and a heightened risk for chronic inflammatory (antibiotic-refractory) Lyme arthritis, thereby linking host genetic variation with dysregulated immunity and severe Lyme borreliosis (I2). Herein, we extended these findings to LNB (Figures 5, 6). As reported previously (I2), 49% of the general population of Europe had both copies of the SNP (GG); distribution was similar in female and male patients. Similarly, 51% of patients with EM had both copies of the SNP, whereas the frequency was slightly higher in patients with LNB (57%), but these differences were not statistically significant (Figure 5). However, when we subcategorized LNB patients by clinical presentation, patients with meningo-radiculoneuritis had a significantly higher frequency of TLR1–1805GG SNP compared to patients with PFP/meningitis (68% vs. 40%; odds ratio 3.1; p = 0.04) (Figure 6). Consent for genetic studies was not obtained from patients with suspected LNB, and they were not included in these analyses.

Discussion
CNS involvement is a common manifestation of Lyme borreliosis in Europe. Although distinct manifestations of LNB are well described clinically, the reasons for these differences are not well understood. Herein, we demonstrated that LNB patients with meningo-radiculoneuritis have a different immune signature than LNB patients without radiculoneuritis. Although both LNB groups (patients with meningo-radiculoneuritis and PFP/meningitis) have broadly elevated innate and adaptive inflammatory immune response that the suspected LNB group does not, we observed the most dramatic differences for cytokines and chemokines associated with adaptive T- and B-cell immune responses in CSF. In particular, patients with meningo-radiculoneuritis had the highest CSF levels of B-cell chemoattractants CXCL12 and CXCL13, as well as T-cell chemoattractants CXCL9 and CXCL10, and a potent Th17 cytokine, IL-17. These inflammatory mediators
were often concentrated 10- to 50-fold in CSF, the site of the disease, and correlated strongly with lymphocyte counts \((p<0.001)\), implying a role in CNS inflammation and disease pathogenesis. In contrast, cytokine and chemokine levels did not correlate with symptom duration, implying that greater immune responses are not caused simply by the longer disease duration in patients with meningoradiculoneuritis. Rather, we postulate that these site-specific T- and B-cell responses likely play a direct role in the pathophysiology of meningoradiculoneuritis and the higher prevalence of systemic symptoms in this condition. T- and B-cell mediators, namely IL-17 and CXCL13, have been implicated in disease pathogenesis in a recent study of patients with LNB or suspected LNB in Sweden (32), although that study did not compare patients with distinct manifestations of LNB. In contrast to findings for CSF, we observed low levels of most of the immune mediators in serum in LNB patients that were comparable to the levels in healthy persons (data not shown). Collectively, these findings support the role of sitespecific T- and B-cell responses in the pathogenesis of borrelial meningoradiculoneuritis.

Because the clinical manifestation of LNB is largely attributable to host immune responses, immune mediators in CSF offer attractive targets as biomarkers for diagnosis as well as markers of disease activity and resolution. LNB diagnosis is limited because...
CSF pleocytosis and clinical signs and symptoms of disease overlap with other neurologic conditions and are thus not unique to LNB. Moreover, borrelia-IT Abs, although highly specific, take weeks to develop and thus offer limited sensitivity during early infection. In addition, once elevated, antibodies against borrelia can persist for years and may not be able to distinguish previous from current infection. Immune mediators such as CXCL13, which are presumably elevated before demonstratable cellular influx necessary for CSF pleocytosis and borrelia-IT-Ab-S, and decrease dramatically after antimicrobial therapy, appear to address these diagnostic limitations (20). Indeed, CXCL13 has been proposed as an adjunct diagnostic marker for LNB (19–26). However, studies in the United States and Europe have demonstrated that the diagnostic utility of CXCL13 is primarily limited to patients with definitive LNB who already meet the standard diagnostic criteria (CSF pleocytosis, Borrelia IT-Bb-Ab-S) (18,33,34). Our findings underscore this point by demonstrating that CSF levels of CXCL13 are below the proposed 162 pg/mL diagnostic cutoff (26) in most patients with suspected LNB (median CXCL13 levels 1 pg/mL) and even in many patients with PFP/meningitis (median CXCL13 levels 53 pg/mL). Our results suggest that CXCL13 diagnostic utility may be constrained to patients with meningoradiculoneuritis, a clinically identifiable condition. Nevertheless, because of their role in pathogenesis, immune mediators remain attractive targets as biomarkers, and new cytokines with greater discriminatory power than CXCL13 have been proposed in LNB (16). As we have demonstrated, diagnostic suitability of these factors will require detailed studies of LNB patients with a range of well-defined clinical manifestations.

In addition to elevated immune responses, patients with borrelial meningoradiculoneuritis had a greater frequency and longer duration of constitutional symptoms than patients without radiculoneuritis. The most prevalent symptoms were sleep disturbance/insomnia, followed by fatigue, concentration disturbance, and memory disturbance, all of which could be attributed to sleeplessness resulting from pronounced radicular pain. However, these patients also had several other symptoms, such as low back pain, arthralgia, myalgia, and paresthesia, that are likely to have other causes, unrelated to sleeplessness. In contrast, patients with suspected LNB more often sought care for nonspecific symptoms such as headache, nausea, malaise, and vertigo. Thus, meningoradiculoneuritis is a distinct clinical entity distinguished not only by radicular pain but also by prevalence of several constitutional symptoms.

Alterations in the human genome have been implicated in maladaptive immune responses and severe Lyme borreliosis (9–12). We previously demonstrated that a SNP (1805GG) in TLR1, a major sensor for Borrelia, alters host immune responses to infection and thereby the clinical course and outcome of Lyme borreliosis (12). This SNP results in a transversion of thymine to guanine (1805T>1805G), causing an amino acid exchange of isoleucine for serine at position 602 in the transmembrane domain of the receptor, a constrained region likely to affect function. Indeed, the SNP results in decreased expression of TLR1 on cell surface and diminished downstream cytokine signaling in response to Pam3CSK4, a specific TLR1/2 agonist (35–37). Paradoxically, in response to B. burgdorferi infection, this SNP is associated with excessive immune responses with marked levels of IFN-γ and IFN-γ-inducible chemokines CXCL9 and CXCL10 in serum of patients with EM and even higher levels in joint fluid of those with postantibiotic (postinfectious) Lyme arthritis. This excessive inflammatory immune response was associated with highly symptomatic early infection in EM patients and with an elevated risk for postantibiotic chronic inflammatory Lyme arthritis (12). In this study, we extended these concepts to patients with various manifestations of LNB. We show that patients with meningoradiculoneuritis have a significantly higher frequency of the TLR1–1805GG SNP (OR = 3.1; p = 0.03) and more symptoms.
(p<0.001) than those with other LNB manifestations. Moreover, patients with meningoradiculoneuritis also have heightened T-cell (namely Th1) and B-cell immune responses, consistent with the role of this SNP in excessive Th1 inflammation and more symptomatic disease in patients with EM or Lyme arthritis.

A limitation of this work is that, because genetic studies require separate consent, the TLR1–1805GG analyses were performed in a different cohort of patients than those in whom the immune responses were tested; thus, we could not directly correlate the SNP genotype with the immune phenotype. Moreover, we based our study on a relatively small number of patients in each group. However, we should point out that this is an initial analysis of these concepts in LNB, and although small, these patient cohorts are well defined and are among the largest available. Finally, although the immune responses were elevated in CSF in patients with meningoradiculoneuritis as a group compared with those with PFP/meningitis, the levels of immune mediators varied in individual patients, and thus not every patient with radiculitis had higher CSF immune responses than did every patient with PFP/meningitis. Despite these limitations, these data support a link between TLR1–1805GG SNP, maladaptive Th1 immunity, and disadvantageous clinical outcomes in several manifestations of Lyme borreliosis, including LNB. An effort is now underway to recruit additional patients to validate these genetic and immunologic results.

In summary, our study demonstrates that meningoradiculoneuritis is a distinct clinical entity with unique immune and genetic pathophysiology. In future studies, we hope to establish the functional link between the TLR1–1805GG SNP, excessive site-specific T- and B-cell immune responses in CSF, and painful meningoradiculoneuritis. The findings from this study provide new considerations for the study of patients with meningoradiculoneuritis and LNB in general; further studies may help to improve accuracy of laboratory diagnosis.

Acknowledgments
We thank Graham Willsey for help with TLR1 genotyping.

This work was supported by the US National Institutes of Health (NIAID no. R21AI144916, and NIAMS no. K01AR062098) and the Massachusetts General Hospital Executive Committee on Research Interim Support Fund to K.S.; and by the Slovenian Research Agency (P3-0296, J3-1744, and J3-8195) to F.S. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or other funding agencies. Funding agencies were not involved in the design, conduct, or interpretation of the findings.

Author contributions: K.S., F.S., and K.O. designed the study. K.S., S.H., and M.K. conducted the experiments and the data analyses for this study. K.O., F.S., P.B., and T.R. examined patients with Lyme neuroborreliosis, made diagnoses, and collected clinical samples and clinical information. S.H., K.S., and K.O. wrote the manuscript. S.H., G.C., M.K., K.S., and L.L. performed the statistical analyses, contributed to generation of tables, and helped with the interpretation of the data. All authors reviewed and approved the manuscript.

About the Author
Dr. Ogrinc is an MD/PhD clinical researcher in the Department of Infectious Diseases at the University Medical Center Ljubljana, Slovenia. Her primary research interest is the clinical evaluation of patients with Lyme borreliosis with a focus on acrodermatitis chronica atrophicans and Lyme neuroborreliosis.

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Address for correspondence: Klemen Strle, Wadsworth Center, New York State Department of Health, 120 New Scotland Ave, Albany, NY 12201-2002, USA; email: Klemen.Strle@health.ny.gov
West Nile virus (WNV) is one of the most widespread arboviruses because of the translocation of the virus by migratory birds (1–3). Since its initial detection in Uganda in 1937 (4), WNV has spread throughout much of Africa (5,6), Europe (7), West Asia (8), Oceania (9), and the Americas (10,11). The enzootic cycle is maintained between birds (the reservoirs) and mainly mosquitoes (the vectors), whereas humans are accidental dead-end hosts (Figure 1). Other mammals such as horses, dogs, camels, and goats are also accidental dead-end hosts for WNV (12). The role of animal monitoring in the surveillance of WNV outbreaks is critical because detecting the virus in animals can help to anticipate its transmission to humans. Moreover, domestic animals such as horses (13) and poultry (14) have been used as sentinels for human cases. Furthermore, wild birds such as crows have been used to define the geographic and temporal limits of WNV in North America (15).

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Because WNV does not produce specific clinical symptoms, WNV infection can be mistaken for other infectious diseases and toxins (16). WNV outbreaks can easily be attributed to other arbovirus diseases that are more common and result in greater human illness in an area. For this reason, any evidence regarding the presence of WNV in an area is important to ensure monitoring of the risk for humans contracting the disease caused by WNV. Thus, all WNV reports should serve as suitable input data for pathogeographic analyses (17) aimed at mapping the areas at risk for WNV transmission to humans.

We conducted a bibliographic review of the detection of WNV in animals in Africa. Next, we applied biogeographic methods to create empirical models on the basis of the virus lifecycle to identify zones that are environmentally favorable for the circulation of WNV in Africa. Moreover, the models were used to ascertain the potential risk for transmission of WNV to animals (epizootic processes) and humans (epidemic processes), even in regions where WNV has not yet been detected.

Materials and Methods

Data Sources and Search Strategy

We performed a literature search in the GIDEON database (18) for 48 countries and territories of Africa (Figure 2), using “West Nile fever” and country names as keywords. For countries that had name changes since 1937, when WNV was first described, we also searched for the ancient names or names that they were otherwise known by; for example, Equatorial Guinea (formerly Spanish Guinea), Saharawi Arab Democratic Republic (Western Sahara), and Côte d’Ivoire (Ivory Coast). We excluded the island countries and territories of Africa from this analysis because WNV probably would be enzootic and independent of the annual movements of migratory birds. The size and isolation of some of these island countries and territories would deserve an independent approach to study WNV (19). We complemented
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the reports obtained from GIDEON with articles acquired through an electronic literature search of the Web of Science (https://clarivate.com/webofsciencegroup/solutions/web-of-science), Scopus (https://www.scopus.com), and Google Scholar (https://scholar.google.com) for all countries in Africa, for which we used different combinations of the following keywords: “West Nile virus,” “WNV,” “West Nile Fever,” “WNF,” and the name of each country. The reports and articles obtained provided a selection of geopositioned reports that described the presence of WNV in vectors (principally mosquitoes), reservoirs (i.e., birds) and dead-end hosts (i.e., horses, dogs, and other mammals, excluding humans). To obtain a robust high-resolution data base, we took into account occurrences of WNV only when the reports referred to specific villages, towns, or cities. We used the names of the localities and the contextual information provided in the information sources to determine the latitudinal and longitudinal coordinates, using Google Maps (https://www.google.es/maps), Google Earth (https://www.google.com/intl/es/earth), Geonames (http://www.geonames.org), and Google Search (https://www.google.com).

Analysis
To reduce the excessive weight of the oversampled areas in the analysis and, thus, autocorrelation caused by sampling bias, we projected the occurrences of WNV onto a grid of equal-sized hexagonal units of 7,742 km². We created a total of 3,970 hexagons by using Discrete Global Grids for R (20). If a report of WNV was located within a hexagon, we considered this report to represent a single presence, regardless of the number of records included, whereas we considered the hexagons that did not contain a report of WNV to represent absences. In this way, the hexagons were operational geographic units (OGUs).

We used a set of environmental variables to identify the areas in Africa that were favorable for the presence of the WNV (Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/28/4/21-1103-App1.pdf). We classified variables as anthropic (human-related) (e.g., infrastructure or agriculture) or non-anthropic (e.g., climate and ecosystem). The ecosystem variables comprised land cover and Ramsar sites (i.e., wetlands of international importance for birds, as determined by a 1971 treaty signed in Ramsar, Iran). These variables could influence the enzootic (reservoirs and vectors) and epizootic (animal dead-end hosts) components of the cycle of WNV, or they could be correlated with drivers of the presence of these components. For each OGU, we calculated an average value for every explanatory variable through the Zonal Statistic as Table tool from ArcGIS Desktop 10.7 software (ESRI, https://www.esri.com). We used biogeographic modeling based on fuzzy logic and machine learning algorithms to separately analyze the environmental

Figure 1. Lifecycle of West Nile virus and schematic elaboration of different models (numbered 1–5) for each component of the cycle of models for Africa. Model 1 (reservoir model) identifies favorable areas for the virus presence in reservoir animals. Model 2 (vector model) identifies favorable areas for the virus presence in vector animals. Model 3 (epizootic model) identifies favorable areas for the virus presence in dead-end hosts. Model 4 (enzootic model) is a fuzzy union of the reservoir and vector models, identifying areas favorable for the virus presence in the reservoir or vector animals. Model 5 (potential risk model) is a fuzzy union of the enzootic and the epizootic models, identifying areas with potential for virus spillovers.
characteristics of WNV in every component of its cycle (Figure 1).

We developed 3 biogeographic models. First was the reservoir model (Figure 1), based on the presence of WNV in reservoir animals, which we intended to detect areas that were favorable to birds becoming infected by WNV. Second was the vector model (Figure 1), which was based on the presence of WNV in vector animals and identified areas favorable to WNV detection in mosquitoes. Third was the epizootic model (Figure 1), based on the presence of WNV in nonhuman mammals, which aimed to detect the areas in which environmental conditions could lead to WNV spillover. To address a comprehensive biogeographic approach to WNV in Africa in the context of reservoir–vector relationships, we identified the areas that are favorable for the presence of WNV in reservoirs or vectors. Accordingly, we joined the reservoir and vector models into a single enzootic model (Figure 1) by calculating their fuzzy union (i.e., the maximum favorability value for any of them \(F\)-reservoir model \(\cup\) \(F\)-vector model). Finally, we merged the enzootic and the epizootic models into a WNV potential risk model (Figure 1), which represented the fuzzy set of areas where the environment is favorable for the presence

![Figure 2. Geoposition of West Nile virus reports in reservoirs, vectors, and nonhuman mammal dead-end hosts, Africa.](image-url)
of WNV in reservoirs, vectors, or dead-end hosts. To this end, we performed a fuzzy union of their favorability values (i.e., F-enzootic model $\cup$ F-epizootic model). Working separately with 3 different models (1 for each component of the WNV cycle), instead of creating a single model based on the detection of WNV in any component of the cycle, enabled us to investigate whether the detection of WNV in the various components could be explained by different drivers. Although the presence of WNV in vectors and dead-end hosts indirectly indicates its presence in reservoirs, this presence may occur at various intensities given the intrinsic characteristics of each of the components of the virus cycle.

Figure 3. Cartographic representation of the biogeographic models (numbered 1–5) based on the different West Nile virus lifecycle components for Africa. Model 1 (reservoir model) indicates environmental favorability for the presence of the virus in birds. Model 2 (vector model) indicates environmental favorability for the presence of the virus in vectors. Model 3 (epizootic model) indicates environmental favorability for the presence of the virus in nonhuman mammals. Model 4 (enzootic model) indicates environmental favorability for the presence of the virus in $\geq 1$ component of the enzootic virus cycle. Model 5 (potential risk model) indicates environmental favorability for potential spillover of the virus.
We produced each model by using several steps. To control the multicollinearity among the environmental variables, we calculated pairwise Spearman correlation coefficients between all variables. If 2 variables belonging to the same subtype of variables (Appendix 1 Table 1) showed a correlation >0.8, we deleted the least explanatory variable. Considering only the remaining variables, we addressed a false discovery rate control to limit the increase in the type I error caused by the number of variables analyzed (21). Hence, we arranged the variables in decreasing order according to their relevance in explaining the presence of WNV. We assessed this relevance according to Rao score tests (22). A variable was used in subsequent steps only if its score-test probability was lower than $i^2q/V$ (where $i$ is the position of the variable in the referred order, $q = 0.05$ is the false discovery rate, and $V$ is the total number of remaining variables).

We used all variables that advanced through the previous filters in a multivariate stepwise logistic regression, a commonly used machine learning algorithm (23), that began with a null model that had no explanatory variables included. We then added a variable at each step if the resulting new regression was significantly improved by the new variable. The result of the multivariate logistic regression was a probability value of WNV being present in each OGU according to the environmental characteristics of the OGU. We transformed the probability values of each OGU into favorability values by using the favorability function (24) (Appendix 1). A more detailed discussion of the procedure has been published previously (25).

We evaluated the discrimination and classification capacities of each model. We assessed the model discrimination capacity by using the area under the receiver operating characteristic curve (26). We estimated the classification power by using the value $F = 0.5$ as a classification threshold through sensitivity, specificity, Cohen’s $\kappa$, the correct classification rate (27), and the overprediction and underprediction rates (28). Finally, we compared the performance of the potential risk model to that of an alternative risk model that was based on the use of the entire set of WNV occurrences (i.e., those occurrences reported in birds, mosquitoes, and mammals) as the dependent variable. Considering that WNV outbreaks in Africa are underestimated because of the generic symptomatology (16,29), an effective model should demonstrate high sensitivity and a low underprediction rate to detect potential risk areas. We projected the distribution of WNV by using the geographic information system ArcGIS Desktop 10.7 and performed logistic regressions by using SPSS Statistics 26 (https://www.ibm.com).

Results

Database

Among 328 articles identified during the literature search, we included 71 in the analysis. We excluded the remainder for any of the following reasons: the survey for WNV was negative for this specific virus, the survey for WNV was positive for the virus but the research was conducted in an entire region, or the survey was conducted to a country level without identifying a specific place.

We collected 189 geopositioned localities where the WNV was present: 33 locations where WNV occurred in reservoirs, 48 locations where WNV was detected in vectors, and 108 locations where WNV occurred in dead-end hosts. These localities were included in 83 of the 3,970 OGUs, and they were distributed across 20 countries in Africa. The presence of WNV in reservoirs (Figure 2; Appendix 2, https://wwwnc.cdc.gov/EID/article/28/4/21-1103-App2.xlsx) involved 52 species and 10 orders of birds. The presence of WNV in vectors (Figure 2; Appendix 2) involved 23 mosquito species and 1 tick species (Argas reflexus hermanni). In certain cases, only the genus of the mosquito pool was identified: Culex and Aedes. Finally, the presence of WNV in dead-end hosts (Figure 2; Appendix 2) mostly involved equids and dogs, although WNV was also detected in bats, buffaloes, camels, monkeys, and elephants.

Biogeographic models

The most favorable areas on the continent for WNV-infected birds were located in Northern Africa (specifically Morocco, northern Algeria, Tunisia, and the Nile Delta), West Africa, and southern Africa (Figure 3). Reservoir zones were characterized climatically by high minimum temperatures ($B = -1.16 \times 10^{-3}$), ecosystemically by being close to Ramsar sites ($B = -0.96$), and having vegetation on regularly flood-ed soil ($B = 5.39$) and anthropically by the presence of croplands ($B = 2.78$) and high densities of poultry ($B = 1.00 \times 10^{-3}$) (Table).

The favorable areas for vectors to become infected with the WNV were not unlike those shown by the reservoir model. Nevertheless, the areas with high environmental favorability ($F > 0.8$) were less extensive, whereas the intermediate-favorability zones ($F = 0.2–0.8$) were wider, including the areas around Lake Victoria where WNV was isolated for the first time.
The potential risk model demonstrated an improved mative cartographic output compared with the single and Angola (Figure 3). The model that defined the areas with a potential risk for WNV transmission to dead-end hosts, included all the areas that were environmentally favorable for WNV to be present in birds or arthropods (Figure 3). Favorable areas in the vector model were characterized by their ecosystemic and anthropic conditions: the closeness to Ramsar sites \((B = -0.54)\), and the proximity to railway tracks \((B = -3.00 \times 10^{-3})\) (Table). The epizootic model, derived from a combination of the reservoir and vector models, included all the areas that were environmentally favorable for WNV to be present in birds or arthropods (Figure 3).

According to the epizootic model, the areas most prone to experiencing epizootic outbreaks were geographically similar to those that were highlighted as favorable by the reservoir and vector models (Figure 3). However, the subtle geographic differences between the epizootic model and the others led to the inclusion of a different pool of variables. Specifically, the most important predictors were anthropic variables, such as a high-density human population \((B = 1.10 \times 10^{-3})\), the proximity to railway tracks \((B = -3.00 \times 10^{-4})\), a high density of poultry \((B = 1.20 \times 10^{-3})\), and a high percentage of irrigated crops \((B = 0.04)\). The short distance to the Ramsar sites \((B = -0.68)\) also explained the distribution of the epizootic cases that were reported (Table).

The model that defined the areas with a potential risk for WNV transmission to dead-end hosts, including humans, comprised all areas that were favorable for the presence of WNV in \(\geq 1\) of the components of the cycle of WNV (Figures 1, 3). The risk extended throughout most of the continent, except the desert areas in the Horn of Africa (Sahara and Kalahari) that are far from oases and a strip that runs north to south along central Africa, between Sudan and Angola (Figure 3).

The potential risk model provided a more informative cartographic output compared with the single model that comprised all WNV detections. In addition, the potential risk model demonstrated an improved sensitivity \((0.87)\) and underprediction rate \((0.0042)\) compared with the alternative model (sensitivity \(0.84\), underprediction rate \(0.0045\)) (Appendix 1 Table 2).

**Discussion**

Our review sought to obtain a broad perspective regarding the geographic distribution of WNV throughout the continent of Africa. Previous studies addressed the distribution of WNV in Africa at a country level \((30–32)\) or considered subcontinental contexts such as the Eastern Mediterranean area \((8)\). Nevertheless, our study analyzed the geography of the potential health risks, derived from the distribution of WNV at a fine \(<8,000 \text{ m}^2\) spatial resolution throughout the entire continent, which elucidates the international risk patterns on this continent. Moreover, our study is useful for understanding the patterns of virus expansion in the continent of Africa and the seasonality patterns that occur in Europe \((25)\).

The geopositioning of the locations in Africa where WNV has been detected (at various stages of its lifecycle) has enabled us to assume different considerations to develop a risk model of the WNV for the entire continent. The presence of WNV in mosquitoes and birds enabled us to develop an enzootic model. We identified the environmental drivers that favor the enzootic circulation of WNV and the most favorable for its circulation. However, WNV can also experience spillover events in mammals (epizootic cycle). Knowing the environmental characteristics that promote these spillovers enabled us to identify where the most susceptible areas to virus transmission are that exceed the enzootic cycle. In addition, by considering the favorable areas for virus transmission in the enzootic and epizootic cycles, we created a potential risk map that highlights the areas where WNV is most likely present \((\geq 1)\) components of the WNV.
cycle) and can ultimately lead to spillover to dead-end mammal hosts, including humans.

The variables involved in the distribution of WNV in Africa are associated with the climate, ecosystems, and human activity (Table). However, the proximity of the Ramsar sites contributed to an explanation of the presence of WNV in each of the components of the virus cycle (i.e., reservoirs, vectors, and mammals). In Tunisia, the proximity to the Ramsar sites was important for explaining the occurrence of WNV in horses (30) and humans (31). Given the protection and conservation status of the Ramsar sites, they offer an ideal habitat for sedentary and migratory birds (which can carry WNV) (33) and for mosquitoes (34). Therefore, we are not surprised that their proximity partially explains the detection of WNV in birds, mosquitoes, and mammals. Except for the proximity to the Ramsar sites, the remaining explanatory variables included in the epizootic model were associated with human activity (Table). Most WNV detections outside the enzootic cycle have been observed in domestic animals, such as horses and dogs. Thus, the favorable areas for the presence of WNV in the epizootic cycle may also reveal the risk for spillover to humans and other mammals.

Because cases of WNV are generally underestimated, we aimed to develop a model with high sensitivity and a low underprediction rate so that potential risk areas would not be ignored. Our potential risk model that resulted from the fuzzy union of the enzootic and epizootic models had a higher sensitivity and lower underprediction rate than the alternative model that considered all the occurrences of WNV presences. This approach demonstrated the convenience of a macro-ecologic perspective that integrates all components of the lifecycle of a pathogen to obtain a comprehensive understanding of risks associated with zoonotic diseases.

In the middle of the Sahara Desert, the favorable zones for WNV (Figure 3) correspond to the National Parks of Ahaggar and Tassili n’Ajjer in Algeria and the oases of Kawar and l’Air in Niger, where inhospitable conditions are less extreme than in the rest of the desert. Moreover, in these areas, closer contact probably occurs between avian hosts and mosquitoes around the remaining water sources, favoring the enzootic cycle (35,36).

Applying biogeographic models to zoonotic diseases helps detect areas that pose a risk for disease transmission. However, these models may have certain limitations. The disease reservoirs may have a great dispersal capacity, especially long-distance migratory birds. In our case, we considered the place where the WNV-positive sample was recorded, although the bird could have been infected in other parts of the continent. WNV is a neglected disease; reports on its detection in vectors, reservoirs, and dead-end hosts are limited. The relatively low number of locations in such a large study area may lead to a map that underestimates the potential risk. However, our model highlighted areas with a high risk for WNV in countries where it has not been detected yet, such as Burundi, Lesotho, Eswatini, The Gambia, Guinea-Bissau, Togo, Benin, and Malawi.

The potential risk model could reveal the risk not only to animals but also to humans because it characterizes the environmental conditions in which spillovers occur. Northwestern Morocco is an area where human WNV cases have occurred repeatedly (37,38) and was highlighted as a high-risk area in our model. The same situation occurs in Tunisia (29,37) and along the Nile River in Egypt (39), particularly in the Nile Delta (40). Our model predicted high-risk areas for WNV in the center and the south of Algeria, in isolated areas that correspond to oases. Furthermore, human cases of WNV occurred in Timimoun (in the center) (34,37) and Djelat and Tamanrasset (in the south) (34). In Uganda (41,42) and South Africa, human cases have also been reported, particularly in Pretoria and Johannesburg (43), which were highlighted in our model as the areas with the highest risk.

Recognizing the conditions that favor the onset of WNV would enable us to optimize resources to prevent the disease. For example, the percentage of irrigation areas (Table) is positively correlated with epizootic episodes. Therefore, during the transmission season, resources to address prevention policies should be put in place in agricultural areas that use irrigation systems. Given the role of bird migration in the spread of viruses, including WNV (2), maintaining a broad spatial perspective and an improved understanding regarding the contribution of the movements of hosts in the spread of the disease is important. Knowing the favorable areas for the presence of WNV in its continent of origin may be of great help for disease prevention at an international level. This knowledge may aid in managing the disease from an intercontinental perspective. Our model may help provide improved medical advice to persons traveling to the area, including screening for WNV upon return to the traveler’s native country, because no vaccines are available for use in humans (44,45).

Areas of North Africa are important stopover sites for migratory birds and are areas of high enzootic risk. Because WNV viremia in birds can last
for up to 7 days (46,47), birds could become infected in these areas and arrive in Europe with a viral load high enough to introduce WNV to Southern and Central Europe. Nowadays, WNV is a priority mosquito-borne pathogen that is spreading in Europe (3,25,48,49). Therefore, knowing the favorable zones for WNV in the wintering and breeding areas of migratory birds may lead to an understanding of the evolution of WNV and help to prevent outbreaks in Europe.

Predicting zoonotic disease outbreaks is one of the ultimate challenges for public health management and the primary goal of preventive medicine (17). Therefore, developing WNV risk maps that account for the dynamic biogeography of birds can help prevent the disease or lead to early management responses to reduce the impact of the disease on humans and domestic animals.

J.M.G.C. received a grant from the Ministerio de Educación, Cultura y Deporte Programa de Formación del Profesorado Universitario (grant no. FPU17/02834). A.-R.M. is supported by project UMA18-FEDERJA-276 (Programa Operativo FEDER, Consejería de Economía, Conocimiento, Empresas y Universidad, Junta de Andalucía). J.O. is supported by project CGL2016-76747-R from the Ministerio de Economía, Industria y Competitividad, and FEDER Funds.

About the Author
Mr. Garcia-Carrasco is PhD student currently working at the Department of Animal Biology in the University of Malaga. His primary research interests are focused on zoonoses biogeography, pathogeography, and spatial ecology.

References


In 2009, a novel phlebovirus, Heartland virus (HRTV), was identified as the cause of illness in 2 severely ill patients from Missouri, USA (1), after exposure to lone star ticks (Amblyomma americanum). HRTV, an RNA virus recently reclassified as belonging to the family Phenuiviridae and genus Bandavirus (2), is closely related to severe fever with thrombocytopenia syndrome virus (SFTSV), which is transmitted mainly by longhorned ticks (Haemaphysalis longicornis) and causes a hemorrhagic fever in Southeast and central Asia (3).

The ecology and natural history of HRTV remain largely unknown (4). The virus was isolated from the initial index cases in Missouri and from ticks collected at nearby sites (1,5). Viral RNA has been detected by molecular tools in immature and mature stages of A. americanum ticks from Missouri (5,6), Alabama (7), Illinois (8), Kansas (9), and New York (10). Antibodies reactive to HRTV have been identified in various wildlife species (11–13) that match the geographic distribution of A. americanum ticks in the United States (14), even in those areas where the presence of the tick is scarce. Nonetheless, viremia in a vertebrate species has not been detected (11,13) and attempts to induce viremia in experimental vertebrate hosts have been unsuccessful (15,16).

Since HRTV was identified in 2009, ≈40 additional human cases of HRTV disease have been identified in Missouri, Kansas, Oklahoma, Arkansas, Iowa, Illinois, Tennessee, Indiana, Georgia, and South Carolina (8,17–20). Most of these cases were reported in persons who had underlying conditions, and their illnesses were predominately severe or fatal (4,21). However, seroprevalence studies in wildlife suggest a broader range of distribution of HRTV than those states from which cases of human disease have been reported (13).

A second novel tickborne arbovirus, Bourbon virus (BRBV), was isolated from a fatal human case (22) and from field-collected arthropods (9) in Bourbon County, Kansas, USA, during 2014. BRBV has a negative-sense RNA genome of 6 segments and represents the only member of the genus Thogotovirus that causes human disease in the Western Hemisphere; a limited number of persons have been infected in the midwestern and southern United States. This virus was also linked to A. americanum ticks in Missouri (23) at a lower infection rate than that for HRTV infection. Wildlife seroprevalence studies suggest a wide distribution of BRBV in the southeastern United States (24), but human disease remains a rare event.

Although A. americanum ticks are widely distributed throughout the southeastern United States, only 1 study, in Alabama, has conclusively identified HRTV in lone star ticks in this region (7).
In Georgia, there is serologic evidence of HRTV infection in white-tailed deer (*Odocoileus virginianus*) dating back to 2001. A single human infection from 2005 was confirmed in 2015 (https://www.cdc.gov/heartland-virus/statistics/index.html). Because *A. americanum* ticks represent ticks most frequently associated with human bites in Georgia (25), we examined *A. americanum* ticks for arboviruses, and specifically for HRTV and BRBV, in a select area in Georgia to better assess the risk for human disease in the region and to increase knowledge of the ecology and genomics of these emerging human pathogens.

**Materials and Methods**

**Study Area**
The study area in Georgia was a 64 km² rural landscape located ≈130 km southeast of Atlanta and situated adjacent to the Piedmont National Wildlife Refuge (latitude 33.117934, longitude −83.413621). This area includes parts of Jones, Baldwin, and Putnam Counties in central Georgia and had a cumulative population of 93,180 inhabitants as of 2010 (US Census Bureau, https://www.census.gov/2010census/data). The area is part of the southern Piedmont ecoregion, comprising predominantly deciduous woodlands (Figure 1). The climate is humid subtropical, has a mean annual high temperature of 31°C and low temperature of 13°C, and has mean annual precipitation of 115 cm.

We selected this study area on the basis of data from a seroprevalence evaluation of white-tailed deer for antibodies to HRTV (12) and from its proximity to the only reported case of human HRTV infection (https://www.cdc.gov/heartland-virus/statistics/index.html) (Figure 1). During 2018, we collected samples from 26 different sites around the area to identify *A. americanum* tick productive sites. During 2019, we focused our efforts in the 2 sites that yielded the highest collections during 2018. All sites were favorable, on a visual inspection, for development of the different stages of *A. americanum* ticks (presence of deciduous forest, open access to diverse fauna, and grass cover). The 2 locations sampled during 2019 were a vacant lot (33.155975, −83.450516) and a private property (33.201552, −83.439257) (the collection activity was approved by their owners), which were 5.41 km apart (Figure 1).

**Tick Collection Strategy and Entomologic Identification**
We collected ticks approximately each week during April–October in 2018 and 2019. We collected host-seeking adults and nymphal ticks by using flannel

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Figure 1. Study area for investigation of Heartland virus ecoepidemiology, Georgia, USA, 2019. Map on the left shows locations of seropositive white-tailed deer and 1 human case; inset shows location of study area in Georgia. Map on the right shows *Amblyomma americanum* tick collection sites during 2018 and the 2 sites during 2019. Circle sizes and colors indicate number of ticks collected.
flags and transported them alive to the laboratory, where we identified them microscopically on a chilled table for sex, species, and life stage by using taxonomic keys (26,27).

**Tick Processing**
We surface disinfected live ticks by sequential immersion for 5 min in cold solutions of 70% ethanol, 10.5% sodium hypochlorite, and 3% hydrogen peroxide, and then rinsed them in distilled water (28). We pooled live specimens by species, collection site, and stage in groups of ≤5 adults and ≤25 nymphs. We added 1 mL of BA-1 diluent (1× medium 199 with Hanks balanced salt solution, 0.05 mole/L Tris buffer [pH 7.6], 1% bovine serum albumin, 0.35 g sodium bicarbonate/L, 100 μg/L streptomycin, 1 μg/mL amphotericin B) to each pool (29) before grinding the pools thoroughly by using a 7-mL glass TenBroeck grinder (Fisher Scientific, https://www.fishersci.com) with alundum Bedding material (Fisher Scientific) as an abrasive. We transferred each homogenate to a sterile 2-mL cryotube and stored the tubes at −80°C for future analysis.

**Molecular Detection and Cell Culture Isolation**
We thawed tick homogenates and centrifuged them at 14,000 rpm for 10 min to clarify before proceeding. We extracted total RNA from each homogenate by using a QIAmp RNA Extraction Kit (QIAGEN, https://www.qiagen.com). We performed a quantitative real-time PCR with a final reaction volume of 25 µL and 1 µL of template by using a Quantitect Probe PCR Kit (QIAGEN), with primer-probe set 1, which was designed for the small segment of the HRTV genome, as described by Savage et al. (6) under the following cycling conditions: 50°C for 30 min; 95°C for 10 min; and 45 cycles with 1 cycle consisting of 95°C for 15 s and 60°C for 1 min. We performed BRBV screening in a separate quantitative real-time PCR by using the primer-probe set NPI, as described (9).

We attempted viral isolation in Vero E6 cells with each tick homogenate pool that yielded a positive result by real-time PCR. We inoculated 100 µL of an undiluted sample into a 12-well tissue culture plate and incubated it at 37°C. We monitored cells daily for cytopathic effect; when noted, we removed 140 µL of medium and processed for RNA extraction. We subcultured and monitored cultures with no demonstrable cytopathic effect by day 11 daily for an additional 7 days for cytopathic effect; if no cytopathic effect was noted, we performed a final subculture and monitored for an additional 11 days. We tested RNA extracted from cell culture supernatants (1 µL) by using a real-time PCR specific for HRTV to confirm viral infection.

**HRTV Genome Sequencing and Analysis**

**Infection Prevalence Estimation**
We estimated the HRTV infection rate in ticks by study site and overall. We calculated this rate by using the minimum infection rate (MIR) per 1,000 ticks (31).

**Results**
We collected 2,960 ticks during 10 collections during April–October 2018, comprising 2,265 nymphs and 646 adults of *A. americanum* ticks, 30 adults of *A. maculatum* ticks, 14 adults of *Dermacentor variabilis* ticks, and 5 adults of *Ixodes scapularis* ticks. We collected 6,470 ticks during 10 collections during April–October 2019, comprising 4,853 nymphs and 1,530 adults of *A. americanum* ticks, 74 adults of *D. variabilis* ticks, 3 adults of *I. scapularis* ticks, and 10 adults of *A. maculatum* ticks. We sorted specimens into 283 pools during 2018 and 677 pools during 2019, by species, stage, sex, and collection site (Table 1). We detected 3 *A. americanum* tick HRTV PCR–positive pools: 1 pool of 5 females (pool 23, cycle threshold [Cₜ] 25.9), 1 pool of 5 males (pool 504, *Cₜ* 29.9), and 1 pool of 25 nymphs (pool 26, *Cₜ* 25.6). Two positive pools originated from site 1 and the third pool from site 2 (Figure 1, Table 2).
Table 1. Collected tick species and life cycle stages, Georgia, USA, 2018 and 2019

<table>
<thead>
<tr>
<th>Tick species and stage</th>
<th>2018</th>
<th>2019</th>
<th>2019, site 1</th>
<th>2019, site 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amblyomma americanum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>646</td>
<td>1,530</td>
<td>790</td>
<td>740</td>
</tr>
<tr>
<td>Nymph</td>
<td>2,265</td>
<td>4,853</td>
<td>2,844</td>
<td>2,009</td>
</tr>
<tr>
<td>Total</td>
<td>2,911</td>
<td>6,383</td>
<td>3,634</td>
<td>2,749</td>
</tr>
<tr>
<td>Pools</td>
<td>272</td>
<td>677</td>
<td>339</td>
<td>338</td>
</tr>
<tr>
<td>Amblyomma maculatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>30</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Pools</td>
<td>6</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Dermacentor variabilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>14</td>
<td>74</td>
<td>57</td>
<td>17</td>
</tr>
<tr>
<td>Pools</td>
<td>3</td>
<td>37</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>Ixodes scapularis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Pools</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

We performed virus isolation in Vero E6 cells on aliquots of each real-time PCR–positive homogenate. Pool 26 showed cytopathic effect of Vero E6 cells on day 3 and was passaged again on day 4. Pools 23 and 504 did not show cytopathic effect after the primary passage (P1) or 2 successive subcultures (P2 and P3). However, the supernatant was positive by real-time PCR for P1 of pools 26 and 504, P2 of pools 26 and 504, and P3 of pool 504. C values for pool 504 were increasing between P1 (C 20), and P3 (C 16); C was 22 for the positive controls. BRBV real-time PCR results were negative for all samples from 2018 and 2019. The MIR of A. americanum ticks from site 1 was 0.35/1,000 ticks during 2019; the MIR for adults of A. americanum ticks from site 2 was 1.35/1,000 ticks during 2019. The MIR for nymphs and 1.26/1,000 ticks for adults

had been obtained from 2 patients in Missouri during 2009, one patient in Tennessee during 2013, and 2 ticks from New York during in 2018 (10) (Appendix Table 2, Figure 1). Overall, there were a greater number of synonymous changes than nonsynonymous changes (Figure 2), and there were 6 amino acid positions at which all 3 samples from ticks differed from all 3 samples from humans: nonstructural protein positions Q233R, G236E, and R238C; glycoprotein position K903R; and polymerase positions N1300S and A1937S.

Discussion

We provide evidence of locally infected A. americanum ticks with HRTV in Georgia. In the southeastern United States, A. americanum ticks are most frequently associated with human bites (32). Rapid range expansion and increasing prevalence of the lone star tick (14), coupled with anthropologic factors that place humans in tick-infested habitats (33), are increasing the human risks for tickborne pathogen spillover. Despite the apparent widespread distribution of HRTV in the southeastern United States, as shown by seroprevalence studies on wildlife (11–13), detecting HRTV in A. americanum ticks has been challenging because of its low infection rate and the typically aggregated nature of arbovirus infections in ticks (34,35). By focusing sampling efforts in an area with reported exposure to HRTV in wildlife and humans and testing for infection in thousands of ticks from multiple sites and physiologic stages, we confirmed the presence of HRTV in Georgia.

Table 2. HRTV-positive Amblyomma americanum tick pools from Putnam and Jones Counties, Georgia, 2019*

<table>
<thead>
<tr>
<th>No. pools</th>
<th>Collection date</th>
<th>Site</th>
<th>No. specimens in pool</th>
<th>HRTV real-time PCR result for homogenate</th>
<th>Vero E6 cells</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Apr 28</td>
<td>1</td>
<td>5</td>
<td>+</td>
<td>CPE</td>
<td>PCR</td>
<td>PCR</td>
<td>PCR</td>
</tr>
<tr>
<td>26</td>
<td>Apr 28</td>
<td>1</td>
<td>25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>504</td>
<td>Jun 14</td>
<td>2</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*CPE, cytopathic effect; HRTV, Heartland virus; P1, primary passage; P2, subculture 2; P3, subculture 3. – negative; +, positive.
Our sampling effort focused on 2 areas that had high tick density and occurred during the peak of seasonal tick activity to ensure sufficient sample size to enable virus detection. Positive samples were detected in mid-April at site 1 and in mid-June at site 2. Adult and nymph specimens of *A. americanum* ticks were found to be infected, consistent with previous reports that showed that both mature and immature stages of the ticks are infected and competent vectors (5,6). The finding of infected adults and nymphs in April, early during *A. americanum* tick seasonality, also suggests that HRTV might be overwintering in these ticks. Estimates of MIR are highly variable between regions, years, and season, but are characteristically low, similar to infection prevalence of the closely related tickborne SFTSV in Asia (36). Our calculated infection rate is among the lowest in the spectrum reported in other states, which coincides with the rarity of the occurrence of clinical cases, although the possibility of underdiagnoses caused by low awareness of the disease must also be considered; studies from Missouri showed an overall MIR of 1.7/1,000 ticks (6), and others from Illinois reported 9.46/1,000 ticks (8). A recent study in New York reported MIRs ≤1.1% (10).

Two of 3 samples that had homogenates positive for HRTV by PCR were successfully isolated in cell lines. The complete lysis of the monolayer was observed only in the first culture (P1) of 1 pool (pool 26). The virus was successfully detected in a subculture (P2) but could not be maintained after subsequent passages. In another pool (pool 504), although no cytopathic effect was seen, the virus was detected in the supernatant of 3 consecutive cell line passages in increasing quantities, suggesting successful viral amplification. One positive homogenate was not detected in culture, which was consistent with the slightly decreased sensitivity of virus isolation compared with molecular methods, although it could also correspond to the presence of nonviable virus. Our quantification of C<sub>t</sub> values throughout passages confirmed viral RNA replication without the need to conduct assays such as immunofluorescent antibody assay or Western blotting. Whereas some investigators might see this result as a limitation, multiple studies confirm the validity of C<sub>t</sub> values for quantifying virus replication (e.g., 37,38). We emphasize the need for attempting viral isolation, which provides a unique source for phenotypic characterization and pathogenesis studies. Detection of HRTV in ticks to confirm virus circulation in an area has the limitation of low sensitivity because of a low prevalence of infection, as reported in other studies showing low infection rate in ticks (6,8,10). Use of complementary tools, such as serosurveys for
vertebrate hosts, could enhance the efficiency in detecting risk areas for human exposure.

Analysis of HRTV genome sequences showed a relatively high degree of conservation between the 3 samples in this study, which were obtained within 2 months of each another and from sites 5 km apart. Because sequencing was performed from pooled tick samples, it is possible that each consensus sequence reflects >1 infected tick. The HRTV genome sequences generated in this study were 2%–5% different from the only 3 other available complete HRTV genome sequences sampled from humans across different states over the preceding decade. This finding reflects a degree of genetic diversity similar to that described for the related Bandavirus SFTSV in South Korea (39). However, further work is needed to characterize the full spectrum of diversity of HRTV in the United States, and in particular to assess whether there are viral genetic features associated with human infection. Our results demonstrate the feasibility of sequencing complete HRTV genomes directly from tick samples, which enables molecular characterization, a critical step in understanding the diversity, evolution, and pathogenesis of the virus.

Our findings confirm the ongoing circulation of HRTV in Georgia. Major knowledge gaps in the biology and epidemiology of HRTV require further efforts to understand which vertebrates or secondary tick species might play a role in the maintenance of the virus in nature. For instance, the presence and ongoing range expansion of the Asian longhorned tick, *H. longicornis*, in the United States (40) could lead to major changes in the transmission ecology of HRTV in areas where this species overlaps with *A. americanum*. Therefore, assessing the current and future risk for HRTV transmission and spillover becomes relevant for disease ecologists and public health practitioners. In the immediate term, knowledge about the presence of HRTV in local ticks would enable improved preventive strategies to mitigate human exposure to ticks, as well as alerting physicians about the presence of this emerging tickborne virus.

**Acknowledgments**

We thank undergraduate and graduate students from Environmental Sciences Department at Emory University who collaborated in field activities, and the Arboviral Diseases Branch, Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, for providing a positive control for a BRBV PCR.

This study was supported by a grant from the Emory University Research Council (project ID 00097272) to G.M.V.-P.

**About the Author**

Dr. Romer is an infectious disease clinician and microbiologist in the Department of Environmental Sciences, Emory University, Atlanta, GA. Her primary research interest is arthropod-borne diseases.

**References**


Address for correspondence: Gonzalo Vazquez-Prokopec, Department of Environmental Sciences, Emory University, Mathematics and Science Center, 5th Fl, Ste E530, 400 Dowman Dr, Atlanta, GA 30322, USA; email: gmvazqu@emory.edu
Chronic wasting disease (CWD) is a naturally-occurring neurodegenerative disease of cervids. Raccoons (Procyon lotor) and meadow voles (Microtus pennsylvanicus) have previously been shown to be susceptible to the CWD agent. To investigate the potential for transmission of the agent of CWD from white-tailed deer to voles and subsequently to raccoons, we intracranially inoculated raccoons with brain homogenate from a CWD-affected white-tailed deer (CWDWtd) or derivatives of this isolate after it had been passaged through voles 1 or 5 times. We found that passage of the CWDWtd isolate through voles led to a change in the biologic behavior of the CWD agent, including increased attack rates and decreased incubation periods in raccoons. A better understanding of the dynamics of cross-species transmission of CWD prions can provide insights into how these infectious proteins evolve in new hosts.

Transmissible spongiform encephalopathies, or prion diseases, are a group of fatal neurodegenerative diseases that include chronic wasting disease (CWD) in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (mad cow disease) in cattle, and Creutzfeldt-Jakob disease and Kuru in humans. As of January 2020, CWD has been reported in free-ranging and farmed cervids in 26 states in the United States and 3 provinces in Canada (1). CWD-affected cervids shed infectious prions into their environment during both the preclinical and clinical stages of disease (2–8), and infectivity persists in soil (9–13), on the surface of contaminated plant leaves and roots (14), and in association with mineral licks (15). Environmental contamination with CWD prions represents a source of infectious material to which noncervid wildlife species, including raccoons and other small mammals, can be exposed.

We previously reported the transmission of the agent of CWD from white-tailed deer (Odocoileus virginianus borealis) and elk to raccoons through experimental intracranial inoculation (16). Raccoons are able to propagate CWD prions from white-tailed deer and elk but with low attack rates (25%) and with disease-associated prion protein distribution restricted to the brain (16).

Successful transmission of the agent of CWD from white-tailed deer to 4 species of native North America rodents has been reported previously, and meadow voles (Microtus pennsylvanicus) were found to be the most susceptible species (17). Meadow voles are known to opportunistically scavenge carcasses and engage in cannibalistic behavior (18), providing a plausible route for exposure to CWD and the possibility of continued disease transmission. Small rodents are a food source for predators and scavengers, including raccoons, and meadow voles and raccoons inhabit overlapping geographic ranges that also overlap with locations undergoing cervid CWD epidemics (Figure 1). Therefore, the potential for direct exposure of meadow voles and raccoons to CWD infectivity in the environment exists. Indeed, studies in Wisconsin have shown that raccoons are present at deer carcasses and gut piles with a high frequency (19). In addition, because raccoons are mesopredators and scavengers, there is the potential for secondary exposure of raccoons through consumption of contaminated rodents.

Increased Attack Rates and Decreased Incubation Periods in Raccoons with Chronic Wasting Disease Passaged through Meadow Voles

S. Jo Moore, Christina M. Carlson, Jay R. Schneider, Christopher J. Johnson, Justin J. Greenlee
To examine the potential for noncervid species to support CWD transmission, we intracranially inoculated raccoons with the agent of CWD from a white-tailed deer or with derivatives of the same inoculum after it had been passaged through meadow voles 1 or 5 times. In this study, we report the successful transmission of the agent of CWD from a white-tailed deer and vole-passaged CWD to raccoons through experimental intracranial inoculation. Our findings suggest passage of the CWD agent through voles results in a CWD agent with altered phenotypic properties.

**Materials and Methods**

We sourced 17 raccoon kits (8 weeks of age) that had no previous history of prion disease from a commercial breeder and challenged them by intracranial inoculation using 0.1 mL of a 10% brain homogenate (20). Brain material from 3 CWD-affected donor animals generated in a previous study (17) were used as inocula: 1 hunter-harvested (year of harvest 2001) CWD-positive white-tailed deer that was heterozygous for glycine and serine at codon 96 of the prion protein (GS96) (CWD<sup>Wtd</sup>), 1 meadow vole that had been inoculated intracranially with the CWD<sup>Wtd</sup> inoculum (first passage, CWD<sup>Vole-P1</sup>), and 1 meadow vole that had been inoculated intracranially with brain material from a fourth passage vole (fifth passage, CWD<sup>Vole-P5</sup>) (Table; Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-0271-App1.pdf). We inoculated raccoons in the negative control groups with brain material from a vole that had been intracranially inoculated with obex tissue from a CWD-negative deer (CWD<sup>Neg</sup>) (Table 1; Appendix). We prepared each inoculum from a single donor animal; no pooling was performed. We monitored raccoons daily and euthanized them when they showed unequivocal signs of prion disease (such as ataxia, inability to climb, or recumbency), when intercurrent illness or injury was present that could not be remedied by veterinary care, or at the end of the experiment at 35 months after inoculation. At raccoon death, we performed a full necropsy on all raccoons. We fixed 1 set of tissue samples in 10% buffered formalin, embedded in paraffin wax, and sectioned at 5 μm for microscopy examination.

**Figure 1.** Overlap of raccoon and meadow vole distributions and chronic wasting disease epidemics, North America. A) Light purple shading indicates raccoon distribution; B) light teal shading indicates meadow vole distribution. Dark green areas and dark purple (A) and teal (B) overlays show known locations of chronic wasting disease in free-ranging cervids (as of March 2020).
after hematoxylin and eosin staining or immunohistochemical staining for detection of disease-associated prion protein (PrPsc) by using a cocktail containing 2 monoclonal antibodies, F89/160.1.5 and F99/97.6.1 (Appendix). We froze the second set of tissues, comprising subsamples of all tissues collected into formalin, and examined selected samples for the presence of disease-associated prion protein (PrPsc) by using a commercially available antigen-capture enzyme immunoassay or in-house Western blotting (Appendix).

**Ethics Statement**
This experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL, USA). The Institutional Animal Care and Use Committee at the National Animal Disease Center reviewed and approved the animal use protocols (approval no. ARS-2778).

**Results**
In the CWDWtd group, 3/4 raccoons demonstrated clinical signs consistent with prion disease (ataxia, inability to climb, recumbency); the average survival time was 23 months postinoculation (mpi) (Table). The remaining raccoon was euthanized at 32 mpi because of bilateral eye lesions; PrPsc was not detected in any tissues examined. We detected PrPsc in all raccoons in the CWDVole-P1 group. Two raccoons were euthanized or found dead because of urinary tract disease, and 1 was euthanized at 22 mpi because of lameness that was not responsive to treatment. The remaining 2 raccoons exhibited ataxia and inability to climb and were euthanized at 22 and 24 mpi (Table). In the CWDVole-P5 group, 2 raccoons were euthanized because of urinary tract disease at 3 mpi (PrPsc not detected) and 17 mpi (PrPsc-positive). During 18–21 mpi, the remaining 3 raccoons demonstrated ataxia and inability to climb; 2 of these animals also showed head tremors (Table). All 4 raccoons in the CWDNeg control group were clinically normal when they were euthanized at the end of the study at 35 mpi (Figure 2). By using antigen-capture enzyme immunoassay, we detected PrPsc in the brains of 3/4 raccoons in the CWDWtd group, 5/5 raccoons in the CWDVole-P1 group,

![Survival curves for raccoons inoculated intracranially with the agent of CWD from white-tailed deer or vole-passaged CWD. CWD, chronic wasting disease; CWDWtd, CWD negative white-tailed deer; CWDVole-P1, first passage (white-tailed deer to vole); CWDVole-P5, fifth passage (vole to vole); CWDNeg, CWD from white-tailed deer.](image-url)
4/4 raccoons in the CWD<sub>Vole-P5</sub> group (not including the raccoon that was euthanized because of urinary tract disease at 3 mpi), and 0/4 raccoons in the CWD<sub>Neg</sub> group (Table).

When we analyzed brain samples by Western blot by using monoclonal PrP antibody P4, migration patterns for all animals within a treatment group were similar to each other and to the original inoculum (data not shown). When we compared samples across groups, migration patterns for vole-passaged groups were similar to each other with the unglycosylated band at ≈19 kDa. The unglycosylated band of the sample from the CWD<sup>Vld</sup> group migrated slightly higher, at 20 kDa, and that of the original donor white-tailed deer migrated slightly higher again, at ≈21 kDa (Figure 3).

We examined hematoxylin and eosin–stained sections to assess pathologic changes in the brain (Figure 4). Immunohistochemical staining for PrP<sup>Sc</sup> was applied to the brain and peripheral tissues to investigate the distribution of PrP<sup>Sc</sup> throughout the body (Figure 4). In raccoons in the CWD<sup>Vld</sup> group, spongiform change of the neuropil was mild caudally (medulla at the level of the obex and midbrain) and moderate rostrally (thalamus and basal nuclei). Spongiform change was not observed in the dorsal motor nucleus of the vagus nerve (Figure 4, panel A) or cerebellum and was mild to moderate in the basal nuclei (Figure 4, panel E) and neocortex. In contrast, spongiform change in the vole-passaged CWD groups was moderate to marked throughout the brain, including in the dorsal motor nucleus of the vagus nerve (Figure 4, panel B), basal nuclei (Figure 4, panel F) and neocortex, and mild in the cerebellum. Intraneuronal vacuolation was only observed in 2 raccoons, both of which were from the vole-passaged CWD groups. A single intraneuronal vacuole was seen in the red nucleus of raccoon 6 (CWD<sub>Vole-P5</sub>) and the dorsal motor nucleus of raccoon 14 (CWD<sub>Vole-P5</sub>).

We detected immunoreactivity for PrP<sup>Sc</sup> in the brain, spinal cord, retina, optic nerve, and/or pituitary in ≥2 raccoons per group (Table). We did not detect PrP<sup>Sc</sup> in any lymphoid tissues sampled but was observed in the enteric nervous system of the stomach, jejunum, ileum and colon of raccoon 3 (CWD<sup>Vld</sup>). In the brains of raccoons in the CWD<sup>Vld</sup> group, the overall amount of PrP<sup>Sc</sup> immunoreactivity was less in the caudal parts of the brain (Figure 4, panel C) and greater in the rostral parts of the brain (thalamus and basal nuclei) (Figure 4, panel G). Extracellular PrP<sup>Sc</sup> accumulation in the neuropil and on neurons was more prominent than intraneuronal accumulation (Figure 4, panels C, G). In contrast, the pattern of PrP<sup>Sc</sup> immunoreactivity was similar in raccoons in the vole-passaged CWD groups and characterized by PrP<sup>Sc</sup> immunoreactivity throughout the brain with intracellular PrP<sup>Sc</sup> accumulation in microglia, astrocytes, and neurons (Figure 4, panels D, H).

To enable objective comparisons of the distribution and severity of spongiform change between inoculation groups, we scored the severity of vacuolation on a scale of 0–4 for 17 neuroanatomical areas and used the score to generate vacuolation lesion profiles as described previously (21). We made modifications to include the red nucleus and dorsal motor nucleus of the vagus nerve, which resulted in a total of 19 neuroanatomical areas examined. The distribution of vacuolation in raccoons was similar in the vole-passaged CWD groups, although the overall severity of vacuolation was greater in the CWD<sub>Vole-P5</sub> group than the CWD<sub>Vole-P1</sub> group (Figure 5). The pattern of vacuolation observed in raccoons in the CWD<sup>Vld</sup> group was different from raccoons in the vole-passaged groups. A trend for less severe vacuolation overall was particularly noticeable in the medulla (Figure 5, neuroanatomical areas 1–4), midbrain (Figure 5, areas 8–9), frontal cortex (Figure 5, area 13), and claustrum (Figure 5, area 17) (Appendix).

**Discussion**

We demonstrated that clinical disease developed in raccoons inoculated intracranially with the agent of CWD from white-tailed deer (CWD<sup>Vld</sup>); the average incubation period was ≈23 mpi. Passage of the CWD<sup>Vld</sup> isolate through meadow voles before inoculation of raccoons with this vole-passaged CWD resulted in slightly shorter incubation periods (≈20 mpi) and different neuropathology and Western blot migration pattern as compared with the original CWD<sup>Vld</sup> isolate.
We previously reported that experimental intracerebral inoculation of raccoons with an inoculum prepared from pooled brainstems from 11 CWD-affected white-tailed deer (CWD\textsuperscript{Wtd\text{-pool}}) resulted in disease in 1/4 raccoons with an incubation period of 73 mpi and restricted distribution of PrP\textsuperscript{Sc} accumulation in the brain (16). The low attack rate and prolonged incubation period produced by the CWD\textsuperscript{Wtd\text{-pool}} inoculum compared with the CWD\textsuperscript{Wtd} inoculum reported in this study could be due to differences in the titer of PrP\textsuperscript{Sc} in the donor inocula. However, we consider this scenario unlikely, because all donor deer used to prepare the CWD\textsuperscript{Wtd\text{-pool}} inoculum and the single donor deer used to prepare the CWD\textsuperscript{Wtd} inoculum.

Figure 4. Patterns of histopathology and immunohistopathology in brains from 2 raccoons inoculated with the agent of chronic wasting disease (CWD). Panels A, C, E, and G show results for raccoon 2, inoculated with the agent of CWD from white-tailed deer; panels B, D, F, and H show results for raccoon 9, inoculated with CWD from a vole that had been inoculated with the 4th vole-passage of the agent of CWD from white-tailed deer. All images original magnification ×20. A–D) Medulla at the level of the obex. A) Raccoon 2 shows no spongiform change in the dorsal motor nucleus of the vagus nerve (DMNV) (above dashed line) or hypoglossal nucleus (below dashed line). Hematoxylin and eosin (H&E) stain. B) Raccoon 9 shows mild to moderate spongiform change in the DMNV. H&E stain. C) Raccoon 2 shows very mild PrP\textsuperscript{Sc} immunoreactivity in the DMNV and no immunoreactivity in neurons of the hypoglossal nucleus. PrP antibodies F89/160.1.5 and F99/97.6.1. D) Raccoon 9 shows moderate PrP\textsuperscript{Sc} immunoreactivity in the neuropil of the DMNV and marked intraneuronal immunoreactivity in the hypoglossal nucleus. PrP antibodies F89/160.1.5 and F99/97.6.1. E–H) Caudate nucleus. E) Raccoon 2 shows moderate diffuse spongiform change. H&E stain. F) Raccoon 9 shows marked diffuse spongiform change. H&E stain. G) Raccoon 2 shows diffuse neuropil PrP\textsuperscript{Sc} immunoreactivity and prominent extracellular PrP\textsuperscript{Sc} accumulation on neurons (arrowheads). PrP antibodies F89/160.1.5 and F99/97.6.1. H) Raccoon 9 shows marked intracellular PrP\textsuperscript{Sc} immunoreactivity in neurons (arrowheads) and glial cells (arrows). PrP antibodies F89/160.1.5 and F99/97.6.1.
inoculum were positive by immunohistochemistry for PrPSc. Another point of difference in disease expression produced by the CWDWtd-pool compared with the CWDWtd inoculum is the pattern of neuropathology observed in the brain: vacuolation of neuronal perikarya was widespread in the brain of the raccoon inoculated with the CWDWtd-pool (16) but was not observed in raccoons inoculated with CWDWtd. The differences in biologic behavior of these 2 CWD isolates are most likely associated with differences in the prion protein (PRNP) genotype of the donor deer. Four PRNP polymorphisms exist in white-tailed deer: Q95H, G96S, A116G, and Q226K (reviewed in S.J. Robinson et al. [22]). At codon 96, the S96 allele is associated with reduced CWD prevalence (23–26) and prolonged incubation periods (27). Donor deer for the CWDWtd-pool inoculum were all GG96 PRNP genotype (16), whereas the donor deer for the CWDWtd inoculum was GS96 PRNP genotype. We did not expect that inoculum containing the S96 allele would produce disease in raccoons more efficiently than inoculum exclusively containing the G96 allele. In addition, sequencing of PRNP from raccoons in a previous study showed that raccoons are homozygous for glycine at codon 96 (GG96) (S.J. Moore, unpub. data). Therefore, our results suggest that patterns of disease susceptibility associated with PRNP polymorphisms at codon 96 in CWD-affected white-tailed deer might not be a useful predictor of disease outcomes in intracranially inoculated raccoons. Further studies are under way to investigate the biologic behavior in raccoons of CWD from a single-source GG96 white-tailed deer.

Cross-species transmission of CWD isolates might result in a change in the biochemical properties of the disease-associated prion protein or the biologic behavior of the prion strain, such as adaptation to its host, or both (16,28–33). The pattern of PrPSc accumulation in the brain of the raccoon inoculated with CWDWtd-pool in our previous study (16) was similar to raccoons inoculated with the CWDWtd inoculum in this study and was characterized by prominent linear and perineuronal PrPSc accumulation, although this comparison is limited by the small number of animals available for examination. Both vole-passaged CWD isolates produced similar disease phenotypes in raccoons with regards to incubation periods, western blot migration patterns, and neuropathology. The patterns of spongiform change and PrPSc accumulation in the brains of raccoons inoculated with vole-passaged CWD isolates were similar to each other and different from those observed in the brains of raccoons inoculated with the CWDWtd isolate. Inoculum-associated differences in western blot migration patterns were observed (i.e., similar patterns for vole-passaged CWD isolates and a different pattern for the CWDWtd isolate). In addition, the migration pattern of the original CWD-affected white-tailed deer donor (Figure 3, deer CWD, unglycosylated band at ≈21 kDa) was different from both the raccoon-pas sage CWD Wtd isolate (Figure 3, white-tailed deer, 20 kDa) and the vole-passaged CWD isolates (Figure 3, vole-P1 and vole-P5, 19 kDa) after passage through raccoons. Therefore, passage of CWDWtd through voles appears to result in a change in the biologic behavior of this prion isolate when inoculated intracranially.
into raccoons. This finding raises the possibility for emergence of novel CWD strains after passage in off-target species through host-driven selection of a strain present in the donor inoculum (29,34). The original inoculum was derived from a white-tailed deer with the GS96 PRNP genotype, and propagation of CWD prions on S96 PrPSc results in the formation of alternative PrPSc conformers (34). We are unsure what role the genotype of the deer in the inoculum might have played in the change in biologic behavior noted after passage through voles. Because intracranial inoculation is not a natural route for exposure of raccoons to CWD infection, oral transmission studies are underway to characterize the biologic behavior of the CWD-Wtd and vole-passaged CWD isolates using a more natural route of exposure.

We observed a single intraneuronal vacuole in the red nucleus of raccoon 6 (CWDVoile-P1 group) and the dorsal motor nucleus of raccoon 14 (CWDVoile-P1 group). Intraneuronal vacuolation was previously reported as an incidental finding in the brainstem (including facial and pontine nuclei), cerebellar roof nuclei, and cerebrum of raccoons (35,36). In those raccoons, no evidence of concurrent neuropil vacuolation, neuronal degeneration, or astrocytosis was seen. In contrast, widespread neuropil vacuolation throughout the brains of raccoons 6 and 14, and strong PrPSc immunoreactivity in vacuolated neurons was evident; therefore, the intraneuronal vacuoles observed in these raccoons are likely associated with prion infection.

Although PrPSc immunoreactivity was widely distributed throughout the brain and spinal cord, we did not generally observe involvement of the peripheral nervous system, with the exception of 1 raccoon (3) inoculated with CWD-Wtd, in which PrPSc immunoreactivity was present in the enteric plexi of the stomach, jejunum, ileum, and colon. The general lack of peripheral nervous system involvement is likely because raccoons were inoculated through the intracranial route that bypasses centripetal spread of PrPSc from the alimentary tract to the brain along parasympathetic nerves, as is observed in orally infected deer (37). Instead, PrPSc immunoreactivity in the enteric nervous system of raccoon 3 was likely the result of centrifugal spread from the central nervous system. Why PrPSc immunoreactivity was observed throughout the spinal cord in all raccoons is unclear, but enteric nervous system involvement was only seen in raccoon 3. Raccoon 3 was the longest surviving raccoon (27 mpi), so the possibility exists that, had other raccoons not succumbed to clinical disease, there might have been time for transport of PrPSc to the enteric nervous system. Inoculation of raccoons by the oral route is needed to improve our understanding of the pathogenesis of CWD in raccoons when exposure occurs by a more natural route.

The longest surviving CWD-inoculated raccoon (4) was euthanized at 32 mpi because of bilateral eye lesions. Histopathologic examination resulted in a diagnosis of multicentric lymphoma, and PrPSc was not detected in any tissues. Clinical disease and widespread PrPSc accumulation at 21-27 mpi developed in all other raccoons in the CWD-Wtd group (n = 3). The reason for the unexpected negative result for raccoon 4 is unclear but could include experimental error or host factors. With regard to experimental inoculation, all inocula were prepared and all raccoons were inoculated on the same day, so the likelihood is very low that this raccoon did not receive the correct inoculum. The strongest determinant of susceptibility to prion diseases is the host PRNP sequence. No unique single nucleotide polymorphisms were detected in the PRNP open reading frame of raccoon 4 (S.J. Moore, unpub. data). It is tempting to speculate that host, genetic, or immunological factors outside of the PRNP open reading frame that contributed to the development of neoplasia might have had a suppressive effect on PrPSc accumulation.

Prion diseases of free-ranging animals do not exist in isolation. Meadow voles and raccoons are widespread in North America, and their habitat ranges overlap with those of CWD-affected white-tailed deer and other cervids. Therefore, a substantial potential for exposure of these or other off-target species to CWD infectivity in the environment exists. We have demonstrated that CWD-Wtd from a GS96 white-tailed deer transmitted readily to raccoons. Passage of this isolate through voles followed by intracranial inoculation of raccoons with vole-derived inoculum resulted in disease with different biologic characteristics and neuropathology than the original CWD-Wtd isolate. These results provide strong evidence for the emergence of a novel strain of CWD after passage in meadow voles and raccoons. Therefore, interspecies transmission of CWD prions between cervids and noncervid species that share the same habitat might represent a confounding factor in CWD-management programs. In addition, passage of CWD prions through off-target species might represent a source of novel CWD strains with unknown biologic characteristics, including zoonotic potential. Characterization of the biologic behavior of CWD isolates after cross-species transmission will help us develop more effective management strategies for CWD-affected populations.
Acknowledgments
We would like to thank Martha Church, Joe Lesan, Leisa Mandell, and Kevin Hassall for excellent technical support. Raccoon and vole range data were provided by NatureServe in collaboration with Bryan Richards, USGS National Wildlife Health Center, and range figures were generated by Andrew Fox (Centers for Epidemiology and Animal Health, Animal and Plant Health Inspection Service, US Department of Agriculture).

This research was supported in part by an appointment (S.J.M.) to the Agricultural Research Service Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the US Department of Energy (DOE) and the US Department of Agriculture. ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract no. DE-SC0014664.

About the Author
Dr. Moore performed this work as a postdoctoral research associate at the National Animal Disease Center, US Department of Agriculture, Ames, Iowa. Her research interests include pathogenesis and pathology of animal diseases with a special interest in neuropathology and interspecies transmission of prion diseases.

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Address for correspondence: Justin Greenlee, National Animal Disease Center, ARS, USDA, 1920 Dayton Ave, PO Box 70, Ames, IA 50010, USA; email: justin.greenlee@usda.gov

EID Podcast
Telework during Epidemic Respiratory Illness

The COVID-19 pandemic has caused us to reevaluate what “work” should look like. Across the world, people have converted closets to offices, kitchen tables to desks, and curtains to videoconference backgrounds. Many employees cannot help but wonder if these changes will become a new normal.

During outbreaks of influenza, coronaviruses, and other respiratory diseases, telework is a tool to promote social distancing and prevent the spread of disease. As more people telework than ever before, employers are considering the ramifications of remote work on employees’ use of sick days, paid leave, and attendance.

In this EID podcast, Dr. Faruque Ahmed, an epidemiologist at CDC, discusses the economic impact of telework.

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The coronavirus disease (COVID-19) pandemic has brought the One Health concept to the forefront of global health. From an infectious disease standpoint, the focus of One Health is often on how human activity expanding and encroaching on wildlife habitats may adversely affect humans through spillover of pathogens from wildlife reservoirs. However, the opposite—transmission of pathogens from humans to wildlife—is also possible in these situations. In Brazil and other countries, destruction and alterations of natural habitats and deforestation driven by human activities such as agricultural and urban expansion force some nonhuman primate (NHP) populations to live in anthropized areas, intensifying interactions between humans and NHP species and increasing the risk for interspecies transmission of agents of infectious diseases (1).

The black-tufted marmoset (Callithrix penicillata) is one example of an NHP now well-adapted to human-altered environments. Marmosets are naturally found in the Brazilian Savanna and Caatinga Biome and are commonly commensal in urban and periurban areas (2); close human–marmoset interactions (i.e., feeding) are common. Because these settings are suitable for interspecies virus transmission with potential spillover not only from animals to humans but also from humans to free-ranging nonhuman primates or other animals.
transmission to new species (3). Human alphaherpes-
viruses (HuAHVs) consist of 2 closely related viruses, 
HuAHV types 1 and 2. Transmission of both HuAHV
types generally requires intimate contact between
actively infected and susceptible persons. Humans
are natural hosts for HuAHV1, also known as herpes
simplex virus 1, an alphaherpesvirus that is endemic
in human populations (4). Natural fatal HuAHV1
infections are well-documented for New World pri-
mates that are in close contact with humans as pets
and in captive conditions such as zoos and biomed-
cal research centers (5–15). In contrast, HuAHV1 in-
fecions in free-ranging New World primates in an-
thropogenic environments are rarely reported and
are limited to isolated outbreaks in urban areas (16)
and in a state conservation park (17).

Captive New World primates are highly suscepti-
table to HuAHV1 infection, and fatal disease frequently
develops, characterized by mucocutaneous, facial,
and oral erosions/ulcerations and meningoencephali-
ritis (5–15). However, reports of HuAHV1 infection
in free-living animals in anthropized environments
are limited. We conducted a retrospective study to
determine pathologic, immunohistochemical, and
ultrastructural features of infection; molecular iden-
tification of the virus; and epidemiologic aspects of
fatal outbreaks and isolated cases of HuAHV1 virus
infection in free-ranging black-tufted marmosets in
anthropized areas of the Federal District and sur-
rounding areas of Brazil.

Methods

Case Selection
To select cases of suspected fatal herpetic infection,
we reviewed 1,042 NHP necropsies performed dur-
ing 2012–2019 and archived by the Veterinary Pathol-
ogy Laboratory at the University of Brasilia, Federal
District, and the Regional Reference Laboratory of the
Brazilian Ministry of Health that performs necropsies
to diagnose yellow fever as part of the National Sur-
vellance Program of Yellow Fever Epizootics in NHP
of the Brazilian Ministry of Health. For all cases, we
retrieved epidemiologic information, clinical data,
pathologic findings, and image data from submission
forms, necropsy reports, archived images, and forma-
lin-fixed paraffin-embedded (FFPE) tissue blocks.

Histopathologic Evaluation
Necropsy tissue samples from 13 NHPs with clinically
suspected emerging infectious diseases were fixed in
10% formalin, embedded in paraffin, sectioned, and
stained with hematoxylin and eosin. We evaluated the
following samples to identify histopathologic changes
consistent with suspected HuAHV infection: brain
(meningitis, perivascular inflammation, neuropil in-
fammation with or without prominent neutrophilic
component, neuronal necrosis, neuronophagy, reactive
gliosis, glial nodules, and inclusion bodies within
neurons and glial cells); tongue (epithelial ballooning
degeneration, acantholysis, ulceration, necrosis, sub-
epithelial inflammation, intranuclear inclusion bodies
within the mucosal epithelium and syncytial cells);
and liver (hepatocellular coagulative necrosis, hepato-
cellular eosinophilic intranuclear viral inclusion bod-
ies, and multinucleated giant cells [syncytia]). We also
recorded histopathologic changes in other organs.

Immunohistochemistry and Transmission
Electron Microscopy
We performed immunohistochemistry (IHC) for
HuAHV types 1 and 2 on FFPE tissues. We used a
polymer-based colorimetric indirect immunoperoxidase
method in deparaffinized tissue sections
after rinsing the tissue sections in 1X Tris–buff-
ered saline with Tween 20 (1X TBS–T; Thermo Fisher
sections were digested with 0.1 mg/mL Proteinase
K (Roche, https://www.sigmaaldrich.com) in 0.6
M Tris/0.1% CaCl₂, for 15 min and blocked in Back-
ground Punisher (Biocare Medical, https://biocare.
net) for 10 min. Slides were incubated with a rabbit
polyclonal anti-HuAHV1 and 2 antibody biological
products maintained at CDC at 1:2,000 dilution, and
attached antibodies were detected with Mach 4 Uni-
versal AP Polymer Kit (Biocare Medical) and Perma-
ent Red Chromogen (Cell Marque/Millipore Sigma,
https://www.cellmarque.com). We counterstained
slides with Mayer hematoxylin (Polyscience,
https://www.polyrned.com) and placed coverslips by
using an aqueous mounting medium (Polysciences,
positive and negative controls were run in parallel.

We also processed FFPE sections from the
brain, liver, and tongue of 3 NHPs with extensive
immunohistochemical evidence of HuAHV for
transmission electron microscopy (TEM) analysis
by using an on-slide technique. In brief, 4-μm sec-
tions of FFPE tissue affixed to glass slides that cor-
related with areas positively labeled for HuAHV
by IHC were deparaffinized in xylene, then rehy-
drated and fixed in 2.5% buffered glutaraldehyde.
Samples were postfixed with 1% osmium tetrox-
ide, stained en bloc with uranyl acetate, dehy-
drated, and embedded in Epon-Araldite resin with
dibutyl phthalate (Electron Microscopy Sciences,
Real-Time PCRs for HuAHV1 and HuAHV2

We conducted real-time PCR for HuAHV 1 and HuAHV2 on FFPE samples with confirmed herpesvirus immunohistochemistry results. We extracted DNA from 16-µm paraffin-embedded brain or liver tissue sections from each animal by using a QIAamp UCP Pathogen Mini Kit (QIAGEN, https://www.qiagen.com) according to the manufacturer’s recommendations. Fluorescence resonance energy transfer (FRET) technology was conducted to discern HuAHV1 from HuAHV2 infections in a real-time PCR targeting the glycoprotein B, UL27 gene. The 2-probed system discriminates HuAHV1 and HuAHV2 according to analysis of the melt curves (19). We considered a sample to be HuAHV1 positive if the melt temperature was 56°C and HuAHV2 positive if 63°C. We included cases positive by FRET PCR for HuAHV1 or HuAHV2 in our study.

To amplify a 147-bp fragment of the glycoprotein B, UL27 gene from the genome of either HuAHV1 or HuAHV2, we used HuAHV1 and HuAHV2 primers (HuAHV-1/2 forward primer, 5’-TTG AAG CGG TCG GCG GCG TA- 3’; HuAHV-1/2 reverse primer, 5’-GTC CAC CTC CTC GAC GAT GC- 3’) along with the detection probe (5’-LC Red 640-GCG ACT GGC GAC TTT G- 3’-phos-cpg) and the anchor probe (5’-GGT AGC CGT AAA ACG GGG ACA TGT A- 3’-fam-cpg). PCR was performed in a 20-µL reaction volume with 0.5 µmol/L of the 50 µmol/L primer stocks, 0.2 µmol/L of the detection probe, 0.1 µmol/L of the anchor probe, and HotStarTaq DNA Polymerase and 1X QuantiTect Probe PCR Master Mix (both from QIAGEN). The PCR had the following cycling conditions: a hot start (95°C), touchdown (10 cycles of denaturation at 95°C, annealing at 62°C, and an extension at 72°C), amplification (40 cycles of denaturation at 95°C, annealing at 52°C, and an extension at 72°C), cooling at 40°C, and 1 melt cycle at 95°C. We considered a sample to be considered positive if the melt temperature was ≈56°C for HuAHV1 and 63°C for HuAHV2. We included cases positive by FRET PCR for HuAHV1 or HuAHV2 in our study.

Results

Real-Time PCR, Epidemiologic, and Clinical Findings

Of the 1,042 retrieved NHP cases, HuAHV fatal infection was morphologically diagnosed for 18 black-tufted marmosets (Table 1). Of these, 5 (27.8%) were from captive conditions and excluded from our study, and for the remaining 13 free-ranging marmosets, HuAHV1 was detected in all tissues tested by real-time PCR. HuAHV2 was not detected in any sample. Marmoset deaths were distributed in urban (38.5%) and peri-urban areas (61.5%) of the Federal District and Goiás State, Brazil (Figure 1). Available information indicated close contact with humans for 7 marmosets. Investigations of the probable infection site frequently showed close contact between marmosets and humans, including local residents feeding fruit to animals.

Two separate HuAHV1 outbreaks during 2012-2019 involved 9 (69%) of the 13 animals, (7 animals in outbreak 1; 2 animals in outbreak 2); the other 4 (31%) had isolated cases. In outbreak 1, an entire family group of marmosets became ill and died. Information about

<table>
<thead>
<tr>
<th>Feature</th>
<th>No. affected/total (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>10/13 (77)</td>
</tr>
<tr>
<td>F</td>
<td>3/13 (23)</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>6/13 (46)</td>
</tr>
<tr>
<td>Adult</td>
<td>7/13 (54)</td>
</tr>
<tr>
<td>Epidemiologic features</td>
<td></td>
</tr>
<tr>
<td>Peri-urban location</td>
<td>7/12 (58)</td>
</tr>
<tr>
<td>Urban location</td>
<td>5/12 (42)</td>
</tr>
<tr>
<td>Outbreak</td>
<td>9/13 (69)</td>
</tr>
<tr>
<td>Isolated case</td>
<td>4/13 (31)</td>
</tr>
<tr>
<td>Known contact with humans</td>
<td>7/13 (54)</td>
</tr>
<tr>
<td>Clinical signs</td>
<td></td>
</tr>
<tr>
<td>Neurologic changes</td>
<td></td>
</tr>
<tr>
<td>Muscular tremors</td>
<td>4/12 (33)</td>
</tr>
<tr>
<td>Depression</td>
<td>3/12 (25)</td>
</tr>
<tr>
<td>Recumbency</td>
<td>2/12 (17)</td>
</tr>
<tr>
<td>Seizures</td>
<td>2/12 (17)</td>
</tr>
<tr>
<td>Anisocoria</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Ataxia</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Not specified (witnessed)</td>
<td>5/12 (42)</td>
</tr>
<tr>
<td>Oral cavity</td>
<td></td>
</tr>
<tr>
<td>Salivation</td>
<td>8/12 (66)</td>
</tr>
<tr>
<td>Bleeding</td>
<td>1/12 (8)</td>
</tr>
<tr>
<td>Gross findings</td>
<td></td>
</tr>
<tr>
<td>Lymphadenomegaly</td>
<td>4/13 (68)</td>
</tr>
<tr>
<td>Nonulcerative glossitis</td>
<td>5/13 (38)</td>
</tr>
<tr>
<td>Ulcerative glossitis</td>
<td>4/13 (31)</td>
</tr>
<tr>
<td>Facial erythema and rash</td>
<td>1/13 (7)</td>
</tr>
</tbody>
</table>

*Denominators indicate numbers of animals for which information was available.
Clinical signs was available for 12 animals. Neurologic signs were most frequently reported and included ataxia (8%), seizures (17%), muscle tremors (33%), nystagmus (8%), and anisocoria (8%). Nonspecific clinical signs were also reported, such as depression (25%), recumbency (17%), salivation (66%), and oral bleeding (8%). Fatal cases affected adults (54%) and juveniles (46%).

Pathology, Immunohistochemistry, and Electron Microscopy Findings
Gross changes retrieved from necropsy reports and photographic documentation revealed multiple cutaneous crusting erythematous erosions and ulcerations in the periorcular region, lip, and tongue (Figure 2). Mandibular and cervical lymph nodes were often markedly enlarged. The most commonly affected organs, of those available for evaluation, were the brain (92%), tongue (69%), and liver (8%) (Table 2). Histopathologic findings in other organs included reactive lymph node hyperplasia (77%) and necrosis of splenic white pulp germinal centers (15%). IHC demonstrated HuAHV1 antigen in the brain of 12 (92%) marmosets, the tongue of 9 (69%), and the liver of only 1 (8%). HuAHV1
immunostaining was not detected in other representative tissue samples available, including spleen (n = 13 animals), lymph node (n = 7), heart (n = 8), kidney (n = 8), lung (n = 7), esophagus (n = 1), gastrointestinal tract (n = 7), trachea (n = 1), adrenal gland (n = 1), pancreas (n = 1), and testicle (n = 4).

Brain
The most frequent neuropathologic finding in the HuAHV1-infected marmosets was necrotizing meningoencephalitis (Figure 3, panel A). Findings included reactive gliosis, glial nodules, neuronal necrosis, neuronophagia (Figure 3, panel B), characteristic herpetic intranuclear eosinophilic inclusion bodies within neurons and glial cells, and prominent perivascular inflammation of the gray and white matter (Figure 3, panel C). Inflammatory infiltrates varied from moderate to severe and consisted of lymphocytes, histiocytes, neutrophils, and a few plasma cells. For 3 animals, marked neutrophilic inflammation in the neuropil was detected (Figure 3, panel D). Swollen reactive endothelial cells and intravascular leukocytosis were also seen in affected areas of neuroparenchyma. Infected neurons and glial cells showed strong cytoplasmic immunostaining for HuAHV1 (Figure 3, panel E). TEM analysis of the brain revealed viral particles morphologically consistent with herpesvirus in the white and gray matter; intranuclear and cytoplasmic viral particles were also observed (Figure 3, panels F and G).

Tongue
Histopathologic lesions in the tongue were multifocal, showing moderate to severe epithelial ballooning degeneration and acantholysis; multifocal to coalescing mucosal ulceration; necrosis with fibrin deposition (Figure 4, panel A); and subepithelial neutrophilic infiltrates with histiocytes, lymphocytes, and plasma cells. Epithelial cells showed rare multinucleation (syncytia formation) with nuclear molding and herpetic intranuclear eosinophilic inclusion bodies (Figure 4, panel B). Necrotic and surrounding ulcerated areas showed epithelial cells with marked cytoplasmic and nuclear viral immunostaining (Figure 4, panel C). Correlating with this viral immunostaining, TEM identified abundant herpesvirus in the nuclei and cytoplasm of epithelial cells (Figure 4, panel D).

Liver
Histopathologic lesions in the liver were found in only 1 of the 13 marmosets. In this animal, necrotizing hepatitis was characterized by multifocal random coagulative necrosis, few neutrophils, and scattered hemorrhage (Figure 5, panel A). Hepatocellular eosinophilic intranuclear viral inclusion bodies and multinucleated giant cells (syncytia) were also observed (Figure 5, panels B). Necrotic foci showed strong immunolabeling for HuAHV1 antigens (Figure 5, panel C). Herpesvirus was found by TEM primarily in the cytoplasm of hepatocytes; a few nuclei also showed the presence of the virus (Figure 5, panels D).

Table 2. Histopathologic features found during necropsy of free-ranging black-tufted marmosets naturally infected with human alphaherpesvirus 1, Brazil, 2012–2019

<table>
<thead>
<tr>
<th>Organ, finding</th>
<th>No. affected/total (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td></td>
</tr>
<tr>
<td>Inclusion bodies within neurons and glial cells</td>
<td>12/13 (92)</td>
</tr>
<tr>
<td>Neuronal necrosis</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>Mononuclear perivascular cuffs</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>Neuronophagia</td>
<td>11/12 (92)</td>
</tr>
<tr>
<td>Reactive gliosis</td>
<td>11/12 (92)</td>
</tr>
<tr>
<td>Neuronal inflammation</td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>10/12 (83)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3/12 (25)</td>
</tr>
<tr>
<td>Intravascular leukocytosis</td>
<td>9/12 (75)</td>
</tr>
<tr>
<td>Nonsuppurative meningitis</td>
<td>9/12 (75)</td>
</tr>
<tr>
<td>Glial nodules</td>
<td>5/12 (42)</td>
</tr>
<tr>
<td>Reactive neurovascular endothelium</td>
<td>5/12 (42)</td>
</tr>
<tr>
<td>Tongue</td>
<td>9/13 (69)</td>
</tr>
<tr>
<td>Acantholysis</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>Epithelial ballooning degeneration</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>Epithelial intranuclear inclusion bodies</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>Subepithelial inflammation</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>Epithelial necrosis</td>
<td>5/9 (55)</td>
</tr>
<tr>
<td>Ulcer</td>
<td>4/9 (44)</td>
</tr>
<tr>
<td>Syncytial cells</td>
<td>2/9 (22)</td>
</tr>
<tr>
<td>Liver</td>
<td>1/13 (8)</td>
</tr>
<tr>
<td>Hepatocellular coagulative necrosis</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Intranuclear viral inclusion bodies</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Multinucleated giant cells</td>
<td>1/1 (100)</td>
</tr>
</tbody>
</table>

*Denominators indicate numbers of animals for which information was available.
Discussion
The expansion of human activity into natural environments can disrupt the One Health equilibrium by increasing opportunities for infectious disease transmission between humans and animals. The effect of this equilibrium on human public health is often emphasized; however, disease transmission can also threaten wildlife and biodiversity in periurban settings. The 13 synanthropic free-ranging marmosets with acute, fatal HuAHV1 infections described here were from across a densely populated, anthropized environment in Brazil. Our findings corroborate those of previous reports of HuAHV1 in captive and pet marmosets and other NHPs, from clinical and pathologic standpoints, expanding the body of knowledge of HuAHV1 infection in free-ranging marmosets and highlighting the value of strategic NHP infectious disease surveillance systems.

The natural reservoir for HuAHV1 is humans, and infection of these marmosets resulted from documented or probable close interactions with humans in urban or periurban environments. HuAHV1 infection occurs by viral host epithelial invasion through direct or indirect contact with mucocutaneous lesions or with bodily secretions/excretions from asymptomatic carriers (3, 6, 8, 20). In at least some of these marmosets, confirmed contact involved humans directly sharing food with marmosets. In the absence of direct sharing, food scraps and garbage are potential sources of HuAHV1 infection for free-ranging marmosets and other animals in anthropized environments such as the Federal

Figure 3. Pathologic changes in brain of free-ranging black-tufted marmosets with fatal human alphaherpesvirus 1 infection, Brazil, 2012–2019. A) Necrotizing meningoencephalitis. Hemotoxylin and eosin (H&E) stain; original magnification ×10. B) Neuronal degeneration and glial nodule. H&E stain; original magnification ×40. C) Neuronal necrosis with microglial proliferation and expansion of Virchow–Robbin spaces by lymphocytes, histiocytes, and few plasma cells. Neurons and glial cells show intranuclear inclusion bodies and prominent margination of the nuclear chromatin. H&E stain; original magnification ×63. D) Prominent neutrophilic inflammation accompanies neuronal necrosis and intranuclear inclusion bodies. H&E stain; original magnification ×63. E) Human alphaherpesvirus 1 immunostaining within neurons (immunohistochemistry; original magnification ×40). F) Intranuclear (arrowhead) and cytoplasmic (arrow) herpesvirus particles in gray matter. Transmission electron microscopy; scale bar indicate 500 nm. Inset: cytoplasmic herpesvirus particles (arrow) white matter, myelinated axon (arrowhead); scale bar indicates 200 nm.
District and surrounding areas. The high number of sick and dead marmosets in 1 family of marmosets in our series and in monkey families reported elsewhere suggests that animal-to-animal transmission of HuAHV1 probably also occurs (5,7–10,13–17). We found numerous viral particles within oral epithelial lesions of marmosets, suggesting high mucosal viral shedding and potential for transmission to other animals (21). Experimental HuAHV1 infection of rhesus macaques also demonstrated ocular, nasopharyngeal, oral, fecal, and urine virus shedding (22).

The clinical disease observed in these animals (neurologic signs, hypersalivation, and oral bleeding) correlated with the gross findings of glossitis and facial erythema with regional lymphadenomegaly and with histopathologic features characteristic of herpes viral meningoencephalitis and stomatitis. Similar clinical and gross findings have been reported for marmosets with fatal and nonfatal HuAHV1 infection, and necrotizing meningoencephalitis and ulcerative glossitis are the histopathologic hallmarks observed in most documented HuAHV1 outbreaks among captive and free-living NHPs (6–9,11–17,23–25).

According to histologic findings, death of these marmosets is largely attributable to the neurologic effects of infection (12,13). HuAHV1 antigen detection by IHC and viral particle detection by TEM within brain lesions confirmed viral neuroinvasiveness in these animals. Animal models have shown that primary HuAHV1 infection originates in the skin or oral mucosa, usually followed by a latent stage in sensory neurons, and finally reaching the brain through the trigeminal nerve or olfactory bulb, causing lethal encephalitis (26). Intracellular HuAHV1 replication triggers direct cytopathic effects such as programmed cell death and necrosis (27). In the 13 marmosets, brain pathology also indicated that a severe inflammatory response to viral infection played a role in disease pathogenesis (28–30). In some HuAHV1 outbreaks among marmosets, however, mortality rates may be elevated even in the absence of neuropathologic changes because marmosets are highly susceptible to infection (6). Necrotizing hepatitis with syncytia and viral inclusions, as seen in the juvenile marmoset with liver tissue available, has only rarely been reported for HuAHV1-infected NHPs (7). Similarly, a severe, disseminated disease with mucocutaneous lesions, hepatitis, and encephalitis occurs only sporadically in human neonates and severely immunocompromised patients (4) as a consequence of high viral load in hosts incapable of limiting replication at mucosal surfaces (31).

As with HuAHV1, other alphaherpesviruses typically cause mild, self-limiting disease in their natural host species but severe, generalized, and often fatal disease when cross-species transmission occurs.

Figure 4. Tongue pathology in free-ranging black-tufted marmosets with fatal human alphaherpesvirus 1 infection, Brazil, 2012–2019. A) Severe necrosis of epithelium. Hemotoxylin and eosin (H&E) stain; original magnification ×10. B) Intranuclear inclusion bodies in epithelial cells at the margin of the lesion and multinucleated syncytial cell (arrow). H&E stain; original magnification ×40. C) Human alphaherpesvirus 1 immunostaining within epithelial cells in the area of necrotizing glossitis. Immunohistochemistry; original magnification ×40. D) Epithelial cell containing accumulations of herpesvirus within the cytoplasm. Transmission electron microscopy; scale bar indicates 600 nm. Inset: higher magnification image of herpesvirus particles with well-defined tegument layer in the cytoplasm; scale bar indicates 400 nm.
Among NHPs, infections of Old World monkeys compared with New World monkeys are generally more self-limiting and fatal cases more rare (23–25, 28, 32, 33). The mechanisms underlying these variations in susceptibility are not fully known but may be associated with species differences in innate immune system function or cellular DNA repair proteins needed for the virus life cycle (34, 35). Cercopithecine herpesvirus 1 (also called B virus) is the most concerning in terms of zoonotic risk, resulting in fatal human infection after transmission from macaques. Human exposure is typically reported in biomedical research settings but could also occur through interactions with macaques in anthropized natural environments (3). Analogous to B virus cross-species infection in humans, our findings reinforce the high susceptibility and severe outcomes of cross-species HuAHV1 infections in marmosets (6).

Viral latency with recrudescence is typical of alphaherpesvirus infections in the natural host, and latency can be associated with asymptomatic virus shedding (20). HuAHV1 is endemic to the human population in Brazil, and seroprevalence studies indicate high rates of infection (36). Serologic testing has also shown the

**Table 3. Primate alphaherpesviruses and interspecies disease manifestations**

<table>
<thead>
<tr>
<th>Alphaherpesvirus ICTV name (common name)</th>
<th>Natural host species</th>
<th>Species with severe generalized disease</th>
<th>Species with self-limiting disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human alphaherpesvirus 1, 2 (herpes simplex virus)</td>
<td>Human</td>
<td>New World primates</td>
<td>Humans, Old World monkeys</td>
</tr>
<tr>
<td>Chimpanzee α-1 herpesvirus†</td>
<td>Chimpanzee</td>
<td>Unknown</td>
<td>Chimpanzees</td>
</tr>
<tr>
<td>Macacine herpesvirus 1 (B virus)</td>
<td>Macaques</td>
<td>Humans, African green monkeys</td>
<td>Macaques</td>
</tr>
<tr>
<td>Papione herpesvirus 2 (herpesvirus papio 2)</td>
<td>Baboon</td>
<td>Unknown</td>
<td>Baboons</td>
</tr>
<tr>
<td>Cercopithecine alphaherpesvirus 2 (simian agent 8)</td>
<td>Vervet, baboon, African green monkey</td>
<td>Unknown</td>
<td>Baboon</td>
</tr>
<tr>
<td>Langur herpesvirus†</td>
<td>Langur</td>
<td>Unknown</td>
<td>Langur</td>
</tr>
<tr>
<td>Saimirine herpesvirus 1</td>
<td>Squirrel monkey</td>
<td>Owl monkeys, marmosets, tamarins</td>
<td>Squirrel monkey</td>
</tr>
<tr>
<td>Ateline alphaherpesvirus 1</td>
<td>Spider monkey</td>
<td>Unknown</td>
<td>Spider monkey</td>
</tr>
<tr>
<td>Human herpesvirus 3 (varicella zoster virus)</td>
<td>Human</td>
<td>Unknown</td>
<td>Humans, great apes</td>
</tr>
<tr>
<td>Cercopithecine alphaherpesvirus 9 (simian varicella virus)</td>
<td>Macaque</td>
<td>African cercopithecines</td>
<td>African cercopithecines</td>
</tr>
</tbody>
</table>

†ICTV, International Committee on Taxonomy of Viruses.
†Not classified by ICTV (https://talk.ictvonline.org).
potential for persistent infection in marmosets (9), implying a potential risk for spillback to humans. HuAHV1 transmission from humans to NHPs and vice versa may therefore be underrecognized; further studies are needed to determine the extent of interspecies transmission in urban and periurban settings.

In Brazil, other enzootic causes of outbreaks and acute deaths among marmosets (e.g., toxoplasmosis, rabies, and yellow fever) are included in the clinical and pathologic differential diagnosis for HuAHV infection (37–39). These other diseases are zoonotic, some of high public health concern, with possible NHP reservoir hosts. This observation exemplifies the value of public outreach and robust One Health-focused epidemiologic and pathologic surveillance programs, such as the National Surveillance Program of Yellow Fever Epizootics in NHP of the Brazilian Ministry of Health, for detecting zoonotic and anthroponotic diseases in free-ranging NHPs (40). Detection, monitoring, and predicting spillover events afforded by these programs enables development of rapid containment measures to prevent development of new public health outbreaks and threats to wildlife populations.

Acknowledgments
We thank Josilene Nascimento Seixas and Demi Rebeneck for sample processing and DNA extraction from FFPE tissues.

The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brazil financed in part (finance code 001) the doctoral scholarship of T.M.W. Special thanks to The National Council for Scientific and Technological Development for financial support (grant no. 310498/2018-0 to M.B.C.).

Sherif R. Zaki, senior author, died before publication of this article.

About the Author
Dr. Wilson is a guest researcher with the Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta, as part of the doctoral scholarship from Capes-PrInt program. Her research interests include investigative and comparative pathology and pathogenesis of zoonotic and human infectious diseases.

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Address for correspondence: Marcio B. Castro, Veterinary Pathology Laboratory, University of Brasilia, Via L4 Norte, Campus Universitário Darcy Ribeiro, Asa Norte, Brasília, DF 70910-900, Brazil; email: mbcastro@unb.br
Molecular Surveillance for Imported Antimicrobial Resistant *Plasmodium falciparum*, Ontario, Canada

Ruwandi Kariyawasam, Rachel Lau, Eric Shao, Katherine Tan, Adrienne Showler, Filip Ralevski, Samir N. Patel, Andrea K. Boggild

Malaria remains the deadliest vectorborne infectious disease worldwide (1). *Plasmodium* spp., most commonly *P. falciparum*, are responsible for ≈229 million cases and ≈500,000 deaths from malaria annually (2). Although malaria incidence and death have decreased over the past decade, emerging antimalarial drug resistance, fueled by counterfeiting, overuse, and underdosing, threatens control and elimination efforts (2). *P. falciparum* resistance mutations have resulted in waning efficacy in multiple antimalarial classes including artemisinins, quinolines, and antifolates (3). Increased international travel and climate change are exacerbating the spread of malarial vectors (4), making drug-resistant *P. falciparum* malaria imported from endemic regions, in particular Africa and Southeast Asia, a growing concern (5). Surveillance of antimalarial drug resistance, particularly among *P. falciparum* specimens, is crucial both to inform universal treatment guidelines and to identify global patterns of emerging resistance. We aimed to identify the prevalence of several resistance markers, including genes that confer resistance to chloroquine, mefloquine, atovaquone/proguanil, and artemisinins, and to quantify the copy number of multidrug resistance genes (*pfmdr1*) in *P. falciparum* isolates from malaria cases imported to Ontario, Canada, over a 10-year period.

**Methods**

**Specimens**

From the malaria biobank at the Public Health Ontario Laboratory in Toronto, Ontario, Canada, we retrieved unique surplus whole-blood clinical specimens containing *P. falciparum* from the years 2008–2009, 2013–2014, and 2017–2018. We confirmed the *P. falciparum* specimens from the biobank as monoinfections using clinical testing, which included Giemsa-stained thick and thin blood film examination by certified medical lab technologists and rapid diagnostic test using the Abbott BinaxNOW malaria kit (https://www.globalpointofcare.abbott/en/index.html).
To eliminate the possibility of recrudescent infections, we excluded specimens from patients who had positive blood smears in the year before our enrollment period.

**Extracting DNA from Whole Blood and Detecting Parasite DNA by Real-Time qPCR**

We extracted DNA from surplus blood (0.4 mL) using the KingFisher Pure DNA Blood Kit (Thermo Fisher Scientific, https://www.thermofisher.com) and stored it in 50-μL aliquots: one at −20°C for working stock and another at −80°C for storage stock. We used included BEI Resources W2 MRA-157 (https://www.beiresources.org) and ATCC 3D7 (https://www.atcc.org) samples as *P. falciparum*-positive controls. We tested samples using 2 species-specific duplex real-time qPCRs (*P. falciparum* and *P. vivax; P. malariae and *P. ovale*) (6,7). Samples were 25 μL total volume, including 12.5 μL Taqman Universal MasterMix (Thermo Fisher) and 5 μL template DNA. To perform all reactions, we used the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher) for 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

**Pyrosequencing**

We analyzed 23 different single-nucleotide polymorphisms (SNPs) across 6 genes: *atpase6* (PIATPase6; SNPs A623E, S769N), *cytb* (cytochrome b; SNP Y268NSC), *dhfr* (dihydrofolate reductase; SNPs A16V, C50R, N51I, C59R, S108N, I164L), *dhps* (dihydropteroate synthetase; SNPs S436FA, A437G, K540E, A581G, A613TS), *mdr1* (multidrug resistance protein; SNPs N86Y, Y184F, S1043CTR, N1042D, D1246Y), and *pfcrt* (chloroquine resistance transporter; SNPs K76T, N75E, M74I, C72S). We performed 16 PCR reactions according to the Pyromark PCR Kit protocol (QIAGEN, https://www.qiagen.com) using 400 nmol/L–concentration primers published elsewhere (Table 1, https://wwwnc.cdc.gov/EID/article/28/4/21-0533-T1.htm). We performed all reactions on ABI Veriti Thermal Cyclers (Thermo Fisher) with adjusted annealing temperatures of 44°C for *dhfr* 164 and 63.8°C for *pfcrt*. We ran 3 μL of PCR product on a 1% agarose gel at 100 V for 30 min to ensure band presence before pyrosequencing.

We performed pyrosequencing using the Pyromark Q24 Vacuum Workstation and Pyromark Q24 Pyrosequencer (QIAGEN) according to manufacturer protocols. We designed 21 pyrosequencing assays using PSQ assay design software version 1.0.6 (https://psq-assay-design.software.informer.com) (Table 1). To determine run-to-run consistency and reproducibility, we calculated the intra-assay coefficient of variation by running the *cytb* assay on 3 samples in quintuplicate. We analyzed samples using Pyromark Q24 version 1.0.10 software (QIAGEN).

**Sanger Sequencing**

We interrogated *kelch13* (kelch protein gene on chromosome 13) for 20 SNPs according to Institut Pasteur protocols using primers published elsewhere (8,9) (Table 1). To validate the results obtained from pyrosequencing, we performed a quality control check of the gene targets using the first 10% of cases from each time period and controls. We designed sequencing primers with Primer 3 (10,11) and performed PCR using primers published elsewhere (8,9,12) that target drug resistance genes according to the Accuprime PfX SuperMix protocol (Thermo Fisher) (Table 1); we adjusted the annealing temperature to 60°C and cycles to 40 for *cytb* and 45 for all *mdr1* and *dhfr* assays. We ran 3 μL of PCR product on a 1% agarose gel at 100 V for 30 min to ensure band presence before sequencing.

We performed sequencing reactions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher) with a volume of 20 μL, including 1 μL of PCR product, 2 μL of Big Dye ready reaction mix, 3 μL of Thermo Fisher sequencing buffer, and 2 μL of 10 mmol/L of primer. We used a Bio-Rad C1000 Thermal Cycler (https://www.bio-rad.com) for 1 min at 96°C, 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. We purified product using an in-house isopropanol protocol including incubating at room temperature using 15 μL of 100% isopropanol for 15 min, centrifuging at 3,000 × g for 30 min at room temperature, centrifuging at 2,000 × g for 10 min after 35 μL of 70% isopropanol had been added, then inverting the plate and centrifuging for 700 × g at room temperature for 1 min. Last, we added 20 μL of Hi-Di Formamide (Thermo Fisher) to reconstitute each sample and sequenced using an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher). We standardized data using a Thermo Fisher sequencing analysis program and used a BioEdit sequence alignment editor version 7.2.5 (https://bioedit.software.informer.com) to analyze the sequence for nucleotide changes at each identified SNP location.

**Pfmdr1 Copy Number Analysis**

We tested specimens for *pfmdr1* copy number using real-time qPCR with primers and probes published elsewhere (12), using a total volume of 25 μL, including 12.5 μL Taqman Universal MasterMix (Thermo Fisher) and 5 μL template DNA. We performed reactions...
on an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher) using the same conditions used to detect parasite DNA. We ran the process in triplicate for specimens and used the 2^ΔΔCT method to calculate relative expression (13).

**Statistical Analyses**

We considered a genotype mutant dominant if the mutant allelic frequency within the specimen was >50% and wildtype dominant if <50%. We calculated median and range pfmdr1 relative copy numbers for each time period and compared all categorical variables (proportions of mutant versus wildtype genotypes) using the Fisher exact test. We calculated median and range for mutant allelic frequencies within specimens and presented them descriptively across time periods to inform surveillance for resistance mutations over time. We compared differences in median pfmdr1 relative copy numbers across time periods using the Kruskal-Wallis H test and set significance at p <0.05. We analyzed data using Stata version 13 software (StataCorp LLC, https://www.stata.com).

**Results**

Over the enrollment period, we retrieved a total of 574 primary blood specimens from the malaria biobank containing ≥1 *Plasmodium* spp. determined using real-time qPCR: 566 (98.6%) specimens contained just 1 species of *Plasmodium* and the other 8 (1.4%) contained 2 *Plasmodium* species. Of specimens with single-species infections, 365/574 (64%) were positive only for *P. falciparum*, 142/574 (25%) only for *P. vivax*, 46/574 (8%) only for *P. ovale*, and 13/574 (2.3%) only for *P. malariae*. Of the specimens with 2 *Plasmodium* species, 3/574 (0.5%) were *P. falciparum–P. ovale* co-infections and 5/574 (0.9%) were *P. ovale–P. vivax* co-infections. Only 243/365 (67%) specimens with *P. falciparum* monoinfection were identified as unique cases, 75 (31%) occurring during July 2008–June 2009, 79 (33%) during July 2013–June 2014, and 89 (37%) during July 2017–June 2018. Of those 243 *P. falciparum*–only specimens from unique cases, 169 (70%) were from male patients, 66 (27%) from female patients, and 8 (3%) had no reported sex; there was no difference in proportions of sex across time periods (p = 0.47) (Table 2). Mean age was 39.2 (range 3–88) years, which did not differ between time periods (p = 0.61) (Table 2). Mean parasitemia was 0.3% (<0.01–24.0%) across all 3 time periods, and we saw no significant differences over time (p = 0.10) (Table 2). A total of 186 (77%) patients had documented travel history; 81 (33%) in West Africa, the most common region, and 40 (16%) in Nigeria, the most common country (Table 2). Only 5/243 (2%) unique *P. falciparum* specimens were from travelers to Southeast Asia, and 1 (0.4%) was from a traveler to the Caribbean (Table 2).

**Prevalence of Mutations in Mutant Dominant Genotypes**

All 243 unique *P. falciparum*–only specimens contained ≥1 resistance mutation. We detected the highest prevalence of mutant genotypes in *dhfr*; 212 (91%) cases demonstrated triple codon mutation N51I, C59R, and
S108N (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/28/4/21-0533-App1.pdf). *dfr* SNP analysis revealed an increase in S108N mutant genotypes from 89% in 2008–2009 to 100% in 2017–2018 (Appendix Table 1). *pfcrt* SNP analysis revealed a gradual decline in K76T mutant genotype from 57% in 2008–2009 to 33% in 2017–2018 (p = 0.011), coupled with a decrease in M74I and N75E from 52% in 2008–2009 to 27% in 2017–2018 (p = 0.010; Appendix Table 2). *dhps* A613T SNP analysis revealed an overall increase in mutant genotypes, from 12% in 2008–2009 to 28% in 2017–2018 (p = 0.024; Appendix Table 3). *mdr1* N86Y and D1246Y analysis revealed a decline in mutant genotypes from 2008–2009 and 2017–2018 (p<0.003; Appendix Table 4). We observed no difference in mutant genotype populations across time periods for *dhps* K540E, A581G, A437G, S436A, S436F *mdr1* Y184F, S1034T, or *atpase6* A623E (Appendix Tables 3–5). Last, we observed no mutant genotype populations for *cvtb* Y268NSC across time periods (Appendix Table 6).

**Mutant Allelic Frequency within Wildtype Dominant Specimens**

We presented summaries of mutant allelic frequencies within wild-type–dominant specimens (i.e., with <50% within-specimen mutation prevalence) for *dfr* (Appendix Table 1), *pfcrt* (Appendix Table 2), *dhps* (Appendix Table 3), *mdr1* and *mdr1* copy number (Appendix Table 4), *atpase6* (Appendix Table 5), and *cvtb* (Appendix Table 6). Many minor changes in allelic frequencies of questionable importance occurred across time periods (Appendix Tables 1–6). Notable findings include that *pfcrt* K76T and C72S mutant allele populations among wild-type infections declined significantly from 2008–2009 to 2017–2018 (Appendix Table 2). Conversely, mutant allele frequency within *mdr1* D1246Y increased from 7% in 2008–2009 to 16% in 2017–2018, despite a decline in overall proportion of mutant-dominant genotypes (i.e., with ≥50% within-specimen mutation frequency) from 17.6% to 3.5% across time periods (Appendix Table 4). Frequency of any allelic mutant among wildtype infections at *cvtb* Y268N increased from 0% in 2008–09 and 2013–2014 to 3% in 2017–2018 (Appendix Table 6).

**mdr1 Copy Number and kelch13 Mutations**

Using the 3D7 comparator reference strain, which has a *pfmdr1* copy number of 1, we found an increase in median *pfmdr1* copy number from 1.1 (range 0.83–1.4) in 2008–2009 to 1.9 (range 0.73–5.4) in 2017–2018 (p<0.001) (Appendix Table 4). *kelch13* SNP analysis of >20 codons revealed no mutations for all analyzed specimens, despite 5 specimens with confirmed *P. falciparum* being from Southeast Asia (data not shown). We observed 2 silent mutations at codon 553 and 561 in the 2017–2018 specimens (sequences deposited to GenBank under accession nos. OM489472 and OM489473); however, travel history was not recorded for those patients.

**Discussion**

We analyzed 243 unique cases of *P. falciparum* importation to Canada using pyrosequencing assays based on previously reported genetic markers known to confer drug resistance. Our results have provided stakeholders with a resource for tracking antimalarial resistance over time. Conventional SNP analysis involves Sanger sequencing and PCR–restriction fragment-length polymorphism, but over the past decade pyrosequencing has added utility for detecting drug-resistance markers (14). Now, pyrosequencing has been partially replaced by more powerful techniques, such as targeted next-generation sequencing, which remain accessible mostly just in reference or research laboratories because of bioinformatics requirements. Our analysis of *P. falciparum* markers of genotypic resistance using pyrosequencing provides evidence of a quantitative advantage over Sanger sequencing because allelic frequencies within wild-type populations can be monitored over time to capture emerging molecular resistance. We observed significant decreases in mutant genotypes for chloroquine resistance genes and an increase over time in the proportion of mutant genotypes for *dfr*, the gene conferring resistance to proguanil. We also observed significant increases in markers of antifolate drug resistance correlating with increases in mutant allelic frequencies within wild-type populations.

The emergence of antimicrobial-resistant *P. falciparum* began in 1957 when the first cases of resistance to chloroquine were observed along the Cambodia–Thailand border (15). *pfcrt* encodes for a transmembrane protein in the digestive vacuoles of *P. falciparum* parasites. Mutations of the *pfcrt* gene, specifically at positions 72, 74, 75, and 76, confer resistance to chloroquine (16–18). *pfmdr1* mutation N86Y combined with increased *pfmdr1* copy numbers, both of which have been documented in many parts of Southeast Asia and recently in sub-Saharan Africa, is also implicated in chloroquine resistance (12,17,19–21). Our data document a decline over time in *pfcrt* mutants from positions 72 and 76 in imported cases of *P. falciparum*, indicating molecular reversion in chloroquine resistance at these codons. This phenomenon may reflect the reduction in chloroquine drug pressure on specimens from West Africa across the 10-year study period, during which oral artemisinin combination therapy has supplanted chloroquine use.
Drugs targeting folate metabolism, including pyrimethamine, sulfadoxine, and proguanil, are common components of fixed-dose combination antimalarials and inhibit purine and pyrimidine biosynthetic pathways. Point mutations in the \textit{dhfr} and \textit{dhps} genes conferring sulfadoxine and pyrimethamine resistance were identified as early as 1988 in Kenya. \textit{dhps} mutations S436AF, A437G, A581G, and A613TS have been identified in sulfonamide-resistant specimens primarily found in Southeast Asia. Higher levels of resistance have been identified in specimens carrying multiple mutations conferring a synergistic effect, especially double mutation S436FA and A613TS. Overall, we documented an increase in all mutant \textit{dhps} genotypes except K540E. Increases in mutant genotypes coincided with overuse of chloroquine alternatives, which have been heavily deployed in sub-Saharan Africa.

\textit{dhfr} S108N is the essential driving force associated with resistance to pyrimethamine. Our data highlight evidence of antifolate resistance over time, which may relate to increasing reliance on antifolate drugs in the face of chloroquine resistance. Additional mutations including \textit{dhfr} N51I, C59R, and I164 have been associated with more pyrimethamine-resistant specimens. We did not identify increases in either N51I or C59R mutations and detected only one I164L specimen in the 2008–2009 time frame, with no subsequent occurrences.

Our findings are limited by a small sample set and incomplete travel history, but this limitation may suggest a decreased prevalence of I164L mutation in sub-Saharan Africa. Of interest, the \textit{dhfr} A16V mutation generally occurs in conjunction with S108N, which is known to cause cycloguanil resistance. However, our data demonstrate no such correlation in the specimens we analyzed. Analyses by many groups over the years have led to the conclusion that greater numbers of point mutations in \textit{dhfr} confer greater resistance, and cross-resistance between pyrimethamine and cycloguanil is a possibility, whereas S108N is the necessary first mutation responsible for pyrimethamine resistance. Our results coincide with previous literature resulting in data on a wave of antifolate resistance markers in specimens of \textit{P. falciparum} imported to Ontario.

Artemisinin derivatives, the most powerful antimalarials used clinically, have been the main focus of concern surrounding recent efforts to mitigate \textit{P. falciparum} antimicrobial resistance. After the initial surge of chloroquine resistance, which was quickly followed by a diminution of antifolate effectiveness, the World Health Organization recommended use of artemisinin-based combination therapies as first-line drugs of choice. However, similar to the experience when generations of antimalarial drugs have been heavily used in other endemic areas, many countries in Africa have seen strains of multidrug-resistant \textit{P. falciparum} appeared within 1 year of introducing artemisinin-based combination therapy formulations, occurring as early as 2003. In Southeast Asia, artemisinin resistance has been associated with mutations in the propeller region of \textit{P. falciparum kelch13}.

Recently, there have been reports of \textit{kelch13}-independent treatment failure with artemether/lumefantrine in \textit{P. falciparum} cases imported to the United Kingdom by persons with travel history to Angola, Liberia, and Uganda. Despite novel mutations, including G112E, outside of the propeller-encoding domain in some patients, no reduced artemisinin susceptibility has been reported in the Greater Mekong region of Southeast Asia. Our data from specimens predominantly from West Africa correlate with these and other findings corroborating an absence of detectable mutations in 20 SNPs identified in the \textit{kelch13} gene between codon 440 and the 3' end of the coding region. On the other hand, \textit{pfATP6}, which is also known as \textit{pfSERCA} or \textit{pfATPase6}, is a calcium ATPase gene involved in calcium ion transport. Point mutations at A623E and S769N in \textit{pfATP6} could be associated with artemisinin resistance. Increased prevalence of these point mutations may be responsible for the rise of resistance in areas such as Cambodia, French Guiana, and Senegal, where artemisinin use is uncontrolled. Our data suggest that artemisinin-resistant \textit{P. falciparum} infection is unlikely to occur in travelers returning to Canada, given that only 1 \textit{atpase6} A623E mutant was detected in both the 2008–2009 and 2013–2014 time frames, and none for the \textit{atpase6} S769N and \textit{kelch13} mutations.

The fixed-dose combination atovaquone/proguanil, an antimarial prophylactic popular among travelers, has been recommended more broadly in response to potential resistance to other antimicrobial classes, including artemisinins. However, documented cases with \textit{cytb} mutations have resulted in treatment failure associated with resistance to atovaquone. We documented an exceptionally high prevalence of \textit{P. falciparum} specimens (83%) with triple codon \textit{dhfr} 51, 59, 108 mutation, which
confer resistance to proguanil, leaving the partner drug, atovaquone, as the sole antimalarial effective in clearing *P. falciparum* (4 of every 5 cases in our setting). Any resistance to atovaquone, conferred by cyt*bd*268 mutations, or impaired atovaquone absorption can lead to treatment failure (37,38); resistance as a result of cyt*bd* Y268NSC has been identified in travelers returning from Kenya, Angola, and many parts of East Africa (22,39–41). We observed no cyt*bd*-dominant mutants in our study, but in some specimens, the mutant allele frequency in the wild-type specimens were close to 20%, suggesting ≈1/5 of the infection could be comprised of mutant strains. Whether having a minor population of allelic mutants instead of dominant mutant allelic frequency equating to an overall mutant genotype can lead to treatment failure or recrudescence is unknown, but nevertheless, that condition may be an early indicator of emerging atovaquone resistance. Identifying subpopulations of resistant mutant strains can help determine appropriate treatment strategies and follow-up; prevalence of resistant strains as low as 10% can double in vitro 50% inhibitory concentration to chloroquine (42). This possibility reiterates the value of pyrosequencing and targeted next generation sequencing, which enable quantification of minor allelic populations.

Our conclusions may be limited by lack of complete data on travel history, history of malaria infection, and treatment strategies and outcomes among study participants. Subtle changes in mutant allelic frequency noted across time periods may be attributable to travel proclivities, period-to-period natural variation in expression, or a true change in prevalence. The technical limitations of developing a pyrosequencing assay targeting positions 74 and 75 only enabled qualitative analysis for pfcrt double mutations at those positions. A technical limitation of pyrosequencing includes diminished accuracy of determining proportions <5%. Although kelch13 remains the only gene target analyzed via Sanger sequencing, minor alleles in mixed populations cannot be detected by Sanger sequencing; thus, pyrosequencing is a more useful and reliable sequencing technology for SNP analysis. Although pyrosequencing has largely been replaced by more effective techniques, such as targeted next-generation sequencing and possibly whole-genome sequencing, those newer techniques are limited by cost and the requirement for bioinformatics infrastructure and expertise, which generally confine their application to large reference or research laboratories. Future studies involving prospective recruitment of *P. falciparum*-infected travelers, with complete demographic, clinical, and treatment outcome data and use of targeted next-generation sequencing to identify other SNPs, risk factors, or confounders of resistance, could fill these knowledge gaps.

Surveillance of SNPs can serve as a sentinel of clinical resistance. Triple codon *dhfr* mutants, for example, were detected years before observation of increased sulfadoxine/pyrimethamine treatment failures in Kenya (43). Robust surveillance analyses over time of known markers conferring drug resistance in *P. falciparum* imported by travelers are lacking. We observed a high percentage of molecular resistance to proguanil and none to atovaquone. We observed an increase in imported antifolate-resistant of *P. falciparum* strains, including a decrease in chloroquine-resistant strains and virtually no signs of artemisinin resistance because of mutations at kelch13. Importation of resistant *P. falciparum* mono-infections highlights the importance of developing better surveillance tools to monitor drug resistance patterns based on time and source region. Such surveillance would also inform clinical decision-making and serve as a resource for tracking antimalarial resistance that clinicians could monitor for possible genetic markers of reduced drug efficacy.

**Acknowledgments**

We thank MR4 for providing us with *Plasmodium* small subunit ribosomal RNA clones contributed by Peter A. Zimmerman.

This study was funded by the Project Initiation Fund of Public Health Ontario. A.K.B. is supported as a clinician scientist by the Departments of Medicine at the University Health Network and University of Toronto.

Author contributions: A.K.B. conceived the study; A.K.B., R.K. and R.L., contributed to study design, data collection, and analysis and interpretation, and were primarily responsible for writing the manuscript. E.S., K.T., F.R., and S.N.P. contributed to data collection and critical appraisal and revision of the manuscript. A.S. contributed to data analysis and interpretation and appraisal and revision of the manuscript.

**About the Author**

Dr. Kariyawasam is a clinical microbiology fellow at the University of Alberta, Edmonton, Alberta, Canada. She completed her PhD on American tegumentary leishmaniasis in the laboratory of Dr. Boggild and has a longstanding interest in other imported vectorborne diseases.
References


Address for correspondence: Andrea K. Boggild, Tropical Disease Unit, Toronto General Hospital, 200 Elizabeth St, 13EN-218, Toronto, ON M5G 2C4, Canada; email: andrea.boggild@utoronto.ca

Having standard biological reference materials, such as antigens and antibodies, is crucial for developing comparable research across international institutions. However, the process of developing a standard can be long and difficult.

In this EID podcast, Dr. Tommy Rampling, a clinician and academic fellow at the Hospital for Tropical Diseases and University College in London, explains the intricacies behind the development and distribution of biological reference materials.
The coronavirus disease (COVID-19) pandemic has affected many areas of public health, including tuberculosis (TB) prevention and response activities (1). TB cases reported to the US National Tuberculosis Surveillance System (NTSS) in 2020 decreased 20% compared with the 2016–2019 average. We examined the correlation between TB medication dispensing data to TB case counts in NTSS and used a seasonal autoregressive integrated moving average model to predict expected 2020 counts. Trends in the TB medication data were correlated with trends in NTSS data during 2006–2019. There were fewer prescriptions and cases in 2020 than would be expected on the basis of previous trends. This decrease was particularly large during April–May 2020. These data are consistent with NTSS data, suggesting that underreporting is not occurring but not ruling out underdiagnosis or actual decline. Understanding the mechanisms behind the 2020 decline in reported TB cases will help TB programs better prepare for postpandemic cases.

We analyzed a pharmacy dataset to assess the 20% decline in tuberculosis (TB) cases reported to the US National Tuberculosis Surveillance System (NTSS) during the coronavirus disease pandemic in 2020 compared with the 2016–2019 average. We examined the correlation between TB medication dispensing data to TB case counts in NTSS and used a seasonal autoregressive integrated moving average model to predict expected 2020 counts. Trends in the TB medication data were correlated with trends in NTSS data during 2006–2019. There were fewer prescriptions and cases in 2020 than would be expected on the basis of previous trends. This decrease was particularly large during April–May 2020. These data are consistent with NTSS data, suggesting that underreporting is not occurring but not ruling out underdiagnosis or actual decline. Understanding the mechanisms behind the 2020 decline in reported TB cases will help TB programs better prepare for postpandemic cases.

Decrease in Tuberculosis Cases during COVID-19 Pandemic as Reflected by Outpatient Pharmacy Data, United States, 2020

Kathryn Winglee, Andrew N. Hill, Adam J. Langer, Julie L. Self

The coronavirus disease (COVID-19) pandemic has affected many areas of public health, including tuberculosis (TB) prevention and response activities (1). TB cases reported to the US National Tuberculosis Surveillance System (NTSS) in 2020 decreased 20% compared with the average number of cases reported during 2016–2019 (2). Although some annual decline is expected on the basis of public health investments in TB control and prevention, TB incidence decreased an average of only 2%–3% annually during the previous 10 years (3). A decline of nearly 20% raises concern that TB cases are being left undetected or unreported to public health agencies. A sharp decline in TB incidence in 2020 is possible, potentially because of control efforts undertaken to combat the COVID-19 pandemic or reduced immigration, leading to fewer cases among persons newly arriving in the United States from regions with higher TB incidence. We therefore sought to determine the extent to which this decline is actual, a surveillance artifact caused by underreporting, or representative of delayed or missed TB diagnoses. Understanding the underlying cause will help TB programs better allocate resources and prepare for TB cases after the pandemic. Analysis of TB-related trends in data sources unlikely to be affected by public health disruptions is a critical way to evaluate the mechanisms behind the reported decline.

One such source is the IQVIA (https://www.iqvia.com) prescription dataset, which captures >88% of all outpatient prescription activity in the United States, including retail, mail, and long-term care channels. These data have been used in public health to answer a variety of questions, including estimating costs of HIV preexposure prophylaxis (PrEP) (4); analyzing the demographics of persons who have been prescribed PrEP (5); identifying opioid prescription patterns (6–8); and assessing naloxone, antibiotic, and hydroxychloroquine prescriptions (9–12). Pharmacy data are particularly valuable for TB disease because of the unique drug regimens used to treat TB. Initial treatment for newly diagnosed drug-susceptible TB disease typically consists of 4 drugs: isoniazid, rifampin, ethambutol, and pyrazinamide (13). All 4 drugs are taken in the first 2 months. If drug susceptibility testing results do not demonstrate resistance to isoniazid or rifampin, this intensive phase is typically followed by a continuation phase consisting of just isoniazid and rifampin for an additional 2–4 months or longer, depending on response to treatment. Rifampin is used to treat multiple diseases, including TB, Neisseria meningitidis, Haemophilus influenzae, leprosy, and endocarditis. Rifampin and isoniazid (alone or in combination)
are also used to treat latent TB infection (LTBI). Ethambutol is used to treat TB and nontuberculous mycobacteria. Isoniazid is used to treat TB disease and LTBI and is rarely used for other diseases. Pyrazinamide is only used to treat TB disease. Thus, we focused on individual isoniazid and pyrazinamide prescriptions, because these prescriptions should generally indicate TB treatment even in the absence of information on concurrent prescriptions (14).

Although most US TB cases are treated in public health clinics that dispense their own medication, some cases are treated by private providers or by clinics that have their TB medications filled by retail pharmacies. Therefore, although it would not be practical to determine overall US TB disease incidence on the basis of outpatient pharmacy dispensing data, assessing the trend in dispensing of TB drugs is possible. We first determined whether the trends in IQVIA’s TB medication prescription data correlated with trends observed in NTSS data before the pandemic (pre-2020). Once this correlation was established, we compared changes in IQVIA TB prescription data with changes in TB cases reported to NTSS in 2020 to assess potential underreporting of TB cases to public health.

Methods

IQVIA Metrics

We used 5 different IQVIA databases in this analysis: National Prescription Audit (NPA), NPA Extended Insights, NPA New to Brand (NTB), NPA Regional, and Total Patient Tracker (TPT) ( Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-2014-App1.pdf). Data used in all pyrazinamide and isoniazid analyses were accessed March 8–11, 2021, so the most recent prescriptions available in all databases were from February 2021. We also selected azithromycin as a control antibiotic to look at the specificity of the analyses to TB data and generated azithromycin data on June 15, 2021.

NTSS Metrics

We used the provisional 2020 NTSS data frozen in February 2021 and reported in March 2021 (2). We removed all TB cases reported by US territories and freely associated states, leaving just cases reported by the 50 US states and the District of Columbia. Cases were aggregated either by treatment start date or case date. Case date was defined as the earliest of treatment start date, drug susceptibility testing date, and report date. If a case was missing the date used for aggregation (7.8% for treatment start date and 1.7% for case date), we excluded it from the analysis.

For comparison to the IQVIA dataset, we tested both including all cases and removing cases with drug resistance (which are unlikely to have been prescribed the drugs of interest). Removing cases with resistance meant removing all cases in which resistance to the drugs of interest was reported on either the first or last isolate for which testing was performed, as well as all cases for which the drug was not part of the initial treatment regimen and was not taken for ≥2 weeks. For isoniazid, we also removed cases that were rifampin resistant, because rifampin-resistant cases are generally not treated with isoniazid. This activity was reviewed by Centers for Disease Control and Prevention and was conducted consistent with applicable federal law and organization policy (e.g., 45 C.F.R. part 46, 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44 U.S.C. §3501 et seq).

Correlation Analyses

We made pairwise comparisons at the national level for all IQVIA databases (except NPA Regional) and metrics for both isoniazid and pyrazinamide against NTSS case counts. NTSS case counts either included all patients or removed cases with resistance and were aggregated either by case date or treatment start date. We tested aggregating the data at the month and quarter level. We used a Pearson correlation coefficient (r) for the aggregated dates available in both datasets to identify the combination (IQVIA database, NTSS aggregation, and month/quarter timeframe) with the strongest correlation (highest r), which was used in subsequent analyses. For visualization, we generated a linear model with IQVIA data as the outcome and NTSS data as the only covariate. We plotted model estimates and 95% prediction intervals with the data.

We conducted similar analyses at the state level by using NPA Regional (data not shown) to explore correlations at smaller geographic levels. On the basis of this analysis, we compared NTSS case counts with a treatment start date in 2019 or 2020 with resistant cases removed to IQVIA isoniazid or pyrazinamide New Prescription (NRx) counts in 2019 or 2020.

Modeling

Seasonal autoregressive integrated moving average (SARIMA) models used to predict 2020 patient counts were built on data from January 2006–December 2019 using all NTSS cases (aggregated by treatment start date month) or TPT projected patient counts for isoniazid and pyrazinamide. SARIMA models have been used previously to explore TB case counts in the United States (15). We fit models by using the auto.arima function with seasonal models from the R package.
We also generated a linear model between the 2 datasets, which we used to predict IQVIA 2020 counts from NTSS 2020 counts and compare those with actual 2020 IQVIA counts (Appendix). We performed all analyses using R version 4.0.2 (18).

Results

Correlation between IQVIA and NTSS Prepandemic

We first examined whether the trends identified in the IQVIA database correlate to trends in NTSS. For both drugs, the strongest correlation occurred when the databases were aggregated by month, NTSS aggregated by treatment start date after removing patients with resistance to the drug of interest and IQVIA aggregated by the TPT projected patient count totals (Appendix Tables 3, 4). This combination had a strong correlation for both drugs ($r = 0.89$ for isoniazid and 0.86 for pyrazinamide; Figure 1). We therefore used monthly aggregation wherever possible. To confirm the specificity of this relationship, we calculated the correlation between NTSS case counts and IQVIA projected patient counts for azithromycin, an antibiotic not used to treat TB (Appendix Figure 1). This $r$ was −0.61, lower in magnitude and opposite in sign compared with TB medications.

We further explored this relationship by comparing additional patient data in both databases. Although the relative proportions of age groups varied, in 2019 and 2020 >50% of patients were >45 years of age in both NTSS and IQVIA (Appendix Figure 2, panel A). NTSS and IQVIA isoniazid had a higher percentage of male than female patients in 2019 and 2020, whereas IQVIA pyrazinamide projected patient counts had a higher proportion of female than male patients. We also compared geographic distributions by using the NPA Regional NRx metric. We found a very high correlation between NTSS patient counts and IQVIA’s NRx for isoniazid ($r = 0.91$) and pyrazinamide ($r = 0.92$) in 2019 and 2020 ($r = 0.92$ and $r = 0.94$; Appendix Figure 3). The correlation between NTSS case counts was weaker but still strong when looking at azithromycin (Appendix Figure 1, panel B).

Large Decline in Both Databases in 2020

We next examined the percent difference each year during 2007–2020 (Figure 2, panel A). Using treatment start date to aggregate the data, we found that NTSS case counts decreased every year except 2007 and 2014. Before 2020, the largest decrease was in 2009, which coincided with the US economic recession (15), but a decrease of 26.7% occurred in 2020. This decrease is larger than reported previously (2), because treatment start date is missing for 2.8% of cases counted in 2019 and 5.2% of cases counted in 2020. Similarly, the isoniazid IQVIA projected patient counts generally decreased each year (except 2014 and 2016), and 2020 had the largest decrease (28.6%). In contrast, the pyrazinamide IQVIA patient counts revealed 4 years (2014, 2015, 2017, and 2019) in which more cases occurred than the previous year. Although 2020 pyrazinamide IQVIA patient counts had a large decrease (15.3%) compared with 2019, data for 2012 showed a larger decrease (17.2%), and the decrease in 2018 (14.4%) was similar. Azithromycin IQVIA data also showed multiple years with more projected patients than the previous year but a large drop in 2020 of −25.9% (Appendix Figure 1, panel C).

To further explore 2020 data, we compared each month of 2020 to the corresponding month in 2019 (Figure 2, panel B). For this analysis, we used NTB prescription data as the IQVIA metric because it has the strongest correlation after projected patient counts but enables users to look across all prescriptions.
Decrease in TB during COVID-19, United States

NTSS data, approximately the same number of cases occurred in January and February 2020 as in January and February 2019 (i.e., the percent difference was near zero). However, starting in March, the percent difference dropped to −10% of 2019 values and stayed below that level for the rest of the year; the lowest percent difference (fewest patients in 2020 compared to 2019) occurred in April (−44%) and May (−42%). IQVIA isoniazid prescriptions followed a similar trend; the decrease began in March and never rose above −10% of 2019 values after March. For isoniazid, the April and May values were −50% and −52% of 2019 values. IQVIA pyrazinamide prescriptions in January and February were around 2019 levels, began to drop in March, and reached their lowest levels in April (−37.5%), May (−39.4%), and June (−35.5%) before increasing again. However, unlike NTSS and IQVIA isoniazid prescriptions, IQVIA pyrazinamide prescriptions came close to 2019 levels and even exceeded 2019 prescriptions in November 2020 before decreasing again in December. The overall IQVIA database (IQVIA no filter) also followed this pattern: there were more prescriptions in 2020 than in 2019 in January and February, a large drop in April and May, and another drop in December. We observed similar trends when using projected patient counts (Appendix Figure 4), although the decreases tended to be larger. Azithromycin prescriptions also largely followed this trend, except the lowest percent difference was in May (Appendix Figure 1, panel D).

Fewer IQVIA Projected Patient Counts in 2020, Similarity to NTSS Trend

We next analyzed whether the large declines in 2020 were within the error of what was expected on the basis of previous trends. NTSS case counts and IQVIA isoniazid and pyrazinamide projected patient counts all demonstrated a general downward trend, with seasonal effects causing regular fluctuations (Appendix Figure 5). Thus, we fit a SARIMA model to each metric (Figure 3). Overall, these models had a good fit, with most of the fitted data within the 95% prediction interval (Appendix Figures 6–8). As we found for the percent
differences by month, the April–December 2020 NTSS monthly case counts and IQVIA isoniazid projected patient counts were all below the lower bound of the 95% prediction interval. In contrast, the IQVIA projected patient counts for pyrazinamide, although lower than the SARIMA model predictions for April–December, were only lower than the 95% prediction interval in April.

We also explored whether the 2020 IQVIA data were within what would be expected from NTSS case counts. As we found for pre-2020 data, we observed a strong correlation ($r \geq 0.83$) between the NTSS patients counts and the IQVIA projected patient counts in 2020 (Figure 4, panels A, B). We used a linear model between the 2 datasets to predict IQVIA 2020 counts from NTSS 2020 counts. If the actual IQVIA counts were outside the prediction interval, this result would have been a sign that although both datasets decreased, other factors could be causing the decline to differ between the 2 datasets. We found that the predictions for the IQVIA isoniazid projected patient counts were lower than predicted for all months in 2020 and below the 95% prediction interval for June–December (Figure 4, panel C). Conversely, the IQVIA pyrazinamide projected patient counts were generally very close to prediction and were within the 95% prediction interval for all months (Figure 4, panel D).

**Discussion**

Both NTSS case counts and IQVIA isoniazid, pyrazinamide, and azithromycin projected patient counts had a large decrease in 2020 compared with 2019; the lowest drops occurred in April and May. These months correspond to when large parts of the country had community mitigation measures in place, which resulted in decreased mobility, including delaying or avoiding of medical care (19,20). Although the percent change in the following months increased compared with values for April and May, changes still tended to be negative, suggesting that persons were not compensating by obtaining these prescriptions later in the year. In fact, based on our SARIMA models, the April–December 2020 declines were lower than expected from previous declining trends.

We found a strong correlation at the national level between NTSS case counts and isoniazid and pyrazinamide IQVIA projected patient counts, suggesting that isoniazid and pyrazinamide prescription data can serve as a proxy for trends in TB cases. In contrast, NTSS case counts and azithromycin (an antibiotic often used to treat chest infections such as pneumonia but not used for TB treatment) had a negative correlation, smaller in magnitude than isoniazid and pyrazinamide correlations. This finding suggests that this relationship is not being driven by general antibiotic prescriptions for respiratory diseases and supports the idea that the isoniazid and pyrazinamide relationship is specific to TB. In contrast, pyrazinamide, isoniazid, and azithromycin all had a strong state correlation, suggesting that the state relationship might be driven more by population than by the number of TB cases.

Differences between isoniazid and pyrazinamide prescriptions might be explained by the fact that isoniazid is prescribed for both LTBI and TB disease while pyrazinamide is prescribed only for TB disease and is generally used for only 2 months of disease treatment (as opposed to 6 months for isoniazid). For example, 2020 pyrazinamide counts were within prediction intervals from 2020 NTSS counts, whereas isoniazid

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*Figure 3. Tuberculosis cases reported to the National TB Surveillance System (NTSS) and IQVIA (https://www.iqvia.com) projected patient counts in 2020 compared with previous years, United States. A seasonal autoregressive integrated moving average model was fit to January 2006–December 2019 data from cases reported to NTSS by treatment start date (A), IQVIA isoniazid projected patient counts (B), or IQVIA pyrazinamide projected patient counts (C) (for model details, see Appendix Figures 6–8, https://wwwnc.cdc.gov/EID/article/28/4/21-2014-App1.pdf). Light gray indicates model with 95% prediction intervals, which was used to forecast 2020 counts with 95% prediction intervals (dark gray). Black dots represent the number of cases (A) or projected patient counts (B, C) each month. Vertical axes in each plot are different because of different scales.*
Decrease in TB during COVID-19, United States

Counts of LTBI treatment were lower. This finding hints at a decline in LTBI treatment as well. However, the large decrease in 2020 compared with 2019, especially in April and May, was not specific to isoniazid and pyrazinamide; it was also seen in the overall database and in azithromycin prescriptions, indicating a general decline in the number of prescriptions dispensed in 2020.

The first limitation of our analysis is that IQVIA data only cover outpatient prescriptions, meaning that they are missing hospital prescriptions and are likely also missing prescriptions from health departments, which is where many TB patients receive their treatment. The proportion of TB cases covered by IQVIA pharmacy data is unknown, but total pyrazinamide TPT counts averaged 36% of NTSS case counts annually in this analysis. However, estimating the number of TB cases from pyrazinamide prescriptions is likely to be inaccurate, given that not all TB patients receive pyrazinamide treatment, doctors may prescribe pyrazinamide for off-label reasons, and treatments spanning multiple years would result in patients being counted twice. Given this limitation, we chose to focus on the trends between

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**Figure 4.** Comparison of National Tuberculosis Surveillance System (NTSS) case counts and IQVIA projected patient counts for isoniazid or pyrazinamide prescriptions, United States, 2020. A, B) Projected patient counts for isoniazid (A) and pyrazinamide (B). Horizontal axis of each plot shows NTSS patient counts aggregated by treatment start date (month), removing patients who had reported resistance. Each point represents a month in 2020. The Pearson correlation coefficient (r) is shown in lower righthand corner of each plot. C, D) A linear model fit to the 2006–2019 data (Figure 1) with quarter as a covariate to predict 2020 IQVIA projected patient counts for isoniazid (C) or pyrazinamide (D). Black line indicates actual data; gray line indicates expected IQVIA counts with 95% prediction intervals. Note vertical axes are different because of different scales for isoniazid and pyrazinamide in the IQVIA dataset. NTSS, National Tuberculosis Surveillance System.
datasets. Despite the lack of hospital and health department data, we still found a strong correlation, suggesting this limitation does not affect the trends between datasets and that the proportion of TB cases in the IQVIA database has remained relatively consistent. Second, we did not analyze concurrent prescriptions (i.e., multiple drugs prescribed at the same time), which could change the results by helping separate TB disease and LTBI treatment. However, IQVIA data indicate only whether patients were prescribed multiple prescriptions at any point during the same year, rather than concurrently prescribed or initiated. Finally, a patient starting treatment at the start or end of the month might be counted in different months in the 2 databases.

If the IQVIA data had followed the NTSS trend for 2006–2019 but had not followed the NTSS trend for 2020, that finding would have suggested the decline in cases reported to NTSS was a surveillance artifact. However, IQVIA outpatient prescription data correlated with NTSS case counts from public health, which suggests that substantial underreporting is not a likely explanation for the decline in TB incidence in 2020. Even so, these analyses are not able to distinguish between a decline in actual TB incidence versus widespread underdiagnosis of TB (because of misdiagnosis as COVID-19 or because of persons with TB symptoms avoiding seeking medical attention out of fear of being exposed to or diagnosed with COVID-19). The COVID-19 pandemic has caused many disruptions to healthcare and public health, and delays in TB diagnosis because of the COVID-19 pandemic have already been reported (21). In fact, in the IQVIA data, the decrease was not specific to anti-TB medications but was seen when analyzing all prescriptions as well as azithromycin, even though azithromycin was not correlated with NTSS cases before 2019. Other studies have also reported large decreases in antibiotic prescriptions (22,23). Combined, this indicates a general overall decline in 2020 in prescriptions and, therefore, diagnoses.

This general decline is further supported by a study that reported that 41% of US adults had delayed or avoided seeking medical care by June 2020 (20). An analysis of health insurance claims from US persons with employer-sponsored insurance also showed large reductions (>20% for most services analyzed) in preventive and elective care, as well as in patient visits in March and April 2020 (24). In contrast, that study found prescription drug use for statins and antidiabetic medications decreased only 2%–3%, whereas asthma medication increased by 11%, although larger decreases in antibiotic prescriptions have been reported elsewhere (22,23). Thus, although healthcare use might have declined overall, this decline is not uniform across diseases. In fact, in the United States, the estimated number of newly diagnosed cancer cases in 2020 increased compared with 2019, but the number of HIV diagnoses decreased (31,670 in 2019 compared with 29,744 in 2020, a 6.1% decrease) (25–28). Thus, the decline in US TB cases and prescriptions was on par with declines in preventive care usage but was larger than the case counts and prescriptions for other diseases that result in longer treatments, although 2020 estimates for many conditions have not yet been reported. Nonetheless, TB cases should continue to be monitored closely, especially given the highly infectious nature of TB and the chance of increased illness and deaths if treatment is delayed.

Our study demonstrates that IQVIA isoniazid and pyrazinamide projected patient counts are strongly correlated with NTSS TB case counts and also shows large declines in 2020, helping to rule out underreporting as the cause of the large decline in reported TB cases in the United States. Evidence of underreporting would have suggested that public health practitioners were not aware of these cases but patients were still receiving timely treatment, resulting in decreased illness, deaths, and infectiousness. However, although we have provided evidence against underreporting, the strong possibility of underdiagnosis means that public health programs should be prepared for a possible rebound in TB cases after the pandemic, because delayed and missed diagnoses could result in increased transmission as patients remain infectious for longer periods of time.

Acknowledgments
We thank Roque Miramontes for his help in formulating the project and Justin Davis for his help with obtaining access to the IQVIA data. We thank Jonathan Wortham, Sapna Bamrah Morris, Laura Vonnahe, and Kristine Schmit for consultation on TB medications. We also thank Elizabeth Gray, Jason Mazzarella, and Anum Siddiqui for their assistance with the IQVIA data.

About the Author
Dr. Winglee is a statistician (data scientist) in the Division of Tuberculosis Elimination, National Center for HIV, Viral Hepatitis, STD, & TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia. Her research interests include statistical analyses of infectious disease data, including bacterial genomics and epidemiologic studies.

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Decrease in TB during COVID-19, United States


Address for correspondence: Kathryn Winglee, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop US12-4, Atlanta, GA 30329-4027, USA; email: nr1@cdc.gov

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 28, No. 4, April 2022
**Durability of Antibody Response and Frequency of SARS-CoV-2 Infection 6 Months after COVID-19 Vaccination in Healthcare Workers**


Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies decay but persist 6 months postvaccination; lower levels of neutralizing titers persist against Delta than wild-type virus. Of 227 vaccinated healthcare workers tested, only 2 experienced outpatient symptomatic breakthrough infections, despite 59/227 exhibiting serologic evidence of SARS-CoV-2 infection, defined as presence of nucleocapsid protein antibodies.

Neutralizing antibodies (nAbs) and binding antibodies (bAbs) appear to be associated with protection against symptomatic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and coronavirus disease (COVID-19) (1,2). Early assessments of the Pfizer-BioNTech (https://www.pfizer.com) BNT162b2 COVID-19 mRNA vaccine observed >95% effectiveness against predominantly Alpha infections (3), but the potential effect of waning post-vaccine neutralizing titers is an ongoing concern (4).

Apparent increases in vaccine-breakthrough infections may result from waning antibody titers, increases in exposure risk, and reduced vaccine effectiveness against Delta and other variants. In mid-2021, Delta became the dominant virus type in the United States (5). Delta appears to cause increased hospitalization rates and has increased transmissibility compared with Alpha and other pre-Delta variants (6; Bolze et al., unpub. data, https://doi.org/10.1101/2021.06.20.21259195). We report bAb and nAb levels as well as clinically overt and asymptomatic breakthrough infections that occurred among US healthcare workers in the Prospective Assessment of SARS-CoV-2 Seroconversion (PASS) study (7), conducted during January–August 2021.

**The Study**

The PASS study protocol was approved by the Uniformed Services University of the Health Sciences Institutional Review Board (Federalwide Assurance no. 00001628, US Department of Defense Assurance no. P60001) in compliance with all applicable federal regulations governing the protection of human participants. Written consent was obtained from all study participants.


DOI: https://doi.org/10.3201/eid2804.212037
For the PASS study, we enrolled and followed generally healthy, adult healthcare workers (HCWs) at Walter Reed National Military Medical Center (Bethesda, MD, USA) who were seronegative for IgG to SARS-CoV-2 spike glycoprotein (spike) and had no history of COVID-19, as previously described (7).

We collected participants’ serum samples monthly and screened them for IgG against SARS-CoV-2 spike and nucleocapsid protein (NP) in multiplex microsphere-based immunoassays, as previously described (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-2037-App1.pdf) (E.D. Laing, unpub. data, https://doi.org/10.1101/2021.02.10.21251518). In addition, we asked participants to obtain nasopharyngeal SARS-CoV-2 PCR testing at a designated COVID-19 testing center if they experienced symptoms consistent with SARS-CoV-2 infection.

To quantify spike IgG bAbs in World Health Organization binding antibody units (BAU), we interpolated IgG levels against an internal standard curve calibrated to the Human SARS-CoV-2 Serology Standard (Appendix Figure 1). We assessed serum samples for nAbs against SARS-CoV-2 wild type and Delta as previously described by using a well-characterized SARS-CoV-2 lentiviral-pseudovirus neutralization assay (Appendix) (8).

Excluding persons infected before January 31, 2021, the study followed 227 participants fully vaccinated

Table. Demographic characteristics of US healthcare worker participants in the Prospective Assessment of SARS-CoV-2 Seroconversion (PASS) study, January–August 2021*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BNT162b2 vaccinated</th>
<th>Vaccinated with 6-mo follow-up bAb</th>
<th>Vaccinated with 6-mo follow-up nAb titers</th>
<th>Unvaccinated</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>227 (100)</td>
<td>187 (100)</td>
<td>49 (100)</td>
<td>17 (100)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>F</td>
<td>159 (70)</td>
<td>131 (70)</td>
<td>33 (67)</td>
<td>13 (76)</td>
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<tr>
<td>M</td>
<td>68 (30)</td>
<td>56 (30)</td>
<td>16 (33)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
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<tr>
<td>Non-Hispanic</td>
<td>209 (92)</td>
<td>175 (94)</td>
<td>47 (96)</td>
<td>16 (94)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>14 (6)</td>
<td>10 (5)</td>
<td>1 (2)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Not reported</td>
<td>4 (2)</td>
<td>2 (1)</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>165 (73)</td>
<td>139 (74)</td>
<td>35 (71)</td>
<td>9 (53)</td>
</tr>
<tr>
<td>Black</td>
<td>26 (11.5)</td>
<td>18 (9.5)</td>
<td>4 (8)</td>
<td>6 (35)</td>
</tr>
<tr>
<td>Asian</td>
<td>23 (10)</td>
<td>19 (10)</td>
<td>8 (16)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>&gt;2 races</td>
<td>7 (3)</td>
<td>6 (3)</td>
<td>1 (2)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Native Hawaiian or other Pacific Islander</td>
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<td>1 (0.5)</td>
<td>1 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Not reported</td>
<td>5 (2)</td>
<td>4 (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Age, y, mean (range)</td>
<td>41.7 (20–69)</td>
<td>42.8 (21–69)</td>
<td>44.7 (26–69)</td>
<td>32.8 (19–50)</td>
</tr>
</tbody>
</table>

*Values are no. (%) except as indicated. bAb, binding antibodies; nAb, neutralizing antibodies.

Figure 1. Vaccine-induced binding and neutralizing antibody responses observed among US healthcare worker participants in the Prospective Assessment of SARS-CoV-2 Seroconversion (PASS) study, January–August 2021. A) MFI levels of vaccine-induced spike IgG binding before and after second vaccination in serum samples diluted 1:400 (n = 227 participants). Horizontal line indicates the positive or negative spike IgG threshold. B) Spike IgG binding antibodies (BAU/mL) quantified from serum samples collected 1 month (mean 36.9 days, range 23–81 days) and 6 months (mean 201.1 days, range 151–237 days) postvaccination (n = 187 participants). Wilcoxon matched-pairs signed rank test performed; y-axis is log2-scale. C) Neutralizing antibody titers against severe acute respiratory syndrome coronavirus 2 wild-type and Delta variant from serum samples collected 1 month (mean 30.8 days, range 28–42 days) and 6 months (mean 200.1 days, range 189–219 days) postvaccination (n = 49 participants). Friedman ANOVA with Dunn’s multiple comparisons performed post-hoc; y-axis is log2-scale. All errors bars represent the geometric mean and 95% CIs. BAU, binding antibody units; IC50, 50% inhibitory concentration; MFI, median fluorescence intensity.
with BNT162b2 vaccine and 17 unvaccinated participants. Participants were generally healthy, had a mean age of 41.7 (range 20–69) years, and were predominantly women (Table). Vaccinated and unvaccinated participants reported similar in-hospital time; >70% of each group worked in the hospital >15 days per month, and had similar rates of direct interaction with COVID-19 positive patients (monthly average of 47% for vaccinated and 45% for unvaccinated participants).

We observed seroconversion in all participants 1 month after the second vaccine dose (Figure 1, panel A). We quantified spike IgG bAbs at 1 and 6 months after full vaccination in the 187 vaccinated participants with serum samples collected at both timepoints. Spike IgG bAbs decreased from a geometric mean of 1,929 BAU/mL (95% CI 1,752–2,124 BAU/mL) at 1 month postvaccination to a geometric mean of 442 BAU/mL (95% CI 399–490 BAU/mL) at 6 months postvaccination (p<0.001) (Figure 1, panel B). Similarly, we observed decay of nAbs between the 1- and 6-month postvaccination timepoints. Peak SARS-CoV-2 wildtype nAbs decreased from a geometric mean titer (GMT) of 551 (95% CI 455–669 GMT) to 98 GMT (95% CI 78–124 GMT) 6 months after full vaccination (Figure 1, panel C). The GMTs of nAbs were significantly higher against wild-type compared with Delta SARS-CoV-2 at both timepoints after vaccination (Figure 1, panel C). In comparison, nAbs against Delta decayed from 279 GMT (95% CI 219–355 GMT) at peak to 38 GMT (95% CI 31–48 GMT) after 6 months. Quantitative IgG bAb (in BAU/mL) correlated with nAb titers (ρ = 0.70; p<0.001), demonstrating comparable decay of IgG bAbs and nAbs (Appendix Figure 2).

In addition to spike IgG bAbs, we also monitored for seroconversion of IgG bAbs to NP. Of vaccinated participants, 26.0% (59/227) had NP seroconversion during March–August 2021 (Figure 2). Only 2 had symptomatic, PCR-positive, vaccine-breakthrough infections, both of which were self-limited, outpatient...
cases. In the unvaccinated cohort, 4 participants had SARS-CoV-2 infection diagnosed: 2 by PCR while experiencing symptomatic infection (1 outpatient case, 1 requiring intensive care) and 2 diagnosed by spike IgG seroconversion and who reported mild symptoms retrospectively. The frequency of NP seroconversions in the vaccinated population correlated with the frequency of SARS-CoV-2 infections diagnosed in the unvaccinated participants (23.5% [4/17]) (Figure 2), suggesting similar exposure rates.

Conclusions
In this prospective cohort study of generally healthy, adult HCWs, we found that SARS-CoV-2 spike IgG bAbs and nAbs induced by BNT162b2 mRNA COVID-19 vaccination waned but remained detectable through 6 months after vaccination, corroborating results of another study (9). Consistent with another report (10), we observed significantly lower vaccine-induced nAb titers against Delta compared to wild-type virus. Asymptomatic infections determined by NP seroconversions were relatively frequent, but symptomatic infection was rare, and severe disease was absent.

We observed 1 of 17 unvaccinated persons have onset of severe COVID-19, versus no severe cases among 227 vaccinated participants. Of vaccinated persons, 2 had symptomatic, PCR-proven breakthrough infections, both of which were managed as outpatient cases. We observed that 26% of vaccinated persons developed antibodies against SARS-CoV-2 NP, suggesting that vaccinated persons experienced exposures to SARS-CoV-2 as frequently as the unvaccinated population, yet rarely had onset of overt clinical disease.

The strengths of the study include frequency of serologic assessments and use of variant specific nAb in addition to multiplexed antigen-specific IgG detection. Use of longitudinal serologic assessments (in addition to PCR testing when participants exhibited symptoms) enabled detection of asymptomatic and pauci-symptomatic SARS-CoV-2 exposures. Although our study was powered to show clear differences in antibody titers over time, limitations include the moderate size of the cohort and the small number of unvaccinated participants. Further, seasonal human coronavirus (HCoV) infections may drive cross-reactive IgG responses against SARS-CoV-2 NP. We mitigated the likelihood of HCoV-driven false-positives by using convalescent serum samples from persons with PCR-confirmed HCoV infections to establish the threshold for SARS-CoV-2 NP IgG positivity, which had a specificity of 94% in our multiplex assay (E.D. Laing et al., unpub. data). In a separate study, NP seroconversion reportedly occurred in only 71% of PCR-confirmed vaccine-breakthrough infections (11); thus, some instances of asymptomatic vaccine-breakthrough infections may have gone unnoticed.

We observed persistence of nAb titers against SARS-CoV-2 wild-type equal to or greater than the lowest dilution tested in 90% (44/49) of healthy adults 6 months after vaccination with BNT162b2. Neutralizing activity against Delta virus was lower; only 47% (23/49) of participants maintained nAb titers above the lowest dilution at 6 months post-vaccination. The decrease in nAb does not necessarily mean that persons have lost protection against severe COVID-19, however, given that nAb titers required for protection remain unknown and virus neutralization is only 1 function of antibodies. In addition, memory B cells and T cells have been detected 8–12 months after SARS-CoV-2 infection, demonstrating that adaptive immune memory can be long-lasting (12,13). Further research is needed to understand the correlates of protection against moderate to severe COVID-19 for known and emerging SARS-CoV-2 variants. Even so, our results suggest that the BNT162b2 vaccine confers protection against severe clinical disease caused by the variants circulating in the United States through August 2021 for ≥6 months in generally healthy adults, even in the face of frequent exposures to the virus and waning antibody titers.

This work was supported by awards from the Defense Health Program and the CARES Act (grant nos. HU00012120067, HU00012120104, and HU00012120094) and the National Institute of Allergy and Infectious Disease (grant no. HU00011920111). The protocol was executed by the Infectious Disease Clinical Research Program, a US Department of Defense program executed by the Uniformed Services University of the Health Sciences through a cooperative agreement by the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. This project has been funded in part by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (interagency agreement no. Y1-AI-5072).

E.D.L., C.D.W., W.W., R.V., T.C., K.M.H.-P., C.A.D., A.M.W.M., T.H.B., C.O., C.C.B., and E.M. are military service members or employees of the US government. This work was prepared as part of their official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the US government. Title 17, U.S.C., §101 defines a US government work as a...
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D.R.T., T.H.B., S.D.P., the Uniformed Services University Infectious Diseases Clinical Research Program (a US Department of Defense institution), and the Henry M. Jackson Foundation were funded under a Cooperative Research and Development Agreement to conduct an unrelated Phase III COVID-19 monoclonal antibody immune-prophylaxis trial sponsored by AstraZeneca. The Henry M. Jackson Foundation, in support of the Uniformed Services University Infectious Diseases Clinical Research Program, was funded by the Department of Defense Joint Program Executive Office for Chemical, Biological, Radiological, and Nuclear Defense to augment the conduct of an unrelated Phase III vaccine trial sponsored by AstraZeneca. Both these trials were part of the US government COVID-19 response. Neither is related to the work presented in this article.

About the Author

Dr. Laing is an assistant professor at the Uniformed Services University, Bethesda. His primary areas of research are infectious and emerging zoonotic pathogens, with a focus on understanding mechanisms of virus virulence and co-evolutionary adaptations of bat immune responses to virus infections.

References


Address for correspondence: Edward Mitre, Department of Microbiology and Immunology, Uniformed Services University, 4301 Jones Bridge Rd, Bethesda, MD 20814, USA; email: edward.mitre@usuhs.edu
In October 2020, the Tennessee Department of Health (Nashville, Tennessee, USA) was notified of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in 3 Malayan tigers (Panthera tigris jacksoni) at a zoo in the state. Felids, including domestic cats and exotic big cats, have greater susceptibility to SARS-CoV-2 infection than other species (1–4). Infected domestic cats can transmit the virus to other cats via respiratory droplets or direct contact (4–6). However, the risk for cat-to-human transmission remains unclear. We investigated the SARS-CoV-2 outbreak in Tennessee to determine its source and provide recommendations to control the spread of infection.

The Study

Tiger 1, the index case, began showing clinical signs of coronavirus disease (COVID-19), including lethargy, anorexia, and nonproductive cough, on October 12, 2020. Subsequent onset of clinical signs occurred in tiger 2 on October 16 and in tiger 3 on October 17. Oropharyngeal swab specimens were collected under sedation on October 19 and October 27 for SARS-CoV-2 diagnostic testing and submitted to the Runstadler Laboratory, Cummings School of Veterinary Medicine at Tufts University (North Grafton, Massachusetts, USA) for initial testing for open reading frame (ORF) 1b-nsp14 (7). Presumptive positive SARS-CoV-2 diagnoses were made in all 3 animals by real-time reverse transcription PCR (RT-PCR) performed by using Mag-Bind Viral RNA Xpress Kit (Omega BioTek, https://www.omegabiotek.com) and UltraPlex 1-Step ToughMix (Quantabio, https://www.quantabio.com). Tiger specimens were then sent to the US Department of Agriculture National Veterinary Services Laboratories (Ames, Iowa, USA) for confirmation by RT-PCR and whole-genome sequencing (3).

We conducted an environmental assessment at the zoo on October 29, 2020. The tiger exhibit has 3 primary outdoor visitor viewing areas: 2 outdoor viewing areas with fencing between humans and animals creating a separation of ≈6 feet, and 1 outdoor overhead viewpoint where visitors could view animals from a platform ≥8 feet above the tiger enclosure. The tiger’s off-exhibit den areas have concrete walls between each animal enclosure and metal interior caging with open airflow that enables keepers to see the tigers. The den areas are directly adjacent to or across from each other.

We observed consistent use of personal protective equipment by zoo employees and veterinary students according to zoo policy. All employees and students in close contact with the tigers before the animals began to show signs of illness wore disposable gloves...
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and cloth facemasks. After the onset of clinical signs of illness and SARS-CoV-2 testing in tigers, persons in the tiger den area wore protective coveralls, disposable gloves, and plastic face shields. At the time of this outbreak in October 2020, fewer data existed for relative mask efficacy, and mask types were not further specified by zoo policy.

Before and after onset of animal illness, cleaning practices in the off-exhibit cages included use of high-pressure water hoses to clean the floors. Staff used disinfectants daily in the den area, and we confirmed disinfectants were on the US Environmental Protection Agency’s List N: Disinfectants for Coronavirus (COVID-19) (https://www.epa.gov/coronavirus/about-list-n-disinfectants-coronavirus-covid-19-0). All zoo employees and veterinary students used a self-reported evaluation tool via mobile phone that screened for COVID-19 symptoms before their shifts. Zoo visitors were encouraged to wear masks; masks were not required in outdoor spaces at the time of this outbreak.

We also conducted an epidemiologic investigation on October 29, 2020. Our investigation focused on the timeframe beginning 2 weeks before onset of index tiger clinical signs (starting September 28) until date of investigation (October 29). We identified 18 zoo employees and veterinary students who prepared food for or were in close contact with the tigers during this timeframe. For this investigation, we defined close contact to tigers as being within 6 feet of any tiger at the zoo for any length of time during the observation period (September 28–October 29). We selected these proximity criteria based on the US Centers for Disease Control and Prevention (CDC) definition of close contact defined (8); however, SARS-CoV-2 transmission can occur from inhalation of virus in the air >6 feet from an infectious source (9,10). During the week after the index tiger showed signs of illness, community transmission of SARS-CoV-2 was at a 7-day average of 101 new cases/day in the county where this zoo is located, and the 7-day test positivity rate was 10.2%.

We identified 2 employees with COVID-19 during the September 28–October 29 timeframe: a tiger keeper and veterinary clinic assistant. Contact tracing identified an additional household contact to the SARS-CoV-2–positive tiger keeper, but no other SARS-CoV-2–positive contacts were identified. We created a timeline comparing signs of animal illness onset and RT-PCR cycle threshold values with dates of zoo employee symptom onset and testing (Figure 1).

We sent specimens from all zoo employees and veterinary students who tested positive for SARS-CoV-2 to CDC for sequencing and genomic analyses. CDC staff performed whole-genome sequencing as previously described (12). We phylogenetically compared tiger sequences with 30 sequences from 834 Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 28, No. 4, April 2022

Figure 1. Timeline of events identified during the epidemiologic investigation of an outbreak of SARS-CoV-2 infection among Malayan tigers and humans at a zoo, Tennessee, USA, October 12–30, 2020. Dates related to tiger events are shown above the timeline; dates related to human events are shown below the timeline. C\textsubscript{T} values for the first positive open reading frame 1b reverse transcription PCR test per animal are shown; methods for extraction and C\textsubscript{T} value calculation were described previously by Sawatzki et al. (11). C\textsubscript{T}, cycle threshold; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
SARS-CoV-2 among Malayan Tigers and Humans, USA

geographically-associated human SARS-CoV-2 cases collected from the county surrounding the zoo during October 29–November 12, 2020. We also compared tiger sequences with 233 statewide background sequences from specimens collected in Tennessee during March 1–November 12, 2020.

Most viral sequences clustered into NextStrain Clade 20G and Pangolin lineage B.1.2, which correspond to the predominant clades observed for human specimens from Tennessee during the time of the outbreak investigation. Sequence analysis showed 3–6 SNP differences between 1 human tiger keeper and all 3 tiger sequences (GISAID accession nos. EPI_ISL_292844–6). Differences are indicated by 1-step edges (lines) between colored dots (individual SARS-CoV-2 sequenced infections). Numbers indicate unique sequences. Phylogenetic relationships were inferred through approximate maximum-likelihood analyses implemented in TreeTime (13) by using the NextStrain pipeline (14). All high-quality genome sequences from Tennessee were downloaded from the GISAID (https://www.gisaid.org) database on March 16, 2021. Pangolin lineages for investigation sequences were assigned on March 16, 2021. Not all analyzed sequences are shown in this figure because some were outside clade 20G. CDC, Centers for Disease Control and Prevention; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SNP, single-nucleotide polymorphism.

Figure 2. Whole-genome phylogenetic analysis from of an outbreak of SARS-CoV-2 infection among Malayan tigers and humans at a zoo, Tennessee, USA, October 2020. The tree shows a close-up view of clade 20G divergence estimates from the SARS-CoV-2 Wuhan-Hu-1 reference genome and sequences from humans living in Tennessee and Malayan tigers sampled during the outbreak investigation. Sequence analysis showed 3–6 SNP differences between 1 human tiger keeper and all 3 tiger sequences (GISAID accession nos. EPI_ISL_292844–6). Differences are indicated by 1-step edges (lines) between colored dots (individual SARS-CoV-2 sequenced infections). Numbers indicate unique sequences. Phylogenetic relationships were inferred through approximate maximum-likelihood analyses implemented in TreeTime (13) by using the NextStrain pipeline (14). All high-quality genome sequences from Tennessee were downloaded from the GISAID (https://www.gisaid.org) database on March 16, 2021. Pangolin lineages for investigation sequences were assigned on March 16, 2021. Not all analyzed sequences are shown in this figure because some were outside clade 20G. CDC, Centers for Disease Control and Prevention; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SNP, single-nucleotide polymorphism.

Conclusions
We describe SARS-CoV-2 infection in captive tigers with respiratory clinical signs and provide additional evidence for nonhuman species as hosts for SARS-CoV-2. Findings of this study support tigers’ susceptibility to the virus and potential for sustained transmission among large cats and a risk for zoonotic transmission to humans. The SARS-CoV-2 sequence from the tiger keeper was 3 SNP's different from tigers 1 and 2 and 6 SNP's different from tiger 3. The close genetic relationship between viruses of the tigers and tiger keeper is consistent with the timing of clinical
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signs of illness and job duties of the tiger keeper, although transmission source or zoonotic transmission cannot be proven from these data alone.

These findings have implications for both the public health and zoologic communities. Zoos should be aware of the possibility of animal infection through incidental exposure by the public or asymptomatic staff members. Humans with known or suspected infection should avoid direct or indirect exposure to susceptible species unless completely unavoidable to avoid potential transmission. Results of this investigation should also prompt zoo and wildlife organizations to reevaluate biosecurity and administrative protocols to minimize risk to and from employees, students, volunteers, and the visiting public interacting with susceptible species.

This work was support by the National Institutes of Health (grant no. HHSN272201400008C/Al/NIAID to K.S.)

About the Author

Dr. Grome is an infectious diseases physician and Epidemic Intelligence Service officer in the Center For Surveillance, Epidemiology, and Laboratory Services of the Centers for Disease Control and Prevention, Atlanta, Georgia. She is currently assigned to the Tennessee Department of Health in Nashville, Tennessee. Her research interests include communicable disease prevention for vulnerable populations.

References


Address for correspondence: Heather N. Grome, Tennessee Department of Health, Communicable Diseases and Emergency Preparedness Division, 710 James Robertson Pkwy, Nashville, TN 37243, USA; email: qds9@cdc.gov
Zika virus (ZIKV) is a flavivirus transmitted through the bite of mosquitoes, principally *Aedes aegypti*. The World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC) on February 1, 2016, by which time autochthonous ZIKV transmission had been reported in 22 countries and territories in Latin America and the Caribbean (1). The PHEIC was prompted by the reporting 2 months earlier of a suspected link between ZIKV infection during pregnancy and subsequent birth defects, most notably microcephaly (2). A high proportion of asymptomatic or mild infections (3) coupled with diagnostic test cross-reactivity (4) obscured the true number of cases during the outbreak. Accounting for these sources of uncertainty recent modeling suggests 132.3 million (95% CI 111.3–170.2 million) persons in the Americas had been infected by the end of 2018 (5).

WHO terminated the emergency in November 2016. During the next 5 years, 2017–2021, infection rates decreased substantially; for example, the United States reported 15 new local cases in 2017 and none since (6). However, ZIKV did not disappear from the region; ≥150,000 cases were reported, unadjusted for underreporting, in the Americas through September 2021 (7). The persistence of ZIKV in this region coupled with its explosive epidemic potential has meant ZIKV remains in the WHO list of priority diseases for research and development in emergency contexts (8). We explored post-PHEIC ZIKV transmission in Brazil, the country with the highest number of infections during the epidemic in the Americas. Our first objective was to analyze post-PHEIC infection trends in Brazil; the second was to identify consistent places and times for ongoing transmission for intervention targeting.

**The Study**

All data were anonymized and reported at the municipality level. We obtained ZIKV infection data from the online database of the Brazilian Ministry of Health’s notifiable diseases information system (Sistema de Informação de Agravos de Notificação, SINAN) (9). We used data from laboratory-confirmed (either by real-time PCR or IgM serology) and clinically diagnosed cases. (Figure 1).

We adapted an autoregressive integrated moving average model to enable direct modeling of the seasonal component of time series data. We tested our seasonal autoregressive integrated moving average (SARIMA) model on monthly cases from January 2017–December 2019 and then validated it with 2020 data before using it for forecasting for 2021–2022. Parameterization involved fitting data to alternative SARIMA models by a modified Powell method and selecting the model with lowest Akaike Information Criterion. We assumed a seasonality lag of 12 time steps (i.e., seasonality repeats every 12 months) for SARIMA models and trained and validated the model for each of the 5 regions of Brazil.

Diagnostic plots for the SARIMA models comprise the standardized residuals, normal Q-Q plot, and correlogram; all generally indicated good model fits for the data from each region (Appendix Figures 1–5, https://wwwnc.cdc.gov/EID/article/28/4/21-1949-App1.pdf). For 2021–2022, we forecasted the following case numbers for each region: North, 291 (95% CI 0–5,334); Northeast, 5,933 (95% CI 251–17,009);
Southeast, 1,228 (95% CI 0–21,793); South, 291 (95% CI 28–208); and Central-West, 733 (95% CI 0–10,388) (Appendix Figure 6). We recorded final model specifications and the root mean squared errors for the 2020 SARIMA predictions (Appendix).

We applied a data filter to 5,570 municipalities in Brazil, and included in our analysis those that have reported ZIKV infections consistently every year since 2017. We generated a kernel density plot (10) for these areas of consistent transmission and weighted the data by minimum annual rate of infection, standardized to local municipality population; the latest census was >10 years ago, so we used 2020 estimates produced by the Brazilian Institute of Geography and Statistics (11). We identified centroid locations of all municipalities that reported ZIKV cases after 2016 and the hotspots of sustained transmission during the post-PHEIC period, in which >40 cases were reported per 100,000 population consistently each year (Figure 2, panel A). We generated contours from kernel density estimates weighted by the minimum annual rate of infection standardized to local municipality population. We noted hotspots in Roraima and Tocantins (North region), Alagoas
(Northeast), Rio de Janeiro (Southeast), Paraná (South) and the border between Rondônia (Northern) and Mato Grosso (Central-West).

We calculated the Moran I statistic (13) for infections at the state level to determine significant disease clustering; to do so, we used a permutation approach because of reduced sensitivity to potential violations of the analytical version’s assumptions, making it a more robust metric. We calculated a reference distribution for the statistic under the null hypothesis of spatial randomness by randomly permuting the observed values over the locations. We computed Moran I for each randomly shuffled dataset; from the statistic, we plotted a reference distribution and a pseudo p value. Because of the high number of municipalities in Brazil with missing data, we estimated global clustering at the state level. Distrito Federal is contained entirely within the state of Goiás, and so data on its cases and population were merged with Goiás. We estimated a Moran I of 0.23; Monte Carlo simulation generated pseudo p value was 0.047, providing support for clustering at this level (Appendix Figure 7). We then assessed clustering for each calendar month (e.g., we included cases from January 2017, January 2018, January 2019, and January 2020 as Jan) (Figure 2, panel B). Monthly Moran I showed clustering persisting throughout much of the year (January–August) but breaking down when case numbers naturally waned during the low mosquito season (September–December).

Conclusions
The objectives of this study were to identify current trends in ZIKV transmission in Brazil and to identify where, and when throughout the year, ZIKV is most consistently reported in the post-PHEIC period. Limitations of this analysis include the high rates of underreporting known to occur for ZIKV infections which, coupled with difficulties in differential diagnosis, make reliable estimates for case numbers a considerable challenge (14). Because of these uncertainties, the forecasts in our analysis should not be viewed as prescriptive predictions of the true numbers of ZIKV infections in Brazil 2021–2022. Instead, forecasts are estimates for cases that will be reported to the notifiable diseases information system; these forecasts have the potential to accelerate public health decisions informed by the information system.

After validating the SARIMA models fitted to monthly regional notification data from Brazil, we forecasted 8,476 new cases for 2021–2022 (95% CI 279–54,732 new cases). Although the Northeast and Southeast regions were likely to continue to have the highest total infection numbers, our consistency-weighted, population-standardized rates highlighted hotspot states within all 5 regions. In the national health system, strategic programs are coordinated and specialized services are delivered at the state level; our results can help inform specific municipalities within hotspot states that exhibit consistently high-level notifications. Identifying hotspots has 2 purposes: first, it provides targets for more intensive vector control efforts to ameliorate disease burden among the worst-affected populations; second, it helps to inform site selection for seroprevalence studies and intervention trials (15). Temporality of clustering statistics indicating clear and consistent transmission seasonality also contributes to both of these purposes.

About the Author
Dr. Yakob is an associate professor of infectious diseases at the London School of Hygiene & Tropical Medicine. He uses computational approaches to help target and strategize disease control.

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Address for correspondence: Laith Yakob, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, WC1E 7HT, UK; email: laith.yakob@lshtm.ac.uk
Legionnaires’ disease is a severe form of acute pneumonia caused by inhalation of aerosols containing Legionella bacteria. Most Legionella infections are related to contaminated artificial water systems. Systems with warm water (35°C), stagnation, and lack of disinfection and maintenance can lead to proliferation of Legionella spp. (1). Cooling towers, warm water systems, and whirlpool spas are well-established sources of infection (2). However, in most sporadic cases, the source of infection remains unknown (3).

Commercial truck drivers are at increased risk for Legionnaires’ disease (4–7). Exposures related to the vehicle are usually considered secondary to outside sources in industrial areas, such as cooling towers, and are seldomly investigated, despite some studies suggesting them as potential sources (4). Using windshield wiper fluid without added screen wash has been identified as a risk factor for Legionnaires’ disease in commercial drivers in a previous case–control study (4). In addition, Legionella spp. can grow in windshield wiper fluid that does not contain screen wash. However, no studies have epidemiologically confirmed the fluid as the source of infection (8). We report 2 cases of Legionnaires’ disease cases diagnosed by urine antigen testing (UAT) linked to detection of the bacteria in the windshield wiper fluid.

The Study
In December 2019, the Public Health Agency of Barcelona (PHAB) received a case report of Legionnaires’ disease in a 59-year-old man. Onset of symptoms had begun a week before diagnosis. On December 13, the patient sought care at a hospital, and a diagnosis of Legionnaires’ disease was made by UAT. A respiratory sample was not available because of the lack of productive secretions. The patient was hospitalized briefly, and his clinical course was unremarkable. After discharge, he completed the remaining course of antimicrobial drug therapy and proceeded favorably to cure.

After the case was reported, public health nurses contacted the patient to complete a structured epidemiologic questionnaire that included demographic data, personal risk factors, activities, and potential exposures during the 14 days before illness onset. The patient smoked and had a medical history of hypertension and type 2 diabetes. He worked as a commercial truck driver, and his driving route included merchandise pickup at an industrial area once a day. The truck used for work was self-owned; the patient had purchased it secondhand ≈1.5 years before illness.
reported that the vehicle had been unused for several months before the purchase and denied using screen wash in the windshield wiper fluid.

No other cases of Legionnaires’ disease reported during the same period were related to his residence or driving areas. Two weeks after the interview, environmental inspectors from PHAB sampled the fluid that remained in the windshield wiper tank of the truck. The 2,000-mL sample of water was collected and stored in a sterile container (Deltalab, https://www.deltalab.es) treated with sodium thiosulfate. The PHAB laboratory analyzed the sample for L. pneumophila. Culture results using Legionella selective media, according to the ISO 11731:2017 protocol (https://www.iso.org), were negative. However, the sample was also analyzed by using real-time PCR (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-0814-App1.pdf). Testing showed that the fluid was PCR positive for L. pneumophila (Table). Laboratory test results and cleaning procedures to follow for the windshield wiper tank were explained to the driver. Public health inspectors also recommended adding screen wash fluid to the windshield wiper fluid regularly.

On September 17, 2020, a new case of Legionnaires’ disease was detected by UAT in commercial truck drivers. A respiratory sample was unavailable because of lack of respiratory secretions. The patient was a 58-year-old man who smoked and had a medical history of recently diagnosed chronic obstructive pulmonary disease. He was hospitalized briefly and was successfully treated with antimicrobial drug therapy. His driving routes included industrial areas frequently located within Barcelona. No further cases that could be epidemiologically linked to this case were identified during the same period.

The patient used the truck daily and denied adding screen wash to the windshield wiper fluid; he also reported that the windshield wiper fluid had not been changed for >6 months. On the basis of previous case experience, we obtained a sample of the windshield wiper fluid. Analysis of the sample was performed in the PHAB laboratory according to the stated standards. Although we were unable to detect L. pneumophila by culture or real-time PCR, Legionella spp. was detected by culture at a concentration of 6.3 × 10³ CFU/L (Table). Consequently, public health inspectors recommended cleaning procedures to disinfect the windshield wiper tank, together with adding screen wash fluid to the windshield wiper fluid regularly.

**Conclusions**

We report 2 sporadic cases of Legionnaires’ disease in commercial truck drivers in which the laboratory findings were consistent with, but not exclusive to, the windshield wiper fluid as the source of infection. The etiologic agent was presumed to be L. pneumophila serogroup 1 (Lp1) because both patients were given a diagnosis by UAT, which detects only Lp1. DNA of L. pneumophila (unknown serogroup) was found in the windshield wiper fluid for the first case, and Legionella spp. was cultured from the fluid for the second case. Although the presence of Lp1 was not confirmed in the windshield wiper fluid for either case, laboratory findings indicated that the fluid was a potential source for both cases.

Previous studies have identified windshield wiper fluid without screen wash as a potential risk factor for Legionnaires’ disease (4). Furthermore, 2 previous studies have identified Legionella spp. in windshield wiper fluid (8,9), confirming that the bacteria can survive in this medium and the fluid as a possible source of infection. These results are consistent with our observations for the windshield wiper fluid for the second case described. Despite these observations, transmission from this source has not been epidemiologically confirmed. Our findings strengthen the epidemiologic connection between windshield wiper fluid as a source of infection for truck drivers.

Several vehicle-related sources have been described as confirmed or potential sources of Legionnaires’ disease, although these sources were infrequently considered and difficult to investigate (3,10,11). Kanatani et al. identified L. pneumophila in road puddles (12) and hypothesized that bacteria reach the windshield wiper fluid tank through road splashes. Alternatively, Legionella spp. could reach

<table>
<thead>
<tr>
<th>Case-patient</th>
<th>Specimen</th>
<th>Test</th>
<th>Test results</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urine</td>
<td>Urinary antigen</td>
<td>Positive</td>
<td>Legionella pneumophila serogroup 1</td>
</tr>
<tr>
<td></td>
<td>Windshield wiper fluid</td>
<td>PCR</td>
<td>Positive</td>
<td>L. pneumophila</td>
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<td></td>
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<td>Culture</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>Urine</td>
<td>Urinary antigen</td>
<td>Positive</td>
<td>L. pneumophila serogroup 1</td>
</tr>
<tr>
<td></td>
<td>Windshield wiper fluid</td>
<td>PCR</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Windshield wiper fluid</td>
<td>Culture</td>
<td>Positive</td>
<td>Legionella spp.</td>
</tr>
</tbody>
</table>

*NA, not applicable.
the tank after a car wash at a contaminated installation or through a contaminated water source used to fill the tank. Warm temperatures or heat radiated from the motor of the vehicle to the water tank, along with lower or absent methanol levels in screen wash fluid, and water stagnation could favor bacterial proliferation in the windshield wiper fluid tank.

Beyond routinely investigating this source, especially in commercial truck drivers, a simple measure of adding screen wash to the fluid can be recommended. This action has the potential benefit of decreasing the risk for infection by inhibiting growth of *Legionella* spp. growth through a bactericidal effect of screen wash components, such as propanol/methanol (9).

In summary, our results indicate that windshield wiper fluid is a potential source of sporadic Legionnaires’ disease, especially in commercial truck drivers, and should be routinely investigated. A simple recommendation of adding screen wash to windshield wiper fluid or emptying the tank when the vehicle is unused for several months is a preventive measure likely to be effective and should be adopted by drivers.

**About the Author**

Dr. Politi is a preventive medicine and public health physician at Parc de Salut Mar, Barcelona, Spain. Her primary research interests are surveillance, prevention, and control of infectious diseases.

**References**


Address for correspondence: Cristina Rius, Epidemiology Service, Public Health Agency of Barcelona, Spain, Pl. de lesseps 1, Barcelona 08023, Spain; email: crius@aspb.cat

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**Bordetella hinzii** Pneumonia in Patient with SARS-CoV-2 Infection

Hend Ben Lakhal, José Bras Cachinho, Pierre Kalfon, Thierry Naas, Zehaira Benseddik

Patients infected with severe acute respiratory syndrome coronavirus 2 might have bacterial and fungal superinfections develop. We describe a clinical case of coronavirus disease with pulmonary aspergillosis associated with **Bordetella hinzii** pneumonia in an immunocompetent patient in France. **B. hinzii** infections are rare in humans and develop secondary to immunosuppression or debilitating diseases.

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) has spread globally and strained health systems with an exponentially increasing number of acute respiratory failures (1). Because severe cases of respiratory distress require ventilator assisted respiration, severe bacterial and fungal co-infections can develop and lead to increased deaths (2,3). **Bordetella hinzii** is a gram-negative, aerobic cocccobacillus initially described as a cause of respiratory infection in poultry and rarely in rodents (4,5). Human infections are rare and occur mostly in immunocompromised persons upon exposure to infected animals (6,7). In humans, these infections were described in 1994 in an HIV-infected patient as a cause of bacteremia (6) and have since been rarely identified in a wide range of infections (8–10). We report a clinical case of SARS-COV-2 infection associated with pulmonary aspergillosis and **B. hinzii** pneumonia.

The Study

This case-patient was identified during routine patient care. Thus, the need for ethics approval was exempted; verbal consent was obtained from the patient.

A 63-year-old man with no notable medical history was admitted for cough, asthenia, and shortness of breath starting 3 days before admission. The patient had a positive result for a SARS-CoV-2 rapid antigen autotest. At the emergency department, COVID-19 diagnosis was confirmed by a positive SARS-CoV-2 RT-PCR result for a nasopharyngeal swab sample and chest computed tomography, which showed bilateral ground-glass opacities (50% involvement) and beginning of consolidation in the lower lobes of the lungs (Figure).

He received dexamethasone (6 mg/d for 10 d), subcutaneous, low molecular weight heparin (2 × 6,000 IU/d during the entire hospitalization), ceftriaxone (2 g/d), and spiramycin (1.5 × 10⁶ IU 3×/d). On day 2 of hospitalization, he was transferred to the intensive care unit, antimicrobial drug treatment was stopped, and awake prone positioning was combined with high-flow nasal oxygen therapy.

On day 9, mechanical ventilation was applied because of acute respiratory distress syndrome, worsening hypoxemia, and gas exchange deterioration. There was no documented bacterial superinfection, and after 48 hours, the patient’s oxygenation level had improved.

On day 13, respiratory function worsened; purulent aspiration and fever developed, and inflammatory markers increased (C-reactive protein 254 mg/L [reference <10 mg/L] and procalcitonin 0.35 ng/mL [reference <0.1 ng/mL]). Four-day intravenous piperacillin/tazobactam treatment (4 g/0.5 g 4×/d) was initiated, and an endotracheal aspirate (EA) showed oropharyngeal flora (10⁶ CFU/mL) and 5 × 10⁵ CFU/mL methicillin-susceptible **Staphylococcus aureus**, 5 × 10⁶ CFU/mL **B. hinzii**, 5 × 10⁵ CFU/mL amoxicillin-susceptible **Escherichia coli**, and 5 × 10⁵ CFU/mL **Candida tropicalis**.

On day 17, another EA showed oropharyngeal flora (10⁷ CFU/mL), decreased methicillin-susceptible **S. aureus** (5 × 10⁵ CFU/mL), increased **B. hinzii** (10⁶ CFU/mL), amoxicillin-susceptible **E. coli** (10⁶ CFU/mL), and **Aspergillus fumigatus** (10⁵ CFU/mL). **A. fumigatus** was considered as an infection because of worsening of respiratory failure despite piperacillin/tazobactam treatment, ventilatory support for severe acute respiratory distress syndrome, an **A. fumigatus**–positive culture on EA (absent on previous EAs), and a computed tomography scan showing cavitating infiltrates (Figure). Voriconazole treatment (400 mg on the first day, followed by 200 mg/12 h) was given for 21 days, in combination with intravenous co-amoxiclav (1 g 3×/d).
EA was repeated on day 25 because of persistence of fever, progressive clinical deterioration, and worsening of radiologic findings and showed $10^6$ CFU/mL *B. hinzii*, $10^5$ CFU/mL amoxicillin-susceptible *E. coli*, and $10^3$ CFU/mL *C. tropicalis*, which was considered as colonization. We switched treatment to piperacillin/tazobactam (4 g/0.5 g 4×/d for 8 d), which resulted in negative results on subsequent EA samples. Testing of rectal swab samples, blood, and urine cultures remained negative throughout hospitalization. The patient was extubated on day 46 and discharged uneventfully from the hospital.

*B. hinzii* grew on horse blood agar (bioMérieux, https://www.biomerieux.com) at 37°C after incubation for 24 hours as smooth, gray colonies. We identified *B. hinzii* by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Biotyper; Bruker, https://www.bruker.com) and confirmed it by using whole-genome sequencing (Illumina, https://www.illumina.com) as described (11).

We initially performed antimicrobial susceptibility testing by using Etest (bioMérieux), confirmed results by using disk diffusion and broth microdilution (Thermo Fisher Sensititer System; Thermo Fisher, https://www.thermofisher.com) (Table), and interpreted results by using the 2021 European Committee on Antimicrobial Susceptibility Testing pharmacokinetic/pharmacodynamic (nonspecies related) breakpoints (12). *B. hinzii* CHAR-1 showed resistance to amoxicillin, cefotaxime, aminoglycosides, and ciprofloxacin; intermediate resistance to amoxicillin/clavulanic acid and ceftazidime; and susceptibility to piperacillin/tazobactam, meropenem, and imipenem. New molecules were also tested and remained susceptible, except for ceftolozane/tazobactam (Table).

We identified β-lactamase activity by using *B. hinzii* CHAR-1 crude protein extracts as described (13). In silico analysis showed a β-lactamase, *Bordetella* lactamase (HBL-1), which had all canonical boxes of a functional broad-spectrum class A penicillinase (13) (Appendix Figure, https://wwwnc.cdc.gov/EID/article/28/4/21-2564-App1.pdf). HBL-1 had 62.7% amino acid identity with BOR-1 β-lactamase from *B. parapertussis* (13). Highly related sequences (99.7%–100% amino acid identity) were identified in genome sequences of *B. hinzii* available in public databases, suggesting

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**Figure.** Computed tomography scans of patient with *Bordetella hinzii* pneumonia and severe respiratory syndrome coronavirus 2 infection. A, B) Scan at admission showing bilateral evidence of extensive areas of mainly crazy paving patterns with some posterior consolidations. C, D) Scan at day 25 showing marked increased extent of consolidation.
that HBL-1–like enzymes might be native to that species (Appendix Figure). MICs of aminopenicillins and carboxypenicillins might be explained by expression of HBL-1, but as suggested for *B. parapertussis*, additional nonenzymatic β-lactam resistance mechanisms, such as impermeability, efflux, or penicillin-binding protein affinities, might be associated for *B. hinzii*. The complete genome and HBL-1 sequences have been deposited in DDBJ/EMBL (accession no. JAJTJL000000000) and GenBank (accession nos. OM212391).

The lung microbiota of deceased patients who had COVID-19 showed complex bacterial and fungal colonization by opportunistic pathogens (14). SARS-CoV-2 infection, antimicrobial drug pressure, alveolar damage, persistent lymphocytic depletion, mechanical ventilation, corticosteroid therapy, and prolonged hospital stays might predispose critically ill COVID-19 patients to opportunistic bacterial or fungal superinfection (2,14). Critically ill COVID-19 patients have the highest percentage of secondary pulmonary infections (34.5%) compared with percentages for severely ill (8.3%) and moderately ill (3.9%) COVID-19 patients (15). COVID-19–associated pulmonary aspergillosis is a worrisome complication in critically ill patients who show increased illnesses and deaths (2). *A. fumigatus* co-infections are frequent among critically ill COVID-19 patients (2,3). Rothe et al. showed that in a group of 50 critically ill COVID-19 patients admitted to the intensive care unit, 34% were co-infected with Enterobacteriales and 18% with *A. fumigatus* (15).

Human cases of *B. hinzii* infection are rare and associated mostly with immunosuppression and anterior poultry exposure (7–10). Our patient reported recent exposure to geese and ducks, which probably led to latent or chronic colonization (digestive or respiratory tract) before infection at a time when he was most vulnerable (e.g., COVID-19 and aspergillosis superinfection). Reports of patients who have *B. hinzii* infections seem to be increasing in recent years, which might reflect emergence of this pathogen or availability of better identification methods, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 16S rRNA gene sequencing, and whole-genome sequencing (8). Among the few *B. hinzii* infections described, none reported *Aspergillus* infections (9).

### Table. Antimicrobial susceptibility of *Bordetella hinzii* isolate from patient with pneumonia and severe respiratory syndrome coronavirus 2 infection, by Etest and broth microdilution

<table>
<thead>
<tr>
<th>Test and antimicrobial drug</th>
<th>MIC for Etest, μg/mL</th>
<th>MIC for BMD, μg/mL</th>
<th>Interpretation</th>
<th>EUCAST PK-PD breakpoint, mg/L†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Routine antibiogram</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>32 ND</td>
<td>R</td>
<td>≤2</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid (2)†</td>
<td>6 ND</td>
<td>I</td>
<td>≤2</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>16 ND</td>
<td>I</td>
<td>≤8</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Piperacillin/tazobactam (4)†</td>
<td>0.5g</td>
<td>&lt;4</td>
<td>S</td>
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<tr>
<td>Ceftoxim</td>
<td>&gt;32 ND</td>
<td>R</td>
<td>≤1</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>2 ND</td>
<td>S</td>
<td>≤4</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;256 &gt;32 R</td>
<td>R</td>
<td>≤4</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Cefepime</td>
<td>4 4 S</td>
<td>S</td>
<td>≤2</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.06 0.25 S</td>
<td>S</td>
<td>≤2</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.75 &lt;1 S</td>
<td>S</td>
<td>≤2</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.75 ND R</td>
<td>R</td>
<td>≤0.25</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.75 ND I</td>
<td>I</td>
<td>≤0.5</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8 R</td>
<td>R</td>
<td>&lt;1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2 ND R</td>
<td>R</td>
<td>&lt;0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>8 R</td>
<td>R</td>
<td>≤0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td><strong>Additional antimicrobial drugs tested</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenem/relebactam (4)†</td>
<td>ND 1 S</td>
<td>S</td>
<td>≤2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Meropenem/vaborbactam (8)‡</td>
<td>ND 0.12 S</td>
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<td>≤8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Ceftazidime/avibactam (4)‡</td>
<td>ND 4 S</td>
<td>S</td>
<td>≤8</td>
<td>&gt;8</td>
</tr>
<tr>
<td>Ceferdocol</td>
<td>ND 1 S</td>
<td>S</td>
<td>≤2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Ceftolozane/tazobactam (4)‡</td>
<td>ND 8 R</td>
<td>R</td>
<td>≤4</td>
<td>&gt;4</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>ND 0.5 S</td>
<td>S</td>
<td>0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Eravacycline</td>
<td>ND 0.12 IE</td>
<td>IE</td>
<td>IE</td>
<td>IE</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>ND &gt;64 IE</td>
<td>IE</td>
<td>IE</td>
<td>IE</td>
</tr>
<tr>
<td>Colistin</td>
<td>ND ≤0.5 IE</td>
<td>IE</td>
<td>IE</td>
<td>IE</td>
</tr>
</tbody>
</table>

*BMD, broth microdilution; EUCAST, European Committee on Antimicrobial Susceptibility Testing; I, intermediate/susceptible with high dose; IC, inhibitory concentration; IE, insufficient evidence; ND, not determined; PK/PD, pharmacokinetic/pharmacodynamic; R, resistant; S, susceptible.

†MIC breakpoints were interpreted by using EUCAST guidelines and PK-PD (nonspecies related) breakpoints (https://www.eucast.org/clinical_breakpoints).

‡Numbers in parentheses indicate the amount of inhibitor used: 2 μg/mL for clavulanic acid; 4 μg/mL for tazobactam, relebactam, and avibactam; and 8 μg/mL for vaborbactam.

§Piperacillin/tazobactam MIC was determined by using the PIP/Tazo UMIC strip (Biocentric, https://biocentricinc.com).
Reported *B. hinzii* isolates were frequently multidrug resistant, including resistance to cephalosporins, aminoglycosides, and quinolones, but remained susceptible to piperacillin/tazobactam, ceftazidime, ticarcycine, and meropenem (9,10). Interpretation of antimicrobial susceptibility testing is not established, and the choice of antimicrobial drugs and treatment duration are not standardized. Cases with documented pneumonia were successfully treated with piperacillin/tazobactam or cefmetazole (9). Our patient was successfully treated with piperacillin/tazobactam, but treatment with amoxicillin/clavulanic acid failed, probably because of intermediate susceptibility of *B. hinzii* to this antimicrobial drug. Our study suggests that *B. hinzii* needs to be taken into account when initiating antimicrobial drug therapy.

**Conclusions**

Increasing reports of invasive *B. hinzii* might indicate its emergence as a pathogen in immunocompromised patients. We describe a *B. hinzii* and *A. fumigatus* co-infection in a SARS-CoV-2-infected immunocompetent patient who had no underlying conditions but had probable transient immunosuppression caused by dexamethasone treatment and SARS-CoV-2 infection. Our study highlights the role of opportunistic infections (by fungal or rare bacterial species) in COVID-19 patients and the need to serially monitor the bacteria/fungi in the lower respiratory tract for timely personalized treatment.

**Acknowledgments**

We thank Panya Wissa and Emna Warzele for providing helpful discussions and the Institut Pasteur PIBNet for performing whole-genome sequencing of the bacterial isolate.

This study was supported by a grant from the Ministère de l’Éducation Nationale et de la Recherche (Université Paris-Saclay), Assistance Publique-Hôpitaux de Paris, and the Centre Hospitalier de Chartres.

**About the Author**

Dr. Ben Lakhal is an intensive care physician at Louis Pasteur Hospital, Le Coudray, France. Her research interests include management of severe clinical manifestations of infectious diseases in critical care.

**References**


Address for correspondence: Hend Ben Lakhal, Service de Réanimation, Centre Hospitalier de Chartres, 4 Rue Claude-Bernard, 28630 Le Coudray, France; email: h.lakhal@gmail.com
Coccidioides immitis and C. posadasii are soil-dwelling fungi that cause the disease coccidioidomycosis, also known as Valley fever (1). Since coccidioidomycosis was first recorded in 1892, the disease has become a public health concern in the United States, and several thousand cases are reported annually (2). Severe cases may involve complicated pneumonia, musculoskeletal disease, and meningitis. Coccidioides arthroconidia, which reside in the soil in dry, arid climates, are endemic in the western United States, as well as in Central and South America (2). In West Texas, a 30-county region in western Texas, the arid climate and prevalence of at-risk occupations in oil, construction, and agricultural enterprises provide conditions for contracting the infection (3). However, epidemiologic and serologic studies about coccidioidomycosis in Texas are limited, and few provide data on patient risk factors, such as occupation and contributing conditions, in part because coccidioidomycosis is not a reportable disease in the state. We retrospectively examined demographics and risk factors related to coccidioidomycosis case-patients seeking treatment at a regional referral center in West Texas.

The Study
We scanned medical records and identified patients diagnosed with coccidioidomycosis during January 1, 2013–December 1, 2019, based on International Classification of Diseases, 9th Revision (ICD-9), and International Classification of Diseases, 10th Revision (ICD-10), codes 114 and B38. To be included, case-patients had to be 9–89 years of age, have a confirmed diagnosis of coccidioidomycosis, and have been diagnosed or treated at the regional referral center during the surveillance period. We separately calculated another set of demographics and risk factors at a regional hospital in another West Texas county (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-1912-App1.pdf). We confirmed diagnoses based on provider notes or laboratory results. We included signs and symptoms considered to be associated with coccidioidomycosis only if present at the time of diagnosis. The list of associated factors was not meant to be exhaustive, and certain risk factors (such as dust exposure) may be relevant only in areas where coccidioidomycosis is endemic. We obtained medical and social history directly from patient charts where possible. We identified at-risk occupations based on National Institute for Occupational Safety & Health status (4). We considered smoking status and travel history associated factors only if specified as such in case records. We defined immunocompromised status as having received chemotherapy or immunosuppressive medication within 3 months of coccidioidomycosis diagnosis, having been diagnosed with immunosuppressive disease, or having a CD4 count <200 cells/mm³. We defined chronic lung disease as having asthma, chronic obstructive pulmonary disorder, pneumonitis, cystic fibrosis, or other medically recognized chronic lung pathology. We defined previous lifetime exposure to an endemic site as travel to or residence in Arizona, California, New Mexico, Nevada, Utah, Mexico, or Texas (if living outside the state at time of diagnosis), all of which are coccidioidomycosis-endemic states.
We determined coccidioidomycosis pathology on the basis of information in pathology or radiology reports or physician notes; for cause of death, coccidioidomycosis had to be specified as the cause of death in a death certificate or other medical record. We recorded any data fields for which we could not verify information from medical records as unknown. We occasionally excluded patient demographic data if records were unclear, missing, or contained contradictory information. We obtained Texas population data used in the model from the US Census Bureau (https://www.census.gov) and calculated workforce percentages using 2020 Census data. We performed statistical analysis using R software (https://www.r-project.org). We ran univariate logistic regression models to explore the association between variables of interest (sex, race and ethnicity, age, and smoking history) and outcomes (multiple risk factors, central nervous system [CNS] pathology).

We identified 73 patients with coccidioidomycosis (Table). Fluctuations in annual case totals were consistent with those previously observed in annual coccidioidomycosis counts (Figure 1) and were possibly related to environmental and climate factors (5). Among case-patients, 3 died from coccidioidomycosis. The most frequent at-risk occupations were oil and gas extraction (8/73, 11.0%) and agriculture (3/73, 4.1%), both industries common in West Texas and eastern New Mexico. Coccidioidomycosis has been associated with professions that involved dust exposure and outbreaks have been associated with exposure at job sites. One study found that more than half of the outbreaks they examined over 75 years involved occupational exposures (6). However, because limited reports of occupation-related cases of coccidioidomycosis exist, in part because standardized surveillance records do not include occupation, determining if the level of association is typical or accurate is difficult. Of note, Texas has the largest oil and gas workforce in the United States (334,400 workers in direct extraction and support services in 2019) (7) and one of the largest agricultural workforces (143,763 hired farm laborers in 2017) (8). The case rates we observed for the oil and gas extraction (11.0%) and agricultural (4.1%) industries were much higher than our calculated estimates for those industries in Texas, 1.15% for direct oil and gas extraction and 0.49% for agricultural farm laborers. These data support previous observations regarding these occupations as being high risk for coccidioidomycosis (4).

Most (55/73, 75.3%) patients were 20–59 years of age. Given that older patients may be more susceptible to severe illness and more likely to have comorbidities, surprisingly few (12/73, 16.4%) were ≥60 years of age; surveillance data have typically shown a larger proportion of patients ≥60 years of age (5). The reasons for this discrepancy are unclear but may include differences in age demographics between regions. As expected, most cases manifested with pulmonary disease, although 14% had a CNS pathology, including meningitis. A study of US Department of Veterans Affairs patients in the

<table>
<thead>
<tr>
<th>Table. Demographics of patients diagnosed with or treated for coccidioidomycosis at a referral center in West Texas, USA, 2013–2019</th>
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</thead>
<tbody>
<tr>
<td>Demographics</td>
</tr>
<tr>
<td>Residence at time of diagnosis</td>
</tr>
<tr>
<td>Texas</td>
</tr>
<tr>
<td>New Mexico</td>
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<tr>
<td>Mississippi</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Age at diagnosis, y</td>
</tr>
<tr>
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<tr>
<td>20–29</td>
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<tr>
<td>30–39</td>
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<td>40–49</td>
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<tr>
<td>F</td>
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<td>Asian/Pacific Islander</td>
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<td>Associated factors</td>
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<td>Incarceration history</td>
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<tr>
<td>Agriculture</td>
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<tr>
<td>Construction</td>
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<tr>
<td>Military</td>
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<tr>
<td>Prison worker or correctional officer</td>
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<tr>
<td>Truck driver</td>
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<tr>
<td>Mining</td>
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<tr>
<td>Lung pathology</td>
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<tr>
<td>Nodule or mass</td>
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<tr>
<td>Pneumonia or consolidation</td>
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<tr>
<td>Cavitation</td>
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<tr>
<td>Effusion</td>
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<tr>
<td>CNS pathology</td>
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<tr>
<td>Meningitis</td>
</tr>
<tr>
<td>Brain abscess or lesion</td>
</tr>
</tbody>
</table>

*CNS, central nervous system; NA, not available
†N = 73 patients in study. Total <73 indicates missing information.
‡Includes only patients for whom a travel history was taken or who resided outside of Texas at time of diagnosis.
1950s documented a meningitis rate of 3.5% associated with coccidiomycosis (9).

Of associated factors, we most frequently observed a smoking history (32/71, 45.1%) and diabetes (25/72, 34.7%). Smoking rate was consistent with the 49.1% observed in one study (10) and lower than the 72.0% observed in another (11). The frequency of diabetes we observed supports surveillance data linking diabetes and coccidioidomycosis (12,13). Thus, the association of diabetes with coccidioidomycosis may be important for populations with high diabetes prevalence and an important consideration for clinicians treating diabetic patients in Coccidioides–endemic regions. Smoking, sex, and race and ethnicity were not significant predictors of CNS pathology. Regional demographics, such as socioeconomic status, may play a role in access to care and diagnosis and treatment of coccidioidomycosis (14), especially in rural regions such as West Texas and eastern New Mexico, where patients may live several hundred miles from facilities providing advanced levels of care. Furthermore, limited access to infectious disease specialists suggests the possibility of delayed diagnoses and increased case severity in this region. Indeed, a 2017 report showed West Texas and parts of eastern New Mexico averaged ≤1 infectious disease physician/100,000 persons (15). Of the patients known to be residing in Texas at the time of diagnosis, 18/39 (46.2%) lived >50 miles from the referral center, which suggests the importance of access to higher levels of care for coccidioidomycosis diagnosis and treatment.

**Conclusions**

We anticipate the need for future studies to provide a longitudinal assessment of coccidioidomycosis in Texas. Retrospective reviews from medical records, although useful, are limited in their ability to thoroughly survey the prevalence of a disease such as coccidioidomycosis within a particular region, suggesting the need for more routine surveillance such as statewide mandatory reporting. Our findings also suggest that access to higher levels of care should be considered when treating populations at risk for coccidioidomycosis.

**Acknowledgments**

We thank the staff of the Clinical Research Institute at Texas Tech University Health Sciences Center and Braden Hale for their support with this project.

**About the Author**

Mr. Peterson is currently a medical student at Texas Tech University Health Sciences Center, School of Medicine in Lubbock, Texas. His research interests include infectious diseases and bibliometric analysis.

**References**


There are only one million pronghorn—hooved animals that resemble antelope—left in North America. Now, outbreaks of *Mycoplasma bovis* threaten to decimate their ranks even further in Wyoming.

With the help of bacterial DNA, researchers are figuring out how this disease, which is usually found only among livestock, emerged in a wildlife species...and whether they can find a solution before it spreads to other populations.

In this EID podcast, Dr. Kerry Sondgeroth, a veterinary bacteriologist at the Wyoming State Veterinary Laboratory and an associate professor at the University of Wyoming, describes the pieces of this genetic puzzle.
Artemisinin-based combination therapies (ACTs) have contributed greatly to the global decline of illness and death from malaria (1). However, the novel emergence of artemisinin resistance in eastern Africa has threatened the effectiveness of these breakthrough treatments (2–4). To avert potential disaster resulting from increased resistant malaria cases, the nature and extent of this resistance in Africa urgently needs to be characterized.

Artemisinin resistance is conferred by some \textit{Plasmodium falciparum} kelch 13 (K13) gene mutations, only a few of which are validated markers of resistance, defined by both in vitro resistance and delayed parasite clearance. We document artemisinin resistance in \textit{P. falciparum} patient isolates from Rwanda carrying K13 R561H, A675V, and C469F mutations.

Artemisinin resistance is conferred by mutations in the \textit{kelch 13} (K13) gene. In Rwanda, K13 mutations have increased over the past decade, including mutations associated with delayed parasite clearance. We document artemisinin resistance in \textit{P. falciparum} patient isolates from Rwanda carrying K13 R561H, A675V, and C469F mutations.

We recruited malaria patients in Huye District, Rwanda, during September–December 2019 and documented patient characteristics and consent, ethical clearance, and K13 variants elsewhere (2). Within 6 hours of sample collection, we cryopreserved all 66 \textit{P. falciparum} isolates in ethylenediaminetetraacetic acid by washing the red blood cell pellet, adding freezing solution (3% sorbitol, 28% glycerol, 0.65% NaCl), and freezing at −80°C. Eight of the 66 isolates carried nonsynonymous K13 mutations (2). We successfully thawed and culture-adapted 4 of the isolates in which we identified K13 mutations: R561H, the current prevalent mutation in Rwanda; A675V, found in 11% of \textit{P. falciparum} samples in Uganda; C469F, another candidate marker; and V555A, which is of unknown significance.

We conducted a 0–3-h postinvasion ring-stage susceptibility assay (RSA) with the active metabolite dihydroartemisinin (6). We exposed ring stages to a 6-h pulse of 700 nmol/L dihydroartemisinin and cultured exposed and nonexposed isolates in vitro in triplicate for 72 h. We counted parasite density per ≥10,000 red blood cells on Giemsa-stained thin blood films and calculated the means of triplicates. Dividing parasite density in dihydroartemisinin-exposed cultures by the density in nonexposed cultures provided the RSA survival rate. We considered results if 72-h growth rates exceeded 1.5× rates in the nonexposed controls and had ≥3 successful independent triplicate experiments per isolate.
also assessed 50% inhibitory concentrations (IC\textsubscript{50}) (7). We exposed synchronized ring-stage parasites for 72 h across a range of dihydroartemisinin concentrations (0–1 µmol/L) in duplicate or triplicate and in 23 independent experiments. We measured growth by SYBR Green I staining (ThermoFisher, https://www.thermofisher.com) and performed photometric assessment using FilterMax F5 microplate readers (Molecular Devices, https://www.moleculardevices.com). We estimated IC\textsubscript{50} using a 4-parameter fit dose-response curve. For artemisinin-susceptible parasites, 2 cultured wild-type isolates from patients in Rwanda grew too poorly for RSA and IC\textsubscript{50} assays and were replaced by artemisinin-susceptible K13 wild-type strain NF54, which is of putative African origin. We assayed isolates in parallel with NF54 and compared IC\textsubscript{50} by Student t-test. We performed analyses using R version 3.6.3, including the drc (dose-response curve) package (https://cran.r-project.org/web/packages/drc/drc.pdf).

RSAs yielded mean ± SE survival rates of 0.2% ± 0.1% for the NF54 strain and 0.3% ± 0.1% for V555A, well below the World Health Organization–accepted 1% resistance threshold (1,6). In contrast, 3 other isolates with K13 mutations had >1% mean survival rates: 4.7% ± 1.5% for R561H (prevalent in Rwanda), 1.4% ± 0.2% for A675V (prevalent in Uganda), and 9.0% ± 1.6% for C469F (Figure 1). Conventional susceptibility testing yielded mean IC\textsubscript{50} of 4.2 ± 0.5 nmol/L for dihydroartemisinin for the NF54 strain and 3.4 ± 0.3 nmol/L for V555A. IC\textsubscript{50} levels were higher in isolates with dihydroartemisinin-resistant RSA findings: 14.1 ± 4.0 nmol/L for R561H, 7.4 ± 3.3 nmol/L for A675V, and 6.9 ± 1.5 nmol/L for C469F (Figure 2).

We determined the regional origin of the 4 tested patient isolates by single-nucleotide polymorphism (SNP) barcoding. We typed 23 SNPs to group into haplotypes associated with geographic origin (7,8). The R561H isolate displayed haplotype 9 and the other isolates haplotype 22 (8), confirming African ancestry.

**Conclusions**

Artemisinin resistance is defined by RSA results and delayed parasite clearance in treated patients. In Africa, abundant K13 variants circulate, but very few have been defined in terms of drug susceptibility (1). The K13 mutation R561H, which has emerged in Rwanda (2,5), confers delayed parasite clearance (3). We found that a patient isolate with the R561H mutation from Rwanda was in vitro artemisinin resistant. Taken together, these results strongly suggest that R561H is a marker of resistance in Rwanda, a finding that needs to be confirmed in larger sample-size research. The same need for confirmation applies to K13 candidate resistance markers A675V, recently characterized in Uganda (4), and C469F (1).

RSA survival rates for K13 R561H P. falciparum in our study concord with levels in multiple gene-edited P. falciparum lines (5,10). Also in line with our findings are high RSA survival rates in A675V isolates from neighboring Uganda, where K13 A675V was found in 11% and C469Y (but not C469F) in 2% of P. falciparum isolates collected during 2017–2019. Both mutations are associated with delayed parasite clearance (4). Isolates with increased survival rates also showed higher dihydroartemisinin IC\textsubscript{50} levels. If this association is confirmed, IC\textsubscript{50} assays that are much less labor-intensive could be useful for flagging isolates deserving additional testing by RSA.

The small number of isolates we evaluated was an obvious limitation of our study. Ideally, we would have compared the effects of individual mutations in wild-type isolates from Rwanda with study isolates, but the few selected performed poorly in vitro.
and were replaced by the artemisinin-sensitive NF54 strain, enabling us to verify that the RSA was working properly. A study strength is the detailed characterization of the susceptibility and ancestry of isolates.

RSA data on suspicious \textit{K13} isolates from Africa are scarce but essential and urgent for the situational evaluation of artemisinin resistance emerging in Africa. \textit{K13} mutations have conferred a wide range of artemisinin susceptibility when introduced in different parasite lines (10). Of note, artemisinin resistance identified in Rwanda and Uganda is of indigenous origin, not imported from Asia where resistance has been prevalent for years (1). These 2 observations argue for the need for local characterization of artemisinin resistance in circulating parasites.

Artemisinin resistance alone does not necessarily lead to ACT treatment failure, and efficacy in Rwanda still is high (3). However, resistance leaves the partner drug unprotected, potentially leading to resistance developing to that component as well. Eventually, this process could result in increased ACT treatment failure, which has already been observed in southeast Asia (11,12). In Africa, this development might be delayed because of prevalent partial immunity contributing to parasite elimination and high transmission increasing the likelihood of resistance allele outcrossing. Nonetheless, in Rwanda, where artemether/lumefantrine is the first-line antimalarial drug combination, a shift in the \textit{P. falciparum} multidrug resistance 1 (\textit{pfmdr1}) genotype pattern over the past decade suggests an increasingly lumefantrine-tolerant phenotype (13,14), although \textit{pfmdr1} is not a validated marker for lumefantrine resistance.

Recent research indicates that the R561H mutation is fitness neutral (10), implying its wider dissemination even without drug pressure. So far, a viable alternative to ACTs is not in sight. Increasing resistance, combined with the lack of effective alternative antimicrobial drugs, suggests a pessimistic scenario for sub-Saharan Africa, considering the region’s high malaria burden. Large-scale monitoring, containment strategies, and early consideration of 3-drug ACTs (15) are required to control widespread artemisinin resistance in Africa.

Acknowledgments

We are grateful to the staff of Sovu Health Centre and Kabutare District Hospital for their collaboration and help. We thank Megan Peedell and Katja Puestow for their support in the laboratory.

The German Research Foundation financially supported this study through grants to W.v.L. (GRK2046) and R.O. and C.B. (GRK2290). The funding bodies had no role in designing the study, collecting, analyzing, or interpreting data, or writing the manuscript.

About the Author

Ms. van Loon is a PhD candidate at the Institute of Tropical Medicine and International Health, Charité Universitätsmedizin Berlin. She is interested in infectious disease epidemiology and public health. This manuscript forms part of her PhD thesis.

References


Address for correspondence: Welmoed van Loon, Institute of Tropical Medicine and International Health, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany; email: welmoed.vanloon@gmail.com

EID podcast
A Decade of Fatal Human Eastern Equine Encephalitis Virus Infection, Alabama

After infection with eastern equine encephalitis virus, the immune system races to clear the pathogen from the body. Because the immune response occurs so quickly, it is difficult to detect viral RNA in serum or cerebrospinal samples.

In immunocompromised patients, the immune response can be decreased or delayed, enabling the virus to continue replicating. This delay gave researchers the rare opportunity to study the genetic sequence of isolated viruses, with some surprising results.

In this EID podcast, Dr. Holly Hughes, a research microbiologist at CDC in Fort Collins, Colorado, describes a fatal case of mosquito-borne disease.

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**Rigidoporus corticola** Colonization and Invasive Fungal Disease in Immunocompromised Patients, United States

Alvaro C. Laga, Jessica W. Crothers, Connie F. Cañete-Gibas, Nathan P. Wiederhold, Isaac H. Solomon

We report 2 cases of *Rigidoporus corticola* (Oxyporus corticola) infection in humans in the United States. Clinical manifestations consisted of angioinvasive fungal sinusitis in 1 patient and pulmonary intracavitary fungus ball in the other patient. These cases illustrate previously undescribed clinicopathologic manifestations of infection by this filamentous basidiomycete in humans.

*Rigidoporus corticola* is a white-rot decay fungus of plants and is widely distributed in North America and Europe (1). *R. corticola* is a filamentous basidiomycete in the Agaricomycotina subphylum, also known as *Oxyporus corticola* because no morphologic differences or type species are in the same phylogenetic clade. *Rigidoporus* is the preferred name because it was described prior to *Oxyporus* (2,3).

Filamentous basidiomycetes occur in the environment as gilled mushrooms and shelf or bracket fungi (4). Filamentous basidiomycetes (other than *R. corticola*) have been documented in immunocompetent and immunocompromised persons (5,6), but *R. corticola* has not been established as an agent of human disease. We describe the clinical, microbiologic, and histopathologic features of 2 cases of human *R. corticola* infection in the United States (Table).

**The Study**

The first patient was a 43-year-old man with chronic granulomatous disease and recurrent acute myeloid leukemia in whom hemifacial pain developed after he received induction chemotherapy. Magnetic resonance imaging showed mucosal thickening and opacification of the patient’s left sinuses (Figure 1, panel A). Nasal endoscopy revealed a black eschar on the middle turbinate, which our clinical colleagues interpreted as highly characteristic of mucormycosis, and we performed surgical debridement. Histologic sections showed numerous thin hyaline septate hyphae (≈5 µm in diameter) with predominantly 90°-angle branching but no conidiogenous cells or conidia (Figure 1, panel B). We noted vascular invasion and infiltrative growth into sinonasal ossicles (Figure 1, panels C, D). Careful examination revealed bulge and hook-like hyphal outgrowths compatible with clamp connections (Figure 1, panels E, F), which are characteristic of filamentous basidiomycetes. Concurrent fungal culture on Sabouraud dextrose agar grew white cottony colonies. Lactophenol cotton blue preparation from subcultures onto potato flake agar revealed thin sterile hyphae. We sent a culture to the Fungus Testing Laboratory (FTL) at the University of Texas Health Science Center (San Antonio, Texas, USA). FTL also noted growth on benomyl agar suggesting a possible basidiomycete. FTL amplified genomic DNA from the isolate and sequenced for the internal transcribed spacer (ITS) and large subregion (D1/D2) of the 28S rRNA gene (BMBC-R/NL4). Compared with sequences from the NCBI database (https://www.ncbi.nlm.nih.gov), most blastn matches were *R. corticola*, 98.53%–100% similarity for ITS and 98.55%–100% for D1/D2; subsequent phylogenetic analysis confirmed *R. corticola* (Appendix, https://wwwnc/cdc.gov/EID/article/28/4/21-1987-App1.pdf). Antifungal susceptibility testing demonstrated similar MIC values for voriconazole (0.5 µg/mL) and isavuconazole (1 µg/mL) but a high MIC (8 µg/mL) for posaconazole. The patient was initially treated empirically with liposomal amphotericin B for 20...
days, then was transitioned to voriconazole after fungal identification and susceptibility testing. However, the patient experienced recurrent episodes of neutropenic fever and died 10 months later of invasive fungal disease. We presumed the infection to be aspergillosis on the basis of increased serum galactomannan on sequential samples, but this assumption was not confirmed by culture or PCR.

The second patient was a 63-year-old man with a history of lung adenocarcinoma and a new 2.4 cm right upper lobe mass resected for possible recurrence (Figure 2, panel A). Histologic sections showed a cavitary lesion with necrotic center and peripheral fibrous capsule (Figure 2, panel B). Sections stained with Gomori methenamine silver showed numerous septate hyphae in the cavity but no invasion into blood vessels or surrounding tissue (Figures 2, panels C, D). Neither conidiogenous cells nor conidia were evident; we did not identify clamp connections in samples after careful examination. The Centers for Disease Control and Prevention (Atlanta, Georgia, USA) identified O. corticola (R. corticola) by PCR from formalin-fixed, paraffin-embedded (FFPE) tissue sections of the wedge resection. We did not attempt culture, and the patient did not receive antifungal therapy. He died 11 months later of recurrent lung adenocarcinoma.

Conclusions
Filamentous basidiomycetes are common environmental fungi increasingly recognized as agents of human disease, causing saprobic colonization of the respiratory tract, allergic disease, and invasive fungal infections (6,7). A review of 218 pathogenic cases reported worldwide found the most common species are Schizophyllum commune (52%), Hormographiella aspergillata (5.9%), and Ceriporia lacerata (5%) (7). Anecdotal reports describe galactomannan and β-d glucan production by filamentous basidiomycetes such as H. aspergillata, suggesting that testing for these polysaccharides might help diagnose invasive disease.

Two cases of R. corticola infection have been reported in canines. One was a disseminated R. corticola infection reported in 2009 in a German shepherd dog,
which was ultimately euthanized 20 months after clinical presentation because of progressive disease and central nervous system involvement despite therapy with itraconazole and terbinafine (1). Another case, in 2012, was reported as generalized lymphadenopathy in a beagle, which was confirmed by sequencing of a culture isolate to be caused by *R. corticola* (8). This dog also was euthanized because of progressive disease despite empiric treatment with itraconazole 14 weeks after clinical presentation (8). Histopathology from both canine cases showed septate branching hyphae with parallel to slightly tapering walls.

We document *R. corticola* as an agent of human disease in 2 immunocompromised patients; the fungus colonized the pulmonary cavity of one patient and caused invasive sinusitis in the other. The spectrum of disease we describe resembles that observed for other filamentous basidiomycete fungi. Our findings align with the literature; these organisms are difficult to identify by in vitro culture because of lack of sporulation and are not yet reliably identifiable by mass spectrometry (9). Standard sequencing of 18S, 28S, or ITS rRNA and other fungal genomic regions can assist in species-level identification, but accurate identification is limited by available sequencing databases (10–12). However, molecular identification of fungi by itself is insufficient to establish pathogenicity because it might not distinguish between an agent of disease and a contaminant, particularly in cases involving common environmental fungi and uncommon species not established as agents of human disease, such as *R. corticola*. The 2 clinical cases we describe were detected by histopathology, confirming a pathogenic role in invasive fungal disease in 1 case and colonization of an existing cavity in the other, according to current guidelines (13); fungal sequencing of a culture isolate and FFPE tissue subsequently confirmed identification. These data support observations that filamentous basidiomycetes are being recognized more frequently in clinical specimens and that identification by fungal cultures and biochemical methods remains challenging. Consequently, filamentous basidiomycetes and their burden of disease likely are underrecognized.

Although we performed susceptibility testing against 1 isolate according to the Clinical and Laboratory Standards Institute M38 reference standard (10), no breakpoints for antifungal agents against this species have been established. Of note, the MIC for posaconazole was elevated compared with other basidiomycetes reported to cause human infections, such as *S. commune*. Although data are limited,
of 5 *R. corticola* isolates in the FTL database with MIC results for posaconazole, 4 had elevated posaconazole MICs (range 2.0 to >16 μg/mL), but voriconazole MICs were lower (range 0.25–1.0 μg/mL). Lack of evidence-based treatment guidelines might have contributed to illness in these patients, both of whom died from complications of their underlying diseases <1 year after seeking care for *R. corticola* infection.

Although filamentous fungi cannot be speculated by histology, detection of the characteristic clamp connections should prompt consideration of filamentous basidiomycetes in the differential diagnosis. Admittedly, this finding might be rare because most clinical isolates are reportedly monokaryons (14), but 1 study documented clamp connections in 78% of clinical isolates of *S. commune* and *C. lacerata* (15).

In summary, our report adds *R. corticola* to the list of increasingly recognized filamentous basidiomycete fungi associated with human disease. Clinicians should consider *R. corticola* when assessing immunocompromised patients for invasive fungal infections or respiratory tract colonization.

About the Author

Dr. Laga is an infectious disease pathology consultant at Brigham and Women’s Hospital in Boston, Massachusetts, USA. His primary research interests include medical mycology and tropical and neglected infectious diseases.

References


Address for correspondence: Alvaro C. Laga, Brigham and Women’s Hospital, Department of Pathology Amory-3, 75 Francis St, Boston, MA 02115, USA; email: alagacanales@bwh.harvard.edu
Consumption of wildlife meat drives emerging infectious diseases (1), often amplified by human encroachment into natural areas and changes in land use. Wildlife trade and consumption have been responsible for outbreaks of diseases such as HIV-1 (2), Ebola (3), and monkeypox (4) and possibly for the coronavirus disease pandemic (5). Wildlife markets bring diverse species into contact, usually in dense and unsanitary conditions, enabling mixing, amplification, and transmission of pathogens among species, including humans (6). Small mammals host diverse pathogenic bacteria and viruses (7), but little investigation of endemic bacteria transmission has occurred. Determining pathogens present in traded wildlife is vital to guide appropriate measures to combat zoonotic diseases and document societal and environmental costs of wildlife trade.

The Study
During December 2014–September 2017, we collected samples from 9 wildlife trade hotspots (8) and 2 roadside stalls (hereafter all referred to as trade sites) in Laos (Figure; Appendix Table 1, https://wwwnc.cdc.gov/EID/article/28/4/21-0249-App1.pdf). In addition, 3 Provincial Offices of Forest Inspection (POFI) collected samples from wildlife confiscated in markets by law enforcement. After identifying wildlife at trade sites (9), we asked vendors for permission to sample their animals. Depending on whether the animal was alive, dead, or butchered, we collected urogenital swabs, urine and blood samples, and kidney, liver, and spleen tissue samples (Appendix Table 2).

We tested animals from wildlife trade sites in Laos for the presence of zoonotic pathogens. *Leptospira* spp. were the most frequently detected infectious agents, found in 20.1% of animals. *Rickettsia typhi* and *R. felis* were also detected. These findings suggest a substantial risk for exposure through handling and consumption of wild animal meat.
We performed descriptive, univariate, and multivariate analyses by using R version 3.6.2 (https://www.r-project.org). We assessed the effect of the wild meat processing status (alive, fresh, or frozen) on the risk for *Leptospira* detection by using a mixed effects logistic regression with species as random effect. Statistical significance was set at $\alpha = 0.05$ (Appendix).

We collected 717 samples from 359 animals (trade sites: 461 samples from 324 animals; POFI: 256 samples from 35 animals); animals sampled were from >37 identifiable vertebrate species from 12 families (Appendix Table 4). Most were Sciuridae squirrels (73.0%, 262/359) and represented 16 species, most frequently Pallas’s squirrel (*Callosciurus erythraeus*) (20.3%, 73/359). From trade sites, 69 animals (21.3%, 95% CI 17.0%–26.2%) had ≥1 samples positive for ≥1 pathogens in 10 of 11 sites (90.9%, 95% CI 57.1%–99.5%) (Appendix Table 5). Of 324 animals tested, 65 (20.1%, 95% CI 15.9%–24.9%) were positive for *Leptospira* spp.; 4/41 were positive for *Rickettsia* spp. (9.8%, 95% CI 3.2%–24.1%), 0 for *O. tsutsugamushi* (0%, 95% CI 0%–10.7%), and 2 for Anaplasmataceae (4.9%, 95% CI 0.8%–17.8%) (Table 1). Positivity was higher among animals collected by POFI; 25/35 (71.4%) animals tested positive for ≥1 pathogens. Of those, 9 were positive for *Leptospira* spp. (25.7%, 95% CI 13.1%–43.6%), 20 for *Rickettsia* spp. (57.1%, 95% CI 39.5%–73.2%), 2 for *O. tsutsugamushi* (5.7%, 95% CI 1.0%–20.5%), and 6 for Anaplasmataceae (17.1%, 95% CI 7.2%–34.3%) (Table 2). Sequencing identified *R. typhi*, *R. felis*, *R. conorii*, *Anaplasma* species (either *A. centrale*, *A. capra*, or *A. marginale*), *A. platys*, *A. bovis*, *A. phagocytophilum*, *Ehrlichia chaffeensis*, *Lactococcus garvieae*, and *Kurthia populi* (Tables 1, 2). No

**Figure.** Wildlife trade sites and POFI sites (black circles) where wildlife samples were collected for study of zoonotic pathogens in wildlife traded in markets for human consumption, Laos. Provinces are labeled with black squares. POFI, Provincial Office of Forestry Inspection.
samples were positive for *C. burnetii* (0/76), flaviviruses (0/359), dengue virus (0/359), or Zika virus (0/358).

Among species for which >10 individual animals were sampled in trade sites, 2 had particularly high proportions of *Leptospirosp.*-positive specimens: the variable squirrel (*Callosciurus finlaysonii*) (13/28; 46.4% 95% CI 28.0%–65.8%) and the common palm civet (*Paradoxurus hermaphroditus*) (10/22; 45.5%, 95% CI 25.2%–67.3%). *Leptospirosp.*-positivity was higher in dry (50/195; 25.6%, 95% CI 19.8%–32.5%) than wet season (15/129; 11.6%, 95% CI 6.9%–18.8%) ($\chi^2 = 8.7$; $p = 0.003$). Data disaggregation by species and province suggested that observed seasonality was driven by results in common palm civets and variable squirrels in Champasak Province. No association was detected between the probability of an animal testing positive for *Leptospirosp.* and the animal being alive (3/22; 14%, 95% CI 3.6%–36%), freshly dead (58/293; 20%, 95% CI 16%–25%; $p = 0.6$), or frozen (4/9; 44%, 95% CI 15%–77%; $p = 0.1$). In a subset of *Leptospirosp.*-positive animals with multiple samples, 75% (18/24; 95% CI 53%–89%) of urogenital swab samples and 50% (9/18; 95% CI 29%–71%) of blood samples were positive ($p = 0.11$ by Fisher exact test). *Rickettsia* spp. were detected exclusively and the animal being *R. typhi*-positive was identified most frequently, found in both palm civet and variable squirrel species. Variable squirrels are commonly traded, often in batches of 2 to 3 squirrels (8); hence, on average, someone purchasing 3 variable squirrels would have an 83% likelihood of buying ≥1 infected squirrel ($p = 1 - (1 - prevalence)^3 = 1 - 0.55^3 = 0.83$). The higher risk for *Leptospirosp.* detection in the dry season is at odds with the typically described correlation of transmission with precipitation and flooding (10), suggesting that much remains to be understood of *Leptospirosp.* ecology. Other

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**Table 1. Zoonotic pathogens detected and animal species and sample types that tested positive in wildlife collected from trade sites, Laos**

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. positive/no. tested</th>
<th>Animals</th>
<th>Species</th>
<th>Samples</th>
<th>Sample types</th>
<th>Sequencing identity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptospirosp.</em></td>
<td>65/324</td>
<td>Callosciurus finlaysonii, 13/28</td>
<td>C. erythraeus squirrel, 8/56</td>
<td>URO, 58/312</td>
<td>SPL, 1/3</td>
<td>NA</td>
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<td></td>
<td></td>
<td>Paradoxurus hermaphroditus civet, 10/22</td>
<td></td>
<td>KID, 2/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. inornatus squirrel, 7/34</td>
<td>Dremomys rufigenis squirrel, 5/35</td>
<td>LIV, 1/40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Menetes berdmorei ground squirrel, 4/29</td>
<td></td>
<td>BLD, 9/85</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rhizomys prininosus rat, 3/21</td>
<td>Articogalidia trivirgata civet, 2/2</td>
<td>URI, 1/15</td>
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<td></td>
<td></td>
<td>Petaurista philippensis flying squirrel, 1/9</td>
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<td></td>
<td></td>
<td>Atherurus macrourus porcupine, 1/1</td>
<td>Belomys pearsonii flying squirrel, 1/12</td>
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<td>Eomystes spelaea bat, 1/3</td>
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<td></td>
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<td>H. phayrei flying squirrel, 1/8</td>
<td>H. spadiceus flying squirrel, 1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muntiacus muntjak deer, 1/1</td>
<td>Paguma larvata civet, 1/2</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Prionailurus bengalensis cat, 1/3</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rhizomys sumatrensis rat, 1/6</td>
<td>Tupaia belangeri treeshrew, 1/3</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Unknown Sciuridae squirrel, 1/2</td>
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<td></td>
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<tr>
<td><em>Rickettsia</em> spp.</td>
<td>1/41</td>
<td>P. philippensis flying squirrel, 1/2</td>
<td>1/68</td>
<td>LIV, 1/40</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td><em>Rickettsia</em> felis</td>
<td>2/41</td>
<td>D. rufigenis squirrel, 1/11</td>
<td>2/68</td>
<td>LIV, 2/40</td>
<td>98–100</td>
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<tr>
<td><em>R. typhi</em></td>
<td>1/41</td>
<td>D. rufigenis squirrel, 1/11</td>
<td>1/68</td>
<td>LIV, 1/40</td>
<td>93</td>
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<td><em>Anaplasma platys</em></td>
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<td>P. hermaphroditus civet, 1/6</td>
<td>1/68</td>
<td>KID, 1/6</td>
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<td><em>A. centrale</em></td>
<td>1/41</td>
<td>M. muntjak deer, 1/1</td>
<td>5/68</td>
<td>KID, 1/6</td>
<td>98.8–99.6 (A. centrale)</td>
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<td><em>A. capra</em></td>
<td></td>
<td></td>
<td>3/40</td>
<td>LIV, 3/40</td>
<td>98.8–99.6 (A. capra)</td>
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</tr>
<tr>
<td><em>A. marginale</em></td>
<td></td>
<td></td>
<td>1/3</td>
<td>SPL, 1/3</td>
<td>98.8 (A. marginale)</td>
<td></td>
</tr>
</tbody>
</table>

*BLD, blood; KID, kidney; LIV, liver; NA, not applicable; SPL, spleen; URI, urine; URO, urogenital swab.
†Organism identified by sequencing of PCR products and identity match given in the right-hand column. All nucleotide sequences were submitted to GenBank under accession nos. MW407963–MW407984 and MW411434–MW411439.
studies have shown higher prevalence in rats (11), and although we are confident of the results from trade sites, storage of animals from POFI sites might have resulted in cross-contamination, which warrants cautious interpretation of results in this subset. Among *Leptospira* spp.-positive animals, detection was more likely in urogenital swab samples, highlighting the risk for transmission through infected urine (10). Although reservoir rodents are characterized by chronic renal infections, septicemia occurs during initial infection (10), and the high proportion of positive blood samples indicates a public health risk in relation to the consumption of uncooked or undercooked meat, organs, and blood. The PCR used to detect leptospires is specific for pathogenic and intermediate species (Appendix Table 3), but we could not confirm their human pathogenicity. The high volume of squirrel trade combined with high infection frequency suggests a high risk for exposure among wildlife consumers. Because leptospirosis is a key cause of fever in rural Laos (12), further work is needed to learn more about the relevance of contact with wildlife through trade and consumption.

The Rickettsiales species identified here are known to cause human infections in Laos (13). *R. typhi* causes murine typhus, a major underrecognized cause of fever (13). *O. tsutsugamushi* is responsible for up to 23% of fever (14), and although commonly associated with ground-dwelling rodents, the vectors (*Leptotrombidium*) parasitize squirrels (15), and *O. tsutsugamushi* has been isolated from *Callosciurus notatus* squirrels in Malaysia (16). Other bacteria identified are reviewed elsewhere (Appendix Table 6).

Although many of the human pathogens identified are transmitted by arthropod vectors, we found few arthropods in the wildlife sampled, probably because vectors leave animals quickly after animal death (17). Therefore, because most market vendors sell dead animals obtained from hunters or intermediaries (8), vendors are less likely to be exposed to disease vectors, and hunters are possibly at greater risk than market vendors or consumers. *O. tsutsugamushi* and *R. typhi* can cause infections through aerosol exposure, bites from infected animals, and needlestick injuries (18), but whether such routes of infection occur at trade sites is unclear. The frequent occurrence of *Leptospira*, which can be transmitted by direct contact with abraded skin and mucous membranes, may pose health risks to hunters, vendors, and consumers.

### Table 2. Zoonotic pathogens detected and animal species and sample types that tested positive in wildlife collected from POFI sites*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Animals</th>
<th>Species</th>
<th>Samples</th>
<th>Sample types</th>
<th>Sequencing identity match, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptospira</em> spp.</td>
<td>9/35</td>
<td><em>Callosciurus finlaysoni</em> squirrel, 1/1</td>
<td>46/256</td>
<td>SPL, 17/69; KID, 14/91; LIV, 14/92; BLD, 1/3</td>
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<td><em>Callosciurus erythraeus</em> squirrel, 4/17</td>
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<td><em>Callosciurus inornatus</em> squirrel, 2/6</td>
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<td><em>Petaurista philippensis</em> flying squirrel, 1/5</td>
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<td></td>
<td><em>Catopuma temminckii</em> cat, 1/1</td>
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<td><em>Orientia tsutsugamushi</em></td>
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<td><em>C. erythraeus</em> squirrel, 2/17</td>
<td>2/252</td>
<td>SPL, 2/252</td>
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<td><em>Rickettsia</em> spp.</td>
<td>12/35</td>
<td><em>C. erythraeus</em> squirrel, 5/17</td>
<td>70/252</td>
<td>LIV, 30/92; KID, 25/91; SPL, 15/69</td>
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<td><em>Paradoxurus hemaphroditus</em> civet, 1/2</td>
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<td><em>Catopuma temminckii</em> cat, 1/1</td>
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<td></td>
<td><em>Ratufa bicolor</em> squirrel, 1/1</td>
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<td><em>Rickettsia conorii†</em></td>
<td>1/35</td>
<td><em>P. philippensis</em> flying squirrel, 1/5</td>
<td>1/252</td>
<td>LIV, 1/252</td>
<td>99</td>
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<td><em>R. felis†</em></td>
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<td>1/252</td>
<td>LIV, 1/252</td>
<td>98</td>
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<td><em>R. typhi</em></td>
<td>6/35</td>
<td><em>C. erythraeus</em> squirrel, 6/17</td>
<td>7/252</td>
<td>KID, 4/91; LIV, 2/92; SPL, 1/69</td>
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<td><em>Anaplasmataceae</em></td>
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<td>KID, 2/252; SPL, 1/69</td>
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<td><em>Anaplasma bovis†</em></td>
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<td>KID, 1/91; LIV, 3/92; SPL, 3/69</td>
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<td><em>A. phagocytophilum†</em></td>
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<td><em>Catopuma temminckii</em> cat, 1/1</td>
<td>4/252</td>
<td>KID, 2/91; SPL, 2/252</td>
<td>98–99</td>
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<td><em>P. philippensis</em> flying squirrel, 1/4</td>
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<td><em>Ehrlichia</em> spp./E. chaffeensis†*</td>
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<td>Unknown Muridae rat, 1/1</td>
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<td>SPL, 1/69</td>
<td>97 (Ehrlichia spp.), 97 (E. chaffeensis)</td>
</tr>
<tr>
<td><em>Kurthia populii†</em></td>
<td>1/34</td>
<td><em>C. erythraeus</em> squirrel, 1/17</td>
<td>1/252</td>
<td>LIV, 1/252</td>
<td>98</td>
</tr>
<tr>
<td><em>Lactococcus garvieae†</em></td>
<td>1/34</td>
<td><em>C. erythraeus</em> squirrel, 1/17</td>
<td>1/252</td>
<td>LIV, 1/252</td>
<td>99</td>
</tr>
</tbody>
</table>

*BLD, blood; KID, kidney; LIV, liver; NA, not applicable; POFI, Provincial Office of Forestry Inspection; SPL, spleen; URI, urine; URO, urogenital swab.
†Organism identified by sequencing of PCR products and identity match given in righthand column. All nucleotide sequences were submitted to GenBank under accession nos. MW407963–MW407984 and MW411434–MW411439.
Acknowledgments
We thank the market managers and vendors for their participation and the Provincial Offices of Forest Inspection and Provincial Livestock and Fishery Section for their assistance. We also thank the Director and staff of Mahosot Hospital and the Microbiology Laboratory, the Wildlife Conservation Society Lao PDR Program, and the Ministry of Health of the Lao PDR for their support. We are very grateful to the late Rattanaphone Phetsouvanh and Manivanh Vongsouvath and Mayfong Mayxay for their support for this work.

Study protocols were reviewed and authorized by the Wildlife Conservation Society’s Institutional Animal Care and Use Committee under permit 15:04, and by the National Animal Health Laboratory, Ministry of Agriculture and Forestry, Laos.

This study was funded by the European Union under the INNOVATE program and the LACANET project (DCIASIE/2013/315-151). This work was funded in whole or in part by the Wellcome Trust (grant number: 220211). M.T.R. and P.N.N. are funded by Wellcome.

About the Author
Dr. Nawtaisong is a postdoctoral researcher specializing in molecular microbiology of zoonotic pathogens and previously worked at the Lao-Oxford-Mahosot Hospital-Instituted Animal Care and Use Committee under permit 15:04, and by the National Animal Health Laboratory, Ministry of Agriculture and Forestry, Laos.

This study was funded by the European Union under the INNOVATE program and the LACANET project (DCIASIE/2013/315-151). This work was funded in whole or in part by the Wellcome Trust (grant number: 220211). M.T.R. and P.N.N. are funded by Wellcome.

References

Address for correspondence: Matthew Robinson, LOMWRU, Microbiology Laboratory, Mahosot Hospital, Quai Fa Ngum, Vientiane City, Vientiane 01000, Laos; email: matthew.r@tropmedres.ac
Toscana virus (TOSV) is an arthropodborne virus, belonging to the genus Phlebovirus, that was first isolated in Monte Argentario in 1971 from the sand flies Phlebotomus perniciosus and P. perfiliewi (1,2). Some years later, TOSV was detected in the cerebrospinal fluid of 2 patients with meningitis (3,4), confirming its role in the etiology of this neurologic disorder. To date, phylogenetic analysis has distinguished 3 genotypes of TOSV (lineages A, B, and C), which are differentially distributed in the countries of the Mediterranean Basin (5). TOSV A is the most common cause of summer viral meningitis in central Italy and France and has a frequency eclipsing that of enteroviruses (6); lineage B is present in France and Spain, and lineage C is present in Croatia and Greece (7). Seroprevalence studies in TOSV-endemic regions indicate that most infections result in mild or self-limited febrile illnesses, whereas neurologic disorders develop in a small percentage of infected persons (6). We report the case of a young man with TOSV meningitis and prolonged persistence of TOSV in blood and semen.

The Study

On October 5, 2019, a 25-year-old man in otherwise healthy condition was admitted to the emergency department of Aurelia Hospital (Rome, Italy), for a history of acute headache, mental confusion, dysarthria, and high-grade fever since October 1. He had been living in Italy since January 27, 2019, after a 4-year period of residence in London, UK, where he abused alcohol and cocaine. During April 23–October 1, 2019, he worked as a waiter in a hotel on Elba Island, Tuscany, where he lived with his girlfriend. Their most recent sexual intercourse occurred on September 25. He reported several mosquito bites related to use of a shower located outside the hotel.

Physical examination revealed signs of meningeal irritation in the absence of focal neurologic deficits. Results of chest radiograph were negative; blood tests showed unremarkable liver and renal function results but indicated lymphocitopenia (total lymphocytes 760/μL), and thrombocytopenia (platelet count 72,000/μL).

Brain computed tomography excluded brain swelling with evidence of elevated intracranial pressure. Examination of cerebrospinal fluid (CSF) revealed pleocytosis (88 cells/μL) consisting primarily of polymorphonuclear cells, normal glucose levels, and increased total protein levels (129 mg/dL). PCR results were negative for neurotropic pathogens (Neisseria meningitides, Streptococcus pneumoniae, Escherichia coli K1, Haemophilus influenzae, Listeria monocytogenes, S. agalactiae, herpes simplex virus types 1 and 2, varicella zoster virus, cytomegalovirus, enterovirus, human...
The patient was transferred to the intensive care unit of the National Institute for Infectious Diseases “Lazzaro Spallanzani” (INMI) in Rome in a state of psychomotor agitation. Orotracheal intubation was performed with mechanical ventilation because of worsening Glasgow Coma scale score (10). Empiric treatment with acyclovir, ceftriaxone, ampicillin, and steroids was started. Epidemiologic evaluation of the case (i.e., season and residence in a TOSV-endemic area) suggested the possibility of an arboviral disease.

Real-time reverse transcription PCR (rRT-PCR) for TOSV (in-house rRT-PCR method targeting the viral medium RNA segment) was positive on CSF collected 5 days after symptom onset (cycle threshold 30.82); molecular assays for Naples, Sicilian, and Cyprus virus and flaviviruses and culture of CSF for bacteria and fungi were negative. Sequencing analysis of the medium gene revealed that the TOSV RNA detected in CSF belonged to lineage A (Figure 1; name: TOSV-CC-INMI1). Specific IgM (1:320) and IgG (1:640) titers were evaluated by indirect immunofluorescence assay (sandfly fever virus Mosaic 1; EUROIMMUN, https://www.euroimmun.com) on serum collected 17 days after symptom onset.

After clinical improvement, the patient was extubated on October 10; 2 days later, he was transferred to the high-isolation unit. Results of magnetic resonance imaging of the brain were unremarkable. The patient was discharged on October 19 in good clinical condition, and a 6-month follow-up examination did not reveal any sequelae.

After signing a written informed consent and upon INMI Ethical Board approval (issue: 14/2015), the patient was enrolled in a study on the tropism of
arboviruses. To this purpose, every 7 or 14 days, and according to the patient’s willingness and ability to reach the INMI, we collected whole blood, serum, saliva, urine, and seminal fluid for viral RNA and specific antibody detection (Figure 2).

Serologic tests demonstrated the presence of both TOSV IgM and IgG from day 17 to the end of the follow-up period at day 107 after symptom onset (Table 1). Afterward, no more visits were scheduled because of the start of the coronavirus disease pandemic. Although TOSV RNA was never detected in serum, saliva, or urine, rRT-PCR results showed an unusual long-term persistence of low-level viremia for >3 months (Table 2).

Of note, viral RNA was detected in seminal fluid from day 17 until day 59 after symptom onset. Results of rRT-PCR on seminal cells demonstrated that TOSV RNA was associated with both spermatozoa and round cells fractions, separated by gradient at the time of collection (PureSperm 40/80; NidaCon International AB, https://www.nidacon.com) (Table 2).

To determine the potential for viral transmission from semen, we inoculated Vero cells (ATCC CCL-81) grown in cell vials with blood, seminal fluid, and seminal plasma collected at day 26 after symptom onset. We were able to isolate TOSV from seminal plasma when the culture showed a cytopathic effect at passage 3. We confirmed TOSV positivity by immunofluorescence on infected cells using anti-nucleocapsid protein serum (data not shown). We extracted total RNA from supernatant of infected cells and amplified the viral genome (8), then sequenced the full-length small and medium segment and part of the large segment by the Sanger method for molecular confirmation (accession nos. MZ643219 for small complete coding sequence [CDS], MZ643217 for medium complete CDS, and MZ643218 for large partial CDS).

Conclusions
Most human TOSV infections are asymptomatic or appear as a nonspecific febrile disease. Neuroinvasive disease can occur, and, although self-resolving in most cases, TOSV infection of the central nervous system can be severe (9). In this study, we describe unusual long-term TOSV viremia and detection and persistence of TOSV in human semen. We could not

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Table 1. TOSV IgM and IgG titer in serum samples of man returning from Elba Island, Italy

<table>
<thead>
<tr>
<th>Day after symptom onset</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>26</td>
<td>320</td>
<td>1,280</td>
</tr>
<tr>
<td>31</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>38</td>
<td>320</td>
<td>1,280</td>
</tr>
<tr>
<td>45</td>
<td>640</td>
<td>1,280</td>
</tr>
<tr>
<td>59</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>74</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>107</td>
<td>320</td>
<td>640</td>
</tr>
</tbody>
</table>

*Titer expressed as reciprocal of serum dilution.
recover infectious TOSV from blood samples, probably because of high levels of specific antibodies; the persistent low-level viremia could also derive from residual virus in the cellular components of blood. We detected viral RNA in both acellular and cellular fractions of semen and isolated infectious TOSV from seminal plasma. Moreover, we detected the viral RNA in both spermatozoa and round seminal cells; both might include potential targets of TOSV infection. Nevertheless, the exact tropism for the genital tract and the origin of TOSV in semen remain to be elucidated. The patient reported no symptoms of genital inflammation, despite persistence of TOSV in genital secretions and the observation of sperm abnormalities (scarce mobility of spermatozoa was observed throughout the study period). To date, there have been few reports on genital involvement in men infected by TOSV; reported manifestations have included testicular pain, orchitis, and epididymitis (10–14). The finding of TOSV in human semen puts TOSV in the long list of viruses detected in this fluid (15), illuminating the potential for sexual transmission as an alternative route of viral spread. In this case, we did not find evidence of TOSV sexual transmission to the patient’s partner, whose serum, blood, saliva, urine, and vaginal fluid never tested positive for TOSV RNA. Moreover, despite the presence of specific IgM, we observed no IgG seroconversion during the 3-month follow-up. All these observations suggest tropism of TOSV for the male genital tract that warrants further investigation.

### Table 2. Ct values of Toscana virus RNA detected in bodily fluids of man returning from Elba Island, Italy*

<table>
<thead>
<tr>
<th>Day after symptom onset</th>
<th>Serum</th>
<th>Whole blood</th>
<th>Urine</th>
<th>Saliva</th>
<th>Seminal fluid</th>
<th>Spermatozoa</th>
<th>Round cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>ND</td>
<td>32.21</td>
<td>Neg</td>
<td>Neg</td>
<td>24.26</td>
<td>27.58</td>
<td>35.14</td>
</tr>
<tr>
<td>26</td>
<td>ND</td>
<td>32.06</td>
<td>Neg</td>
<td>Neg</td>
<td>24.20</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>31</td>
<td>ND</td>
<td>33.94</td>
<td>Neg</td>
<td>Neg</td>
<td>26.13</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>38</td>
<td>ND</td>
<td>35.11</td>
<td>Neg</td>
<td>Neg</td>
<td>28.80</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>45</td>
<td>ND</td>
<td>35.31</td>
<td>Neg</td>
<td>Neg</td>
<td>31.65</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>59</td>
<td>ND</td>
<td>35.04</td>
<td>Neg</td>
<td>Neg</td>
<td>38.20</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>74</td>
<td>ND</td>
<td>36.00</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>107</td>
<td>ND</td>
<td>38.08</td>
<td>Neg</td>
<td>Neg</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

*C, cycle threshold; ND, not detected by reverse transcription PCR; neg, negative; NT, not tested.

### About the Author

Dr. Matusali is a biologist working in the Laboratory of Virology of the National Institute for Infectious Diseases “Lazzaro Spallanzani” IRCCS (INMI), Rome. Her research is primarily focused on emerging viruses’ tropism and on immune response to viral infections. Dr. D’Abramo is a medical doctor working in the High Isolation Unit of the National Institute for Infectious Diseases “Lazzaro Spallanzani” IRCCS (INMI), Rome. She has extensive experience in the field of antimicrobial and antiviral therapy and clinical management of infectious/tropical diseases and highly infectious diseases.

### References


Address for correspondence: Concetta Castilletti, National Institute for Infectious Diseases “Lazzaro Spallanzani” IRCCS, Via Portuense 292, 00149, Rome, Italy; email: concetta.castilletti@sacrocuore.it

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Multisystem Inflammatory Syndrome in Adult after First Dose of mRNA Vaccine

Yusuke Miyazato, Kei Yamamoto, Gen Yamada, Shuji Kubota, Masahiro Ishikane, Masaya Sugiyama, Mikako Ueno, Akihiro Matsunaga, Tohru Miyoshi-Akiyama, Yukihito Ishizaka, Norio Ohmagari

Author affiliations: National Center for Global Health and Medicine, Tokyo, Japan (Y. Miyazato, K. Yamamoto, G. Yamada, S. Kubota, M. Ishikane, M. Ueno, A. Matsunaga, T. Miyoshi-Akiyama, Y. Ishizaka, N. Ohmagari); National Center for Global Health and Medicine, Chiba, Japan (M. Sugiyama)

DOI: https://doi.org/10.3201/eid2804.212585

A 32-year-old man from France living in Tokyo was admitted to the National Center for Global Health and Medicine after experiencing shortness of breath and fever. He had received the first dose of the BNT162b2 (Pfizer-BioNTech, https://www.pfizer.com) vaccine 5 days before admission. After vaccination, he experienced a fever, systemic joint pain, nausea, and vomiting. The patient sought care because of these persistent symptoms.

At admission, the patient was experiencing dyspnea as well as chest and back pain that worsened during inhalation. The patient was obese (body mass index 42.1 kg/m²). He had no history of smoking, illegal drug use, or international travel. When he received the vaccine, Japan was experiencing its largest coronavirus disease (COVID-19) surge, but he had no known exposure to patients with COVID-19. At admission, he had a body temperature of 38.1°C and peripheral oxygen saturation (SpO₂) of 95% on room air (Table). He had no notable jugular venous dilation, chest crinkles, peripheral edema, or rashes.

Laboratory test results showed an elevated inflammatory response and cardiac enzymes (Table). Chest computed tomography (CT) showed smooth interlobular septal thickening, mixed lesions with ground-glass opacity, and infiltrates in the bilateral lower lobes (Figure, panel A). Electrocardiography showed slight ST segment elevations in leads I, aVL, V1, and V2. Echocardiography showed no pericardial effusion, myocardial edema, or decreased wall motion. Real-time PCR results were negative for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Loop-mediated isothermal amplification did not detect Legionella pneumophila. We used FilmArray version 1.3 (bioMérieux, https://www.biomerieux.com) to conduct a respiratory panel on respiratory specimens and a meningitis/encephalitis panel on serum specimens to detect herpesvirus, enterovirus, and cytomegalovirus; results of both panels were negative.

One day after admission, the patient’s dyspnea and hypoxemia worsened, and he experienced profuse cold sweats. His SpO₂ dropped to 90% despite 10 L/min of oxygen supply. We suspected severe respiratory failure resulting from COVID-19 vaccine–related systemic inflammation and congestive heart failure. Therefore, we treated the patient with intravenous methylprednisolone at a dose of 1 mg/kg/day (125 mg/d) and with diuretics and noninvasive ventilation (NIV). The next day, his symptoms and hypoxemia greatly improved. He tapered off both treatments; he no longer needed ventilation 2 days after treatment and completed the course of steroids by the day of discharge, 7 days after admission. One month after discharge, CT was performed to confirm the improvement in the lung lesions (Figure, panel B).

Testing showed that SARS-CoV-2 spike IgG and neutralizing activities were significantly elevated 5 days and 23 days after the first COVID-19 vaccination dose had been administered (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/4/21-2585-App1.pdf). Moreover, SARS-CoV-2 nucleocapsid IgG in the serum was positive 5 days after COVID-19 vaccination. On the basis of these findings, we hypothesize that the patient had an asymptomatic or mild SARS-CoV-2 infection before vaccination. After his discharge, we measured a panel of 67 cytokines and chemokines from the patient and 3 healthy controls for comparison (Appendix Table, Figure 2).

This case emphasized 2 clinical issues. First, severe respiratory failure can occur after COVID-19 vaccination, and steroids effectively alleviated this complication. Second, multisystem inflammatory syndrome in adults (MIS-A) can occur after COVID-19 vaccination in a previously infected patient and can manifest as respiratory distress. In cases of respiratory failure after the vaccination, a previous SARS-CoV-2 infection should be considered.
# Table. Clinical features and laboratory results of a patient who experienced multisystem inflammatory syndrome in an adult after a coronavirus vaccination, Japan, 2021

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hospital day 1</th>
<th>Hospital day 2</th>
<th>Hospital day 3</th>
<th>Hospital day 5</th>
<th>Day of discharge (day 8)</th>
<th>1 month after discharge</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical features</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum body temperature, °C</td>
<td>38.1</td>
<td>39.1</td>
<td>36.8</td>
<td>36.8</td>
<td>36.8</td>
<td>36.0</td>
<td>NA</td>
</tr>
<tr>
<td>Maximum respiratory rate, breaths/min</td>
<td>20</td>
<td>35</td>
<td>26</td>
<td>22</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Maximum heart rate, bpm</td>
<td>126</td>
<td>128</td>
<td>120</td>
<td>111</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Minimum blood pressure, mm Hg</td>
<td>102/81</td>
<td>105/85</td>
<td>113/88</td>
<td>141/85</td>
<td>135/85</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Laboratory results</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SARS-CoV-2 real-time PCR</td>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Positive (day 19)</td>
</tr>
<tr>
<td>SARS-CoV-2 spike IgG</td>
<td>Positive</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Negative</td>
</tr>
<tr>
<td>Leukocytes, cells/µL</td>
<td>12,790</td>
<td>16,330</td>
<td>14,280</td>
<td>13,380</td>
<td>17,680</td>
<td>16,780</td>
<td>3,300–8,600</td>
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<tr>
<td>Platelets, × 10³/µL</td>
<td>166</td>
<td>217</td>
<td>240</td>
<td>294</td>
<td>341</td>
<td>208</td>
<td>158–348</td>
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<tr>
<td>Creatinine, mg/dL</td>
<td>1.02</td>
<td>1.14</td>
<td>1.26</td>
<td>1.09</td>
<td>0.95</td>
<td>1.07</td>
<td>0.65–1.07</td>
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<tr>
<td>LDH, U/L</td>
<td>210</td>
<td>228</td>
<td>225</td>
<td>227</td>
<td>214</td>
<td>213</td>
<td>124–222</td>
</tr>
<tr>
<td>Troponin I, ng/mL</td>
<td>0.371</td>
<td>1.102</td>
<td>1.306</td>
<td>0.295</td>
<td>0.094</td>
<td>0.07</td>
<td>0–0.026</td>
</tr>
<tr>
<td>BNP, pg/mL</td>
<td>129.3</td>
<td>490.5</td>
<td>NA</td>
<td>NA</td>
<td>68.0</td>
<td>NA</td>
<td>0–18.4</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>30.73</td>
<td>35.82</td>
<td>33.34</td>
<td>10.35</td>
<td>1.98</td>
<td>0.08</td>
<td>0–0.14</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>880.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>21–282</td>
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<tr>
<td>ESR, mm/h</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>49</td>
<td>NA</td>
<td>2–10</td>
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<td>IL-6, pg/mL</td>
<td>NA</td>
<td>NA</td>
<td>99.29</td>
<td>0</td>
<td>0 (day 9)</td>
<td>0 (day 44)</td>
<td>0</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Oxygen delivery devices</td>
<td>Nasal cannula</td>
<td>NIV</td>
<td>NIV</td>
<td>Nasal cannula</td>
<td>None</td>
<td>None</td>
<td>NA</td>
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<tr>
<td>Corticosteroids</td>
<td>None</td>
<td>mPSL 125 mg/d (1 mg/kg/d) IV</td>
<td>mPSL 125 mg/d (1 mg/kg/d) IV</td>
<td>None</td>
<td>None</td>
<td>NA</td>
<td></td>
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<tr>
<td>Diuretics</td>
<td>Furosemide 20 mg orally</td>
<td>Furosemide 40 mg IV</td>
<td>Furosemide 40 mg IV</td>
<td>None</td>
<td>None</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial drugs</td>
<td>LVFX 500 mg orally</td>
<td>LVFX 500 mg orally</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*BNP, brain natriuretic peptide; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IL-6, interleukin-6; IV, intravenous; IgG, immunoglobulin G; LDH, lactate dehydrogenase; LVFX, levofloxacin; mPSL, methylprednisolone; NA, not applicable; NIV, noninvasive ventilation; PSL, prednisolone; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

**Figure.** Chest computed tomography (CT) images of a male patient in Japan who was hospitalized with multisystem inflammatory syndrome. A) CT performed at hospital admission revealed infiltration in bilateral lower lobes. B) Chest CT performed a month after discharge revealed that most of these lesions had resolved.
Postvaccination myocarditis has been reported as more common in male than in female patients (1). Bozkurt et al. described mild cases (1); however, severe cases have also been reported (2). Although our patient’s myocardial damage was not severe, we suspected myocarditis based on his elevated troponin I level after COVID-19 vaccination. Vaccine-related myocarditis typically develops after the second vaccination, but it has been reported after the first vaccination of patients who had COVID-19 previously (1). Therefore, we considered the possibility of myocarditis after the first vaccination in this patient, because his serology results suggested a history of COVID-19. Moreover, his respiratory failure, severe inflammation, and serologic test results strongly suggesting a history of COVID-19 led us to suspect MIS-A, as reported by Morris et al. (3). Although the association between the COVID-19 vaccine and MIS-A development is unclear (4), the patient in our case fulfilled the clinical criteria of severe cardiac illness, hypotension, vomiting, and fever. In addition, his laboratory results showed elevated C-reactive protein levels, ferritin levels, interleukin-6 levels, and erythrocyte sedimentation rate. He also exhibited serologic positivity for SARS-CoV-2. These findings were consistent with the definition of MIS-A (5). This case showed that vaccination was a possible trigger of MIS-A in a patient who had a history of COVID-19.

The treatment for postvaccination myocarditis and MIS-A has not been standardized. As demonstrated in our case, immunosuppressive therapy, particularly corticosteroids, improved the prognosis. Intravenous immunoglobulin, anakinra, and infliximab have been used to treat multisystem inflammatory syndrome in children (6,7); a previous case report documented their role in treating MIS-A (8).

Acknowledgments
We thank Hitomi Igarashi for providing technical assistance.

This research was supported by the National Center for Global Health and Medicine Intramural Research Fund (grant no. 21A006) and Japan Agency for Medical Research and Development Research Program on Emerging and Re-emerging Infectious Diseases (no. JP20fk0108416).

About the Author
Dr. Miyazato is a clinical fellow in the Disease Control and Prevention Center at the National Center for Global Health and Medicine. His primary research interests are sexually transmitted diseases and clinical infectious diseases.

References

Address for correspondence: Kei Yamamoto, Disease Control and Prevention Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan; email: kyamamoto@hosp.ncgm.go.jp
Recurrent SARS-CoV-2 RNA Detection after COVID-19 Illness Onset during Pregnancy

Isabel Griffin, Kate R. Woodworth, Romeo R. Galang, Veronica K. Burkel, Varsha Neelam, Samantha Siebman, Jerusha Barton, Susan E. Manning, Kathryn Aveni, Nicole D. Longcore, Elizabeth M. Harvey, Van Ngo, Deborah Mbotha, Sarah Chicchelly, Mamie Lush, Valorie Eckert, Paula Dzimira, Ayomide Sokale, Miguel Valencia-Prado, Eduardo Azziz-Baumgartner, Adam MacNeil, Suzanne M. Gilboa, Van T. Tong

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (I. Griffin, K.R. Woodworth, R.R. Galang, V.K. Burkel, V. Neelam, E. Azziz-Baumgartner, A. MacNeil, S.M. Gilboa, V.T. Tong); Minnesota Department of Health, St. Paul, Minnesota, USA (S. Siebman); Georgia Department of Public Health, Atlanta (J. Barton); Massachusetts Department of Public Health, Boston, Massachusetts, USA (S.E. Manning); New Jersey Department of Health, Trenton, New Jersey, USA (K. Aveni); New York State Department of Health, Albany, New York, USA (N.D. Longcore); Tennessee Department of Health, Smithville, Tennessee, USA (E.M. Harvey); Los Angeles County Department of Public Health, Los Angeles, California, USA (V. Ngo); Washington State Department of Health, Tumwater, Washington, USA (D. Mbotha); Kansas Department of Public Health, Wichita, Kansas, USA (S. Chicchelly); Nebraska Department of Health and Human Services, Lincoln, Nebraska, USA (M. Lush); California Department of Public Health, Sacramento, California, USA (V. Eckert); Pennsylvania Department of Health, Harrisburg, Pennsylvania, USA (P. Dzimira); Philadelphia Department of Public Health, Philadelphia, Pennsylvania, USA (A. Sokale); Puerto Rico Department of Health, Bo. Monacillos Río Piedras, Puerto Rico, USA (M. Valencia-Prado)

DOI: https://doi.org/10.3201/eid2804.212354

The Surveillance for Emerging Threats to Mothers and Babies Network conducts longitudinal surveillance of pregnant persons in the United States with laboratory-confirmed severe acute respiratory syndrome coronavirus 2 infection during pregnancy. Of 6,551 infected pregnant persons in this analysis, 142 (2.2%) had positive RNA tests >90 days and up to 416 days after infection.

Detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in respiratory specimens that recurs over extended time intervals might indicate viral RNA persistence, continued viral replication, reinfection, or sample testing error (1). Although SARS-CoV-2 infections are generally acute, persistent detection of RNA in upper respiratory specimens has been described with a mean duration of 17 days and detectable RNA for up to 12 weeks after symptom onset among recovered patients (2–5). Virus detection has been reported in severely immunocompromised patients beyond 20 days and up to 143 days after an initial positive SARS-CoV-2 test result (3,4). SARS-CoV-2 reinfections documented through whole-genome sequencing are rare (6).

Immunologic changes during pregnancy might increase risk of SARS-CoV-2 infection, susceptibility to severe illness, and viral shedding (7). A cohort study identified several pregnant persons who tested positive 90 days after an initial positive test (8). The objective of our study was to describe demographic and clinical characteristics overall and by recurrent test–positive status in a convenience sample of pregnant persons with SARS-CoV-2 infection laboratory confirmed by molecular amplification detection testing (9). This exploratory analysis includes data reported from 21 jurisdictions to the Surveillance for Emerging Threats to Mothers and Babies Network; first positive reverse transcription PCR (RT-PCR) results during pregnancy occurred during March 29, 2020–December 31, 2020, with data reported through September 3, 2021.

We enrolled pregnant persons who met our inclusion criteria (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-2354-App1.pdf). We defined persons with an RT-PCR–positive respiratory specimen collected >90 days after symptom onset as recurrent positive (RP) independent of the presence of any intermittent RT-PCR–negative specimens. Persons who did not meet the RP definition were labeled not recurrent (NR). However, not all persons received follow-up testing, and additional laboratory results were voluntarily reported. Duration of RT-PCR positivity was defined as the number of days from symptom onset until the last known positive RT-PCR in a respiratory specimen. Duration of RNA shedding was defined as the number of days from symptom onset until the second consecutive negative SARS-CoV-2 result by RT-PCR among pregnant persons reported with a follow-up test. Testing was not routine, and this factor likely will overestimate length of RNA shedding.

Among 6,551 pregnant persons (median age 29 years; 39.7% non-Hispanic White) in our analysis, 17.5% had first trimester infections, 35.5% second, and 47.0% third (Table). Median duration of RT-PCR positivity was 3 days (range 0–416 days). Overall, we collected 9,985 respiratory specimens with RT-PCR results (which could...
Table. Symptomatic pregnant persons with SARS-CoV-2 infection detected in respiratory specimens by recurrent positive status, based on RT-PCR positive test result >90 d after symptom onset. Surveillance for Emerging Threats to Mothers and Babies Network, United States, March 29–December 31, 2020*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total, N = 6,551</th>
<th>Not recurrent, n = 6,409</th>
<th>Recurrent positive, n = 142</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median duration of RT-PCR positivity (range), d</td>
<td>3 (0–416)</td>
<td>3 (0–90)</td>
<td>127 (91–416)</td>
</tr>
<tr>
<td>Median duration of RNA shedding (range), d†</td>
<td>135 (0–441)</td>
<td>148 (0–441)</td>
<td>143 (18–412)</td>
</tr>
<tr>
<td>Median age at first positive result (IQR), y</td>
<td>29 (25–33)</td>
<td>29 (25–33)</td>
<td>29 (25–32)</td>
</tr>
<tr>
<td>Age at initial infection, y‡</td>
<td>&lt;25</td>
<td>1,547 (23.6)</td>
<td>1,512 (23.6)</td>
</tr>
<tr>
<td></td>
<td>25–29</td>
<td>2,034 (31.0)</td>
<td>1,990 (31.0)</td>
</tr>
<tr>
<td></td>
<td>30–34</td>
<td>1,857 (28.4)</td>
<td>1,809 (28.2)</td>
</tr>
<tr>
<td></td>
<td>&gt;35</td>
<td>1,113 (17.0)</td>
<td>1,098 (17.1)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White non-Hispanic</td>
<td>2,599 (39.7)</td>
<td>2,554 (39.8)</td>
<td>45 (31.7)</td>
</tr>
<tr>
<td>Asian non-Hispanic</td>
<td>275 (4.2)</td>
<td>270 (4.2)</td>
<td>5 (3.5)</td>
</tr>
<tr>
<td>Black non-Hispanic</td>
<td>1,197 (18.3)</td>
<td>1,171 (18.3)</td>
<td>26 (18.3)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2,077 (31.7)</td>
<td>2,029 (31.7)</td>
<td>48 (33.8)</td>
</tr>
<tr>
<td>Multiple or other non-Hispanic</td>
<td>210 (3.2)</td>
<td>200 (3.1)</td>
<td>10 (7.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>193 (2.9)</td>
<td>185 (2.9)</td>
<td>8 (5.6)</td>
</tr>
<tr>
<td>Trimester of SARS-CoV-2 infection§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>1,146 (17.5)</td>
<td>1,114 (17.4)</td>
<td>32 (22.5)</td>
</tr>
<tr>
<td>Second</td>
<td>2,327 (35.5)</td>
<td>2,231 (34.8)</td>
<td>96 (67.6)</td>
</tr>
<tr>
<td>Third</td>
<td>3,078 (47.0)</td>
<td>3,064 (47.8)</td>
<td>14 (9.9)</td>
</tr>
<tr>
<td>Underlying conditions¶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4,945 (74.4)</td>
<td>1,478 (25.6)</td>
<td>10 (7.0)</td>
</tr>
<tr>
<td>No</td>
<td>1,488 (22.7)</td>
<td>4,184 (72.4)</td>
<td>131 (92.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>118 (1.8)</td>
<td>117 (2.0)</td>
<td>1 (0.7)</td>
</tr>
</tbody>
</table>

*Values are no. (%) except as indicated. IQR, interquartile range; RT-PCR, reverse transcription PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
†Duration of RNA shedding was available for only 458 persons.
‡Age in years at initial infection was calculated based on date of first SARS-CoV-2–positive test result during pregnancy or symptom onset if date of positive is unavailable.
§Trimester of SARS-CoV-2 infection is calculated based on date of last menstrual period and either date of first positive SARS-CoV-2 results or symptom onset if date of positive unavailable.
¶Presence of any underlying condition was defined as any of the following: obesity, chronic lung disease, hypertension, diabetes, cardiovascular disease, or immunosuppressive conditions (conditions that weaken the immune system [e.g., HIV or AIDS, some cancers such as leukemia] or medications [e.g., chemotherapy or radiation treatment, chronic corticosteroids, or other immunosuppressive medications]).

include multiple positive, negative, or indeterminate results per person); 12.5% of specimens tested 90 days after initial symptom onset were positive (158/1,257 specimens) and 9.2% of specimens tested 330 days after initial symptom onset were positive (6/65 specimens). Median duration of documented RNA shedding per person was 130 days (range 0–441 days; n = 458).

Overall, 142 persons (2.2%) from 14 jurisdictions met the RP definition. Among 6,409 NR persons, 727 (11.3%) were RT-PCR-negative after 90 days; 5,682 (88.6%) did not have any known RT-PCR results after 90 days. Several RPs were positive by RT-PCR in respiratory specimens for up to 330 days after symptom onset (Figure). Comparing RP to NR persons identified small differences in age group, race/ethnicity, and trimester of infection (Table). A higher percentage of RPs (33.8%) than NRs (28.2%) were 30–34 years of age. Non-Hispanic White persons made up 39.8% of RPs compared with 31.7% of RPs; most RPs were Hispanic (33.8%) and non-Hispanic Black (18.3%). Most RPs had second trimester infections (67.6%), whereas most NRs had third trimester infections (47.8%).

Our study’s first limitation is that SARS-CoV-2 genetic sequencing was not reported; reinfection could not be distinguished from recurrent viral shedding. A positive RT-PCR result alone without other information (data on symptom onset, cycle threshold, or viral culture) cannot distinguish infectious virus from noninfectious genomic fragments. Second, health departments were not required to send laboratory testing beyond the first positive result that occurred during pregnancy; these findings are from a convenience sample of pregnant persons with varied duration of follow-up and do not estimate the actual extent of recurrent positivity. Furthermore, persons with infections earlier in pregnancy might be more likely to be classified as RP, given that they can be followed longer and receive additional COVID-19 testing compared with persons with initial symptom onset later in pregnancy. Last, only 31.4% of our cohort (n = 2,062) had multiple test results, representing a small proportion of pregnant persons.

The findings of this report suggest that specimens from pregnant persons diagnosed with symptomatic SARS-CoV-2 infections might be recurrently positive for up to 416 days after symptom onset. Future prospective cohort studies among pregnant persons with SARS-CoV-2 testing should be performed over
consistent lengths of time, distinguish infectious viral shedding from noninfectious recurrent positive PCR results, and examine risk of reinfection during pregnancy given the recent emergence of new coronavirus disease variants. Longitudinal surveillance of pregnant persons with COVID-19 can be used for hypothesis generation, in addition to monitoring the impact of infection on pregnancy and infant outcomes.

Acknowledgments
We thank our Surveillance for Emerging Threats to Mothers and Babies Network (SET-NET) jurisdictional partners: California Department of Public Health (Valorie Eckert, Barbara Warmerdam, Similoluwa Sowunmi, Olga Barer), Georgia Department of Public Health (J. Michael Bryan, Cristina Meza, Victoria Sanon, Teri’ Willabus, Cynthia Carpentieri, Michael Andrews, Sashawn Lawrence, Camille Millar), Houston Health Department, Iowa Department of Public Health, Kansas Department of Public Health, Los Angeles County Department of Public Health (Umme-Aiman Halai, Caleb Lu, Emily Barnes, Alison Ryan, Nina Mykhaylov), Massachusetts Department of Public Health (Eirini Nestoridi, Hanna Shephard, Mahsa Yazdy, Catherine Brown), Michigan Department of Health and Human Services, Minnesota Department of Health, Nebraska Department of Health and Human Services, Maryland Department of Health and Human Services, New Jersey Department of Health and Senior Services (Shoshana Merzel), New Hampshire Department of Health and Human Services, New York State Department of Health (Zahra S. Alaali, Nadia Thomas, Pauline Santos), Pennsylvania Department of Health, Philadelphia Department of Public Health (My-Phuong Huynh), Puerto Rico Department of Health (Camille Delgado-López, Mariam Marcano-Huertas, Leishla Nieves-Ferrer), South Carolina Department of Health and Environmental Control, Tennessee Department of Health (Lindsey Sizemore, Heather Wingate), US Virgin Islands Department of Health, and Washington State Department of Health. We would also like to acknowledge our colleagues.
Hantavirus Pulmonary Syndrome in a COVID-19 Patient, Argentina, 2020

Rocio M. Coelho,1 Natalia Periolo,1 Carolina Perez Duhalde, Daniel O. Alonso, Carla M. Bellomo, Marisa Corazza, Ayelén A. Iglesias, Valeria P. Martinez

About the Author

Dr. Griffin is a contracted epidemiologist with Eagle Global Scientific, LLC, in the National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, in Atlanta, Georgia, USA. Her primary research interest is emerging infectious diseases.

References


1. These authors contributed equally to this article.

We describe a patient in Argentina with severe acute respiratory syndrome coronavirus 2 infection and hantavirus pulmonary syndrome (HPS). Although both coronavirus disease and HPS can be fatal when not diagnosed and treated promptly, HPS is much more lethal. This case report may contribute to improved detection of co-infections in HPS-endemic regions.

The current coronavirus disease (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in substantial illness and death rates worldwide. Orthohantaviruses are zoonotic viruses responsible for another severe respiratory infectious disease in the Americas, hantavirus pulmonary syndrome (HPS). Although humans generally become infected with HPS through inhaling excreta generated by infected rodents, person-to-person transmission has been well documented in Argentina and Chile (1–3). Humans become infected with SARS-CoV-2 and orthohantaviruses in similar ways, through inhaling contaminated aerosols, and can have onset of similar respiratory syndromes. Despite these similarities, the incubation period is shorter in COVID-19 patients (2–14 days) than in HPS patients (7–45 days). Furthermore, at the time the case we describe was reported, the cumulative case-fatality rate for COVID-19 in Argentina was 2.7% (4); for HPS, it was 22%–40% (5).

HPS is characterized by the onset of symptoms such as fever, myalgia, cough, dyspnea, diarrhea, and sweating. Rapid progression to shock or respiratory distress can occur within hours. Symptom-based...
therapy with oxygen and ventilatory or circulatory support is needed (6,7).

We describe a case of SARS-CoV-2 and Andes virus co-infection in central Argentina. The patient, a 22-year-old woman without relevant pathologic records, sought care at a local hospital in November 2020 for fever, headache, myalgia, and gastrointestinal manifestations. A nasopharyngeal swab sample tested positive for SARS-CoV-2 by reverse transcription PCR at the Instituto Biológico “Tomás Perón” (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-1837-App1.pdf). Five days after the onset of fever, the patient’s clinical status worsened, and she was admitted to the hospital. Clinical laboratory findings at admission indicated thrombocytopenia, high leukocyte count, lymphopenia, and elevated hepatic enzymes (Appendix). Computed tomography revealed bilateral pleural effusion associated with interstitial infiltration, and capillary filtration with slight peripheral pulmonary ground-glass opacity (Figure).

Within a few hours after admission, the patient had onset of marked respiratory distress. She was then referred to the intensive care unit for orotracheal intubation and treated with ampicillin/sulbactam and azithromycin. The epidemiologic investigation established that the patient resided in a hantavirus-endemic area. Consequently, HPS was suspected, despite the COVID-19–positive diagnosis. According to the confirmation criteria used by the Hantavirus National Reference Laboratory (8), Andes virus infection was confirmed by the detection of specific IgM and IgG by ELISA and genomic viral RNA by quantitative reverse transcription PCR in blood (Appendix).

Three days after the co-infection was confirmed, the patient was extubated and progressed favorably. Twenty days after onset of symptoms, she was discharged from the hospital.

To determine the viral genotype of Andes virus, we conducted a nucleotide sequence analysis from 2 partial fragments of viral small (496-bp) and medium (611-bp) segments, and we submitted the sequences obtained to GenBank (accession nos. OL840325 and OL840326). The highest nucleotide identities matched previous published sequences corresponding to Plata genotype of Andes virus (GenBank accession nos. EU564720 [96% identity] and AY101185 [97.8 identity]). This viral genotype is one of the prevalent pathogenic orthohantaviruses circulating in central Argentina and Uruguay (9).

Because the incubation period for HPS is longer than that for COVID-19, we might speculate that hantavirus infection occurred before coronavirus infection. The respiratory distress syndrome appeared 5 days after the onset of fever, which coincided with the characteristic prodromal period described for HPS. This condition, during the incubation period of HPS, could have induced a higher susceptibility to COVID-19. Because HPS can evolve rapidly to respiratory failure in most patients with severe disease, resulting in high case-fatality rates, alerting health-care workers from HPS-endemic areas is warranted to detect co-infections in the context of the COVID-19 pandemic. In particular, at least 2 genotypes of Andes virus can be transmitted person-to-person, and these species are prevalent in 2 of the 3 hantavirus-endemic regions of Argentina (10).

In conclusion, we detected co-infection with SARS-CoV-2 and Andes virus causing HPS in a patient from a hantavirus-endemic area. Clinicians should be aware of the possibility of co-infection for patients originating, residing, or traveling in hantavirus-endemic areas.
Early Circulation of SARS-CoV-2, Congo, 2020


DOI: https://doi.org/10.3201/eid2804.212476

To determine when severe acute respiratory syndrome coronavirus 2 arrived in Congo, we retrospectively antibody tested 937 blood samples collected during September 2019—February 2020. Seropositivity significantly increased from 1% in December 2019 to 5.3% in February 2020, before the first officially reported case in March 2020, suggesting unexpected early virus circulation.

After coronavirus disease (COVID-19) was reported in China in December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly spread around the world; most countries officially reported their first cases within the first 3 months of 2020. However, reports from China show a possible earlier first case on November 17, 2019, detected retrospectively in Hubei Province (1). Furthermore, phylogenetic analysis places the date of emergence as sometime during October–December 2019 (2). These data suggest possible virus spread outside China before the first officially reported case in December 2019. Indeed, several retrospective studies that analyzed stored respiratory samples and wastewater for RNA detection, as well as serologic studies, suggest that SARS-CoV-2 may have been circulating in France, Spain, and Italy (3–7) before December 2019, months before the first official cases were reported.

In central Africa, the first cases were officially reported during March 6–April 6, 2020; in Congo, the first case was reported on March 14, 2020. However, a
serologic study in Kenya suggested that the virus was present in January 2020, two months before the first official case was reported (8). Similar retrospective studies have not been conducted in Central Africa, meaning that the time of SARS-CoV-2 introduction in this region remains unknown.

To provide a more accurate date for the arrival of SARS-CoV-2 in Congo, we retrospectively examined serum samples collected from persons with HIV (PWH) as a part of the national HIV program. These samples were collected during July 2019–February 2020 in Brazzaville and Pointe-Noire, the 2 biggest cities in Congo (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-2476-App1.pdf). The study was conducted with approval of the Comité Technique de la Riposte à la Maladie à Coronavirus COVID-19, of which F.R.N. is president of the commission laboratory and research, and the Programme National de Lutte Contre le SIDA, led by the National Public Health laboratory of Congo, of which F.R.N. is director.

We tested 1,212 plasma samples for SARS-CoV-2 IgG by using a microsphere immunoassay with beads coupled with receptor-binding domain antigen. We used 275 samples collected during July–August 2019 as negative controls and to establish the seropositivity cutoff value of our test (Appendix). The remaining 937 samples were collected September 2019–February 2020. Overall, 28/937 (3.0%) samples were positive: 22/655 (3.3%) from women, 5/241 (2.1%) from men, and 1/41 (2.4%) from a patient for whom sex was not reported. SARS-CoV-2 seropositivity rate was 1.7% (10/563) in Brazzaville and 4.8% (18/374) in Pointe-Noire. However, the Pointe-Noire samples were all collected in 2020 and compared with those from Brazzaville from the same period (5.4%; 6/110) did not differ significantly (p = 0.8). Although seropositivity was very low from September through November, seropositivity subsequently increased linearly, reaching 5.3% by February 2020 (Figure). Furthermore, seropositivity was significantly higher in January–February 2020 (p = 0.0002) than in the preceding 4 months of 2019 (Table). We also observed a significant increase between samples collected in Brazzaville in 2019 and those collected in Brazzaville in 2020 (p = 0.0052).

Our results suggest increased SARS-CoV-2 circulation during January–February 2020 in Congo, indicating that the virus arrived in the country in December 2019. Our findings align with those of a serologic study of an asymptomatic general population in Congo, conducted in April 2020, which found 1.7% seropositivity for IgG and 2.5% for IgM (9). The higher seropositivity found before April in our study may result from the higher sensitivity of the microsphere immunoassay as compared with that of rapid tests (9). Moreover, the PWH in our study may be more exposed to the virus than the randomized general population tested by Batchi-Bouyou et al. because PWH must regularly visit healthcare centers as part of their treatment. A recent study of participants with and without HIV tested during January–March 2020 in Kenya reported 3%–4% positivity was significantly higher in January–February 2020 (p = 0.0002) than in the preceding 4 months of 2019 (Table). We also observed a significant increase between samples collected in Brazzaville in 2019 and those collected in Brazzaville in 2020 (p = 0.0052).

Our results suggest increased SARS-CoV-2 circulation during January–February 2020 in Congo, indicating that the virus arrived in the country in December 2019. Our findings align with those of a serologic study of an asymptomatic general population in Congo, conducted in April 2020, which found 1.7% seropositivity for IgG and 2.5% for IgM (9). The higher seropositivity found before April in our study may result from the higher sensitivity of the microsphere immunoassay as compared with that of rapid tests (9). Moreover, the PWH in our study may be more exposed to the virus than the randomized general population tested by Batchi-Bouyou et al. because PWH must regularly visit healthcare centers as part of their treatment. A recent study of participants with and without HIV tested during January–March 2020 in Kenya reported 3%–4%

### Table. Seropositivity of SARS-CoV-2 IgG among persons with HIV, Congo, July 2019–February 2020*

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. tested/no positive (% positive)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>655/22 (3.3)</td>
<td>0.38</td>
</tr>
<tr>
<td>M</td>
<td>241/5 (2.1)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>41/1 (2.4)</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazzaville 2019</td>
<td>453/4 (0.8)</td>
<td>0.0052</td>
</tr>
<tr>
<td>Brazzaville 2020</td>
<td>110/6 (5.4)</td>
<td></td>
</tr>
<tr>
<td>Pointe-Noire 2020</td>
<td>374/18 (4.8)</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2019 September–December</td>
<td>453/4 (0.9)</td>
<td>0.0002</td>
</tr>
<tr>
<td>2020 January–February</td>
<td>484/24 (4.9)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>937/28 (3.0)</td>
<td></td>
</tr>
</tbody>
</table>

*NA, not applicable; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
seropositivity, which did not differ between these populations (8). Early circulation of SARS-CoV-2 has also been found in France, Spain, and Italy; seropositivity estimates in France increased from 1.3% in November 2019 to 6.7% in February 2020 (6).

There is some concern that seropositive samples may reflect possible cross-reactions with other coronaviruses that infect humans (human coronaviruses NL63, 229E, OC43, and HKU1 and Middle East respiratory system coronavirus) (10). Although cross-reaction may explain the very low SARS-CoV-2 seropositivity in September–October 2019, the significant increase in seropositivity from the end of 2019 to the beginning of 2020 argues in favor of actual detection of antibodies directed against SARS-CoV-2. The early introduction of SARS-CoV-2 in Congo, and more generally in Africa, probably results from the intense trade activities that link Africa to China, leading to frequent exchange of persons between these countries.

Determining early circulation patterns of SARS-CoV-2 in Africa or other countries requires retrospective testing of as many samples as possible from existing national sample repositories. Such studies will help enrich knowledge of the propagation of pathogens in the context of globalization of human and material exchange. To better evaluate the epidemiology of future pandemics, international organizations should help reinforce and develop repositories in low- and middle-income countries.

Start// Acknowledgments
We are grateful to the study participants. We also thank the healthcare workers in all the treatment centers in Congo and Kurt McKean for the English editing of the manuscript.

The study was funded by the World Organisation for Animal Health through the European Union EBO-SURSY.

About the Author
Mr. Bobouaka Bonguili is a master’s student under the direction of co-authors F.R.N., E.M.L., and M.F. He is a member of the SARS-CoV-2 response team established by the National Laboratory of Public Health. Dr. Fritz is a postdoctoral researcher in the unit Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle at the Institute for sustainable development. He develops serologic techniques to detect zoonotic viruses.

References

Start// Address for correspondence: Fabien Niama, Laboratoire National de Santé Publique, Avenue du General de Gaulle, Brazzaville, Congo; email: fabien.niama@gmail.com
Amplification Artifact in SARS-CoV-2 Omicron Sequences Carrying P681R Mutation, New York, USA

Adriana Heguy, Dacia Dimartino, Christian Marier, Paul Zappile, Emily Guzman, Ralf Duerr, Guiqing Wang, Jonathan Plitnick, Alexis Russell, Daryl M. Lamson, Kirsten St. George


DOI: https://doi.org/10.3201/eid2804.220146

Of 379 severe acute respiratory syndrome coronavirus 2 samples collected in New York, USA, we detected 86 Omicron variant sequences containing Delta variant mutation P681R. Probable explanations were co-infection with 2 viruses or contamination/amplification artifact. Repeated library preparation with fewer cycles showed the P681R calls were artifactual. Unusual mutations should be interpreted with caution.

The recently emerged Omicron variant of severe respiratory syndrome coronavirus 2 (SARS-CoV-2) (1) is highly transmissible and partially immune evasive (2). Omicron contributes to the recent surges in coronavirus disease (COVID-19) case numbers, even in locations with highly vaccinated populations and vaccination requirements for indoor dining and events, such as New York, New York, USA. During our ongoing SARS-CoV-2 genomic surveillance using full-genome sequencing with random sample selection from positive cases at NYU Langone Health, a large metropolitan healthcare system, we observed a rapid rise in Omicron and displacement of Delta. We analyzed a subset of 379 Omicron sequences, which we deposited in GISAID (https://www.gisaid.org), all BA.1 sublineage, from cases detected during November 30, 2021–January 5, 2022.

Detailed methods were recently described (3). In brief, we used the xGen SARS-CoV-2 Amp Panel 96rxn (Integrated DNA Technologies [IDT], https://eu.idtdna.com) and 18 or 24 cycles for the multiplex PCR step of library amplification. Of the 379 samples, amplified using 24 cycles regardless of cycle threshold (Ct), we detected 86 with P681R, a key Delta mutation associated with increased transmissibility, fusogenicity, and pathogenicity (4), distinct from the P681H mutation of Omicron. The presence of P681R in Omicron was cause for concern because it could be associated with higher pathogenicity/transmissibility.

Closer examination indicated that the Omicron sequences with P681R contained varying numbers of P681H reads (median frequency of P681R call, a G at nucleotide position 23604, was 0.79 [range 0.43–0.98]). This observation could indicate either co-infection with Omicron and Delta or contamination/artifact of sample processing and library preparation. We also observed that sequences with P681R were from samples with higher Ct according to real-time detection assays compared with those with P681H, (open reading frame, TaqPath COVID-19 Combo Kit, Applied Biosystems, https://www.thermofisher.com) (median Ct = 22 for P681H and 28 for P681R; p<7.0803 × 10−37). We also observed that coverage of nt 23604 was higher when the call was G (Delta context) than when the call was A (Omicron context) (Figure). These observations are consistent with contamination of Omicron samples with lower Ct by Delta sequences, possibly exacerbated by over-amplification and preference of the polymerase for the specific amplicon flanked by primers at positions 23534 and 23641 (covid19 genome_200–29703_s20720_D_32 in the IDT xGen kit).

To investigate further, we repeated the library preparation and sequencing on 13 random samples previously assigned as P681R and 13 assigned as P681H as controls and changed 2 parameters: reverse transcriptase (RT) and PCR cycles. We prepared 10 samples with SuperScript IV Reverse RT (recommended by IDT xGen kit) and 3 samples with Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, https://www.thermofisher.com). To exclude the possibility that a high number of cycles could exacerbate cross-contamination of samples with low viral load, we ran either 18 or 24 cycles for the multiplex PCR step of library amplification. We also sent residual portions of 12 nasopharyngeal swab specimens to the New York State Department of Health (Albany, NY, USA) for comparative sequencing with a different platform and chemistry. The Department of Health performed RNA extraction with an easyMAG (bioMérieux, https://www.biomerieux.com) and library preparation and sequencing with an Ion Chef and Ion S5 XL System, using the Ion AmpliSeq SARS-CoV-2 Insight Research Assay with 27 cycles (ThermoFisher Scientific, https://www.thermofisher.com). To exclude the possibility that a high number of cycles could exacerbate cross-contamination of samples with low viral load, we ran either 18 or 24 cycles for the multiplex PCR step of library amplification. We also sent residual portions of 12 nasopharyngeal swab specimens to the New York State Department of Health (Albany, NY, USA) for comparative sequencing with a different platform and chemistry. The Department of Health performed RNA extraction with an easyMAG (bioMérieux, https://www.biomerieux.com) and library preparation and sequencing with an Ion Chef and Ion S5 XL System, using the Ion AmpliSeq SARS-CoV-2 Insight Research Assay with 27 cycles (ThermoFisher Scientific, https://www.thermofisher.com).
Repeating the library preparations and resequencing by different methods produced sequences with no P681R calls, except for 2 samples that showed P681R with 24 PCR cycles and P681H with 18 cycles (Table), indicating that a high number of PCR cycles can introduce false mutation calls. Our combined experiments also confirmed that these were not errors induced by reverse transcriptase during the cDNA synthesis step.

We then reprocessed the remaining 61 samples with P681R, using the xGen kit with 18 cycles of amplification. A total of 59 samples showed P681H on this repeated testing; only 2 still showed P681R, 1 at 0.52 frequency (down from 0.98) and 1 at 0.94, exactly as the previous sequence, suggesting a true P681R call, possibly co-infection with Delta, because the C<sub>t</sub> for this sample was low (C<sub>t</sub> = 17). We performed an additional RNA extraction, library preparation, and sequencing for this sample; the P681R persisted at frequency 0.84, suggesting that this sample represents co-infection.

We conclude that the foremost reason for detecting P681R in our Omicron samples was contamination with Delta amplicons and artifactual mixed base pair calls, resulting from preferential coverage of that specific position and amplicon in the context of Delta but not Omicron. Although non-amplicon-based approaches such as capture-hybridization libraries using SARS-CoV-2 baits generally lead to more even coverage, and amplicon-based methods are known to result in dropouts because of new mutations in different variants, amplicon methods have been widely adopted by genomic surveillance laboratories under pressure for faster turnaround.

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**Table.** Results of repeated library preparations and sequencing of 25 random SARS-CoV-2 samples with Omicron variant and mutation previously assigned as P681R, including an alternative chemistry and sequencing platform*  

<table>
<thead>
<tr>
<th>Location</th>
<th>NYULH Genome Technology Center</th>
<th>NYSDOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol</td>
<td>xGen SARS-CoV-2 Amp Panel 96x†</td>
<td>AmpliSeq SARS-CoV-2 Insight‡</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>SuperScript IV</td>
<td>Maxima H Minus</td>
</tr>
<tr>
<td>PCR cycles, no. P681R calls</td>
<td>18, 0</td>
<td>18, 0</td>
</tr>
<tr>
<td></td>
<td>24, 1</td>
<td>24, 1</td>
</tr>
</tbody>
</table>

*NA, not applicable; NYSDOH, New York State Department of Health, Albany, NY, USA, which performed ThermoFisher sequencing chemistry on 12 samples; NYULH, NYU Langone Health, New York, NY, USA, which performed Illumina sequencing chemistry on 13 different samples; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

§Invitrogen (https://www.thermofisher.com).
and high volumes, especially during large waves of infection. We urge laboratories to confirm unusual mutation findings by repeating libraries and sequencing or by using alternative protocols, or both, to avoid artifacts and ensure accurate sequences in databases such as GISAID, which are used by the global scientific community.

Acknowledgments
We thank the clinical laboratory technicians, especially Joanna Fung, for assistance with testing, saving, and retrieving specimens. We also thank Joan Cangiarella for her continuous support of genomic surveillance for SARS-CoV-2 at NYU Langone Health, including provision of institutional funding for this study. In addition, we thank Benjamin Rambo Martin, Kristine Lacek, and John Barnes for helpful comments and Katarzyna Wilk for verifying our coverage findings around the P681 position.

About the Author
Dr. Heguy is director of the Genome Technology Center at NYU Langone Health and professor at the Department of Pathology, NYU Grossman School of Medicine. She has been involved in SARS-CoV-2 genomic surveillance since the start of the pandemic, and her laboratory has submitted >6,000 sequences to GISAID.

References

Address for correspondence: Adriana Heguy, Department of Pathology, NYU Grossman School of Medicine, Genome Technology Center, NYU Langone Health, 550 First Ave, MSB 294A, New York, NY 10016, USA; email: adriana.heguy@nyulangone.org

Tularemia Transmission to Humans, the Netherlands, 2011–2021

Jolianne M. Rijks,1 Anna D. Tulen,1 Daan W. Notermans, Frans A.G. Reubsaet, Maaike C. de Vries, Miriam G.J. Koene, Corien M. Swaan, Miriam Maas

Author affiliations: Dutch Wildlife Health Centre, Utrecht University, Utrecht, the Netherlands (J.M. Rijks); European Programme for Intervention Epidemiology Training (EPIET), European Centre for Disease Prevention and Control, Stockholm, Sweden (A.D. Tulen); National Institute for Public Health and the Environment, Bilthoven, the Netherlands (A.D. Tulen, D.W. Notermans, F.A.G. Reubsaet, M.C. de Vries, C.M. Swaan, M. Maas); Wageningen Biodiversity Research, Wageningen University and Research, Lelystad, the Netherlands (M.G.J. Koene)

DOI: https://doi.org/10.3201/eid2804.211913

We used national registry data on human cases of Francisella tularensis subspecies holarctica infection to assess transmission modes among all 26 autochthonous cases in the Netherlands since 2011. The results indicate predominance of terrestrial over aquatic animal transmission sources. We recommend targeting disease-risk communication toward hunters, recreationists, and outdoor professionals.

Francisella tularensis subspecies holarctica bacteria are the main causative agent of tularemia in Europe (1). The pathogen can be transmitted to humans from animals, vectors, food and water, or the environment, through broken skin or via conjunctival, oral, or respiratory routes. The clinical manifestation of tularemia in humans can be ulceroglandular, glandular, ocuglandular, oropharyngeal, pneumonic, or typhoidal. The bacterium has a complex ecology and 2 interconnected lifecycles: a terrestrial lifecycle associated primarily with lagomorphs, small rodents, ticks, and tabanids; and an aquatic lifecycle associated with mosquitoes, semiaquatic animals such as beavers, contaminated water, and mud (1). The relative contribution of these lifecycles to human tularemia varies among countries (1).

In the Netherlands, no autochthonous human cases were reported during 1953–2010 (2), even though notification was mandatory during January 1976–April 1999. However, since 2011, multiple autochthonous human tularemia cases caused by F. tularensis subsp. holarctica infection have been detected

1 These first authors contributed equally to this article.
(2–5) and systematically registered; mandatory notification was reinstated in 2016. In addition, the bacterium has been detected since 2013 in European brown hares (Lepus europaeus) and Eurasian beavers (Castor fiber), as well as in surface water (6–8). No wildlife cases were reported during 1953–2012 (6).

To target preventive measures and communication regarding human tularemia requires insight into the main transmission modes and identification of the lifecycle. We assessed the distribution of transmission modes in autochthonous human tularemia cases in the Netherlands, using the national registry of tularemia cases. We extracted data from all autochthonous human cases from 2011–2021 from the National Public Health Institute database. Public health authorities identified the most probable transmission mode of each case at the time of diagnosis on the basis of the clinical presentation of the disease combined with information on exposure, either occupational or nonoccupational, obtained from standardized interviews with each patient https://lci.rivm.nl/sites/default/files/2018-10/LCI-richtlijn%20Tularemie%20-%20bijlage%203%20vragenlijst%20Osiris.pdf. We aggregated cases per transmission mode and allocated them to either the terrestrial or aquatic lifecycle of F. tularensis subsp. holarctica. We considered the transmission mode confirmed if the source was an animal carcass that tested positive for F. tularensis subsp. holarctica by quantitative PCR or culture; otherwise, the mode remained probable. We included clinical manifestations and basal clade data of cases in the overview if available.

In total, we analyzed 26 human cases from across the country, all but 2 in male patients. Median age was 52 (range 1–78) years. In 23 cases, the source was confirmed (n = 2) or probable (n = 21). Of these, 16 cases were allocated to the terrestrial lifecycle, and 7 to the aquatic lifecycle. In 3 cases, the transmission mode was unclear; we excluded these cases from further analysis (Table).

Occupational exposure was likely in 4/23 cases: 1 case-patient was probably infected while tending to cattle in pasture, the other 3 while performing vegetation maintenance, and 2 of those 3 had the pneumonic tularemia, which was reported in no other patients (Table). The strain from 1 pneumonic case-patient had been characterized previously as belonging to

<p>| Table. Overview of autochthonous human tularemia infections reported in the Netherlands, 2011–2021 |
|---------------------------------------------------|----------|---------|-----------------|--------|</p>
<table>
<thead>
<tr>
<th>Life cycle</th>
<th>Transmission mode</th>
<th>Probable or confirmed mode</th>
<th>Year</th>
<th>Occupational exposure</th>
<th>Clinical manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terrestrial</td>
<td>Aerosols from contaminated vegetation</td>
<td>Probable</td>
<td>2016</td>
<td>Yes</td>
<td>Pneumonic</td>
</tr>
<tr>
<td></td>
<td>Contact with (or consumption of) infected hare carcass</td>
<td>Probable</td>
<td>2017</td>
<td>Yes</td>
<td>Pneumonic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2014</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confirmed</td>
<td>2014</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2016</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2017</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2019</td>
<td>No</td>
<td>Ulceroglandular and oropharyngeal</td>
</tr>
<tr>
<td></td>
<td>Mouse bite</td>
<td>Probable</td>
<td>2021</td>
<td>No</td>
<td>Glandular</td>
</tr>
<tr>
<td></td>
<td>Tick bite</td>
<td>Probable</td>
<td>2021</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td>Insect bite while on land</td>
<td>Probable</td>
<td>2020</td>
<td>No</td>
<td>Glandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Confirmed</td>
<td>2013</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable†</td>
<td>2016</td>
<td>No</td>
<td>Glandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2021</td>
<td>Yes</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td>Aquatic</td>
<td>Contact with contaminated water/mud</td>
<td>Probable†</td>
<td>2016</td>
<td>No</td>
<td>Glandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2016</td>
<td>Yes</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2016</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable†</td>
<td>2015</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td>Contact with contaminated water or insect bite</td>
<td>Probable†</td>
<td>2021</td>
<td>No</td>
<td>Ulceroglandular and ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2011</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td>Insect bite while on water</td>
<td>Probable</td>
<td>2016</td>
<td>No</td>
<td>Glandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2018</td>
<td>No</td>
<td>Ulceroglandular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Probable</td>
<td>2018</td>
<td>No</td>
<td>Glandular</td>
</tr>
</tbody>
</table>

*Data are for 26 infections caused by Francisella tularensis subspecies holarctica. ND, not determined.
†Water, sampled within 6 weeks from waterbodies in the area where infection was assumed to have occurred, tested positive for F. tularensis subsp. holarctica by quantitative PCR, indicating presence of the bacterium in the local environment around the time of infection and highlighting the interconnection between lifecycles (7).
basal clade B6 (4), supporting previous associations found between pneumonia and basal clade B6 in both humans and hares (8,9).

Nonoccupational exposure through contact with infected terrestrial mammals was likely in 9 cases. Of those, 8 were assumed or confirmed to be infected by contact with infected hares, mainly through skinning and rarely through consumption. These case-patients were mostly hunters (n = 7) who showed diverse clinical symptoms; 2 cases were related to the same hare (Table). The ninth case concerned an ulceroglandular infection from a mouse bite (Table), a mode previously described in Switzerland (10). Nonoccupational exposure through arthropod bites, contaminated water, or mud was likely in the remaining 10 case-patients, who contracted tularemia while performing recreational outdoor activities in a terrestrial (4/10) or aquatic environment (6/10) (Table).

These results support the need for ongoing tularemia risk and prevention communication to hunters, and they identify a need for communication to outdoor (water) recreationists and to professionals such as grounds maintenance workers and foresters. Physicians must be aware of these risk groups and the diversity of clinical presentations for early identification and treatment.

The relative importance of the terrestrial lifecycle as a source of human infections in the Netherlands is consistent with the rare and sporadic occurrence of cases; human tularemia cases from aquatic sources are more likely to occur as large outbreaks (1). Nevertheless, local disease ecology can change over time, and the Netherlands is a low-lying, water-rich country in which favorable conditions for F. tularensis, such as floodplains and meandering waterways, are promoted to buffer excess rainfall due to climate change. It is therefore relevant to continue monitoring the transmission routes in human tularemia cases for early detection of shifts in tularemia lifecycle contributions, which may require adaptation of risk and prevention communications.

Acknowledgments

We thank the medical microbiological laboratories in the Netherlands for submitting diagnostic material or strains and clinical, and epidemiological information, and the Dutch municipal health services for obtaining clinical and epidemiological information. We thank Amber Hendriks, Maaik van den Beld, Airien Harpal, Marion Sunter, Ramón Noomen, and Dienieke Hoeve-Bakker for assistance in confirmatory laboratory diagnosis.

This work was funded by the Dutch Ministry of Health, Welfare and Sport; the Dutch Ministry of Agriculture, Nature and Food Quality; and Utrecht University.

About the Author

Dr. Rijks is a postdoctoral researcher at the Dutch Wildlife Health Centre in Utrecht, the Netherlands. Her primary research interests are wildlife diseases and epidemiology.

References


Address for correspondence: Jolianne Rijks, Dutch Wildlife Health Centre, Utrecht University, Androclus kamer O.177, Yalelaan 1, 3584CL Utrecht, the Netherlands; email: j.m.rijks@uu.nl
Streptobacillus notomytis Bacteremia after Exposure to Rat Feces

Akira Kawashima, Satoshi Kutsuna, Akira Shimomura, Lubna Sato, Honami Ando, Tsutomu Tanikawa, Maki Nagashima, Tohru Miyoshi-Akiyama, Takeshi Inagaki, Norio Ohmagari

Author affiliations: National Center for Global Health and Medicine, Tokyo, Japan (A. Kawashima, S. Kutsuna, A. Shimomura, L. Sato, H. Ando, M. Nagashima, T. Miyoshi-Akiyama, T. Inagaki, N. Ohmagari); Yamazaki Professional College of Animal Health Technology, Tokyo (T. Tanikawa)

DOI: https://doi.org/10.3201/eid2804.204965

To determine the source of *Streptobacillus notomytis* bacteremia in a woman in Japan with signs of rat-bite fever, we examined rat feces from her home. After culture and PCR failed to identify the causative organism in the feces, next-generation sequencing detected *Streptobacillus* spp., illustrating this procedure’s value for identifying causative environmental organisms.

Rat-bite fever, characterized by fever, rash, and arthritis, is caused by *Streptobacillus moniliformis* and *Spirillum minus*, which are gram-negative, filamentous, bacilli transmitted by rat bites or ingestion of water or food contaminated with rat feces or urine (1). In 2015, *S. notomytis* was reclassified from *S. moniliformis* to the new species (2). We reported a case of rat-bite fever in Japan that was caused by *S. notomytis*, identified by 16S rRNA sequencing after blood culture and most likely transmitted by rat feces.

A 70-year-old woman was admitted to the National Center for Global Health and Medicine (Tokyo, Japan), reporting left back pain, lower leg myalgia without arthralgia, and a 3-day fever with abdominal discomfort. At the time of admission, she was fully conscious with the following parameters: temperature 37.9°C, blood pressure 132/68 mm Hg, heart rate 112 beats/min, respiratory rate 24 breaths/min, and oxygen saturation 98%. She reported that rats often entered her apartment and that she had noticed the smell of urine over the previous 2 weeks. She had no rash or bite wounds, and her conjunctiva appeared normal. Chest radiographs showed no signs of pneumonia.

Laboratory tests revealed leukocytosis (9.0 × 10^8 cells/L) and elevated C-reactive protein concentration (16.9 mg/dL). Urinalysis revealed hematuria but no other signs of pyelonephritis. Contrast-enhanced computed tomography of the trunk revealed no kidney abnormalities.

Because hematuria led us to suspect pyelonephritis, we initiated ceftriaxone treatment (2 g/d), after which, the patient’s fever rapidly resolved. On day 2, diffuse purpura appeared on both her legs but rapidly resolved. Blood culture revealed gram-negative bacilli (Figure, panel A). On the basis of her history of rat contact and blood culture results, we provisionally diagnosed rat-bite fever but continued administering ceftriaxone.

Bacterial culture of the patient’s blood yielded small, smooth colonies on 5% sheep blood agar and 5% horse blood agar (both Nissui Pharmaceutical, https://www.nissui-pharm.co.jp) with addition of healthy serum (provided by one of the authors of this article) and incubation at 35°C under 7% carbon dioxide for 48 h (Figure, panel B). We evaluated the isolate by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with the Bruker MALDI BioTyper software version 6.903 library database (Bruker Daltonik GmbH, https://www.bruker.com). *S. moniliformis* was a candidate,
but its score of 1.49 was considered too low. We determined antimicrobial susceptibility patterns by using broth microdilution. The MIC of oxacillin was <0.12 µg/mL; ampicillin, <0.12 µg/mL; cefazolin, <0.5 µg/mL; imipenem, 0.25 µg/mL; vancomycin, <0.5 µg/mL; clindamycin, <0.06 µg/mL; and levofloxacin, <0.5 µg/mL. To further characterize the isolate, we performed 16S rRNA gene sequencing by using a universal primer pair: 5’-TTGGAGAGTTTGATCTGGCCTC-3’ and 1485B (5’-TACGGTGTTACGAC-3’). The sequence showed 100% identity (1,465/1,465 bp) with S. notomytis (GenBank accession no. KR001919).

Therefore, on day 6, we changed treatment to intravenous ampicillin (2 g 4x/d). On day 7, treatment was changed to oral amoxicillin (500 mg 3x/d), and the patient was discharged. She completed a 14-day course of ceftriaxone, ampicillin, and amoxicillin and showed no further signs of bacteremia.

After the patient was discharged, we visited her home and collected rat feces samples. We saw a rat in her home but were unable to capture it. The rat feces were dry, and the target bacteria could not be cultured. Because the causative organism could not be identified by wide-range 16S rRNA PCR due to the large number of species of bacteria, we performed next-generation sequencing of DNA extracts as described previously (3), prepared library NEBNext Ultra II DNA Library Prep Kit (New England BioLabs, https://www.neb.com) according to the manufacturer’s instructions, and analyzed sequence data by using MiSeq (Illumina, https://www.illumina.com). Results confirmed the presence of Streptobacillus spp. in the rat feces. We used the S. notomytis genome as a reference sequence (GenBank accession no. GCA_00161275.1) for comparison of sequence reads. We performed mapping with a 99% identity setting. Of the 844,070,6 genes read, 177 were mapped and possibly belong to S. notomytis.

Three cases of S. notomytis infection in humans have been reported; the pathogen was detected in the blood, skin pustules, and synovial fluid (4–6). In the case we report, S. notomytis was identified in the blood by using 16S rRNA sequencing. S. moniliformis and S. notomytis may also cause Haverhill fever, which is epidemic arthritic erythema transmitted by inhalation or ingestion of food and drink contaminated with rat feces and urine (7–10). Because the patient reported here had no history of rat bites, the most likely mode of transmission was aerosol inhalation of dried rat feces or water contamination at the patient’s home.

S. moniliformis infection is usually treated with penicillin, cephalosporins, or tetracyclines (1). S. notomytis is also reportedly sensitive to these drugs, but its sensitivity to amoxicillin is reportedly intermediate (4,5). For this patient, ceftriaxone and ampicillin were prescribed. In summary, after conventional methods (culture and PCR) failed to identify the causative organism for this patient’s infection, we detected Streptobacillus spp. in rat feces by using next-generation sequencing, thereby illustrating the value of this procedure for identifying causative organisms in the environment.

This work was supported by the Health, Labour, and Welfare Policy Research Grants, Research on Emerging and Re-emerging Infectious Diseases and Immunization (grant no. 20HA1006).

About the Author
Dr. Kawashima is a senior resident at the Disease Control and Prevention Center, National Center for Global Health and Medicine, Tokyo, Japan. His primary research interest is the diagnosis of infectious diseases.

References
Leishmaniasis, a neglected tropical disease of humans and animals, is estimated to affect ≤1 million persons annually (https://www.who.int/health-topics/leishmania). The disease is caused by intracellular protozoan parasites of the genus Leishmania (Trypanosomatida: Trypanosomatidae), which are vectored by female sand flies of the genera Phlebotomus in the Old World and Lutzomyia in the New World (1). Although leishmaniasis is endemic to several resource-poor countries in eastern, western, and northern Africa, there is a dearth of information on the epidemiology of the disease in southern Africa, largely caused by weak surveillance systems (1,2).

In Zambia, human visceral leishmaniasis was reported in 1973 in the Eastern Province (3) and subsequently in 1976 in the same area (4). In 1994, a case of canine visceral leishmaniasis was reported in a dog in Lusaka Province (5). We report detection of canine leishmaniasis caused by Leishmania infantum, suggesting possible reemergence or reintroduction of the disease in Zambia.

The study was approved by the Department of Veterinary Services, Government of the Republic of Zambia. In June 2021, two female mixed-breed shelter dogs (case 1 and case 2) rescued in Southern Province of Zambia during 2020 were brought to a veterinary clinic in Lusaka. These 2 dogs had chronic weight loss, generalized alopecia, and ulcerative and exfoliative dermatitis (Figure, panel A). The dogs had been previously treated for tickborne and helminth infections but showed no improvement. Physical examination showed that the precapillary and popliteal lymph nodes were enlarged. Case 1 had onychogryphosis (Figure, panel A) and focal corneal opacity in the left eye (Figure, panel B).

Biochemistry profiles for both dogs showed increased levels of serum proteins (>93.3 g/L) and hyperglobulinemia (>74.9 g/L) and hypoalbuminemia (<14.7 g/L), which are common manifestations suggestive of canine leishmaniasis. Giemsa staining of fine-needle lymph node aspirate identified Leishmania spp. amastigotes (Figure, panel C).

We performed serologic analysis of Leishmania antibodies by using L. donovani soluble lysate antigen from cultured promastigotes and recombinant...
LinJ14.1160r4 antigens as described (6). Both dogs had high antibody titers, >1.0 optical density units (Appendix Figure, https://wwwnc.cdc.gov/EID/article/28/4/21-2378-App1.pdf). Among in-contact dogs from Southern Province that had no clinical signs (n = 6), 1 dog (case 3) had high antibody titers for both assays, and 2 dogs (dogs 6 and 7) had high antibody titers for LinJ14.1160r4 only. All control serum samples (n = 39) from Central Province were negative for *Leishmania* antibodies on both assays (Appendix Figure). For the purpose of disease control and the absence of antileishmanial agents in Zambia, the dogs (cases 1 and 2) were euthanized by rapid intravenous infusion of pentobarbitone sodium (0.7 mL/kg body weight). Case 3, an in-contact dog that showed high antibody titers for both assays, was euthanized 1 month after clinical disease was detected and the initial diagnosis.

At necropsy, we aseptically harvested spleen tissue and processed this tissue for genomic DNA extraction by using the QuickGene DNA Tissue Kit (Kurabo, https://www.kurabo.co.jp) according to the manufacturer’s protocol. We performed PCRs targeting the partial small subunit ribosomal RNA gene (7) and internal transcribed spacer (ITS) 1 and ITS 2 genes (8). PCR for 3 dogs showed expected band sizes, which we purified and sequenced on a 3500 Genetic Analyzer (Applied Biosystems, https://www.thermofisher.com). Sequences obtained were 100% identical with the *L. infantum* reference strain (JPCM5) isolated in Spain (9). The ITS sequence type was type A, which was assigned according to 12 microsatellite regions in ITS1 and ITS2 within the *L. donovani* complex (8). ITS type A is the dominant *L. infantum* type reported mainly from the Mediterranean basin, and types D, E, F, and G are associated with *L. donovani* from eastern Africa (8). Nucleotide sequences from this study were deposited in the DNA DataBank of Japan (GenBank accession nos. LC652643–LC652645).

Our study confirmed the presence and probable emergence of leishmaniasis and *Leishmania* parasites in Zambia. An in-contact, seropositive dog that did not have clinical signs had clinical disease develop 1 month after the initial diagnosis. However, the probable route of infection remains unclarified.

Although the geographic distribution of vector sandflies has not been described in Zambia, neighboring countries have reported presence of *Phlebotomus* spp. sand flies (10). In addition, although the extent of disease distribution in the country, including Southern Province, is yet to be determined, autochthonous leishmaniasis cases reported in Zambia (3–5) suggests the presence of an infection foci. To further clarify the epidemiology of leishmaniasis in Zambia, there is need for improved understanding of the epidemiology of the disease in dogs, vector distribution, and the risk for human infection, particularly in high-risk populations, such as immunocompromised persons.

This study was supported in part by the Department of Veterinary Services under the Ministry of Fisheries and Livestock of the Government of the Republic of Zambia, the Japan Agency for Medical Research and Development (JP21wm0125008), and the Lusaka Animal Welfare Society.

**About the Author**

Dr. Squarre is a state wildlife veterinarian in Lusaka, Zambia. His research interests include molecular epidemiology of emerging and reemerging zoonosis in free-ranging wildlife and domestic animals and their interaction at the human-wildlife–livestock interface.
We report a pseudo-outbreak of *Bordetella parapertussis* in the Department of Pediatrics in Rijnstate, an 809-bed teaching hospital in the Netherlands. The department provides level II care to infants, neonates, and preterm infants. In March 2021, we diagnosed *B. parapertussis* in 3 infants hospitalized for respiratory symptoms by using an in-house PCR against insertion sequences (IS) IS481 and IS1001 (1). During calendar week 21 (Figure), we identified more *B. parapertussis* cases in the same department, bringing the total case count to 5 in neonates, 1 in a toddler, and 6 in infants. Several of these patients were born prematurely.

PCR-positive case-patients had pertussis-like complaints, and we confirmed *B. parapertussis* in the patients or their siblings. We traced all positive tests to the Department of Pediatrics. Because we suspected nosocomial transmission, we started contact tracing investigations among parents and healthcare workers (HCWs) and identified *B. parapertussis* in another 4 patients and in 3 HCWs.

Cases among HCWs were particularly unexpected. Because of the coronavirus disease (COVID-19) pandemic, all HCW were using type IIR surgical masks and keeping ≥1.5 m distance from each other. In addition, all patients had private rooms, and we observed no increase in other respiratory pathogens.
Because we discovered additional *B. parapertussis*–positive cases, we upgraded HCW masks to FFP1, the recommended type for pertussis (2). We also confirmed instructions regarding continued HCW social distancing, including during lunch breaks, to prevent further *B. parapertussis* spread. We then implemented extended screening for asymptomatic cases among all HCWs and relatives of *B. parapertussis*–positive case-patients. Among 22 HCWs tested, 72% (16/22) tested IS1001-positive.

Parallel to actions in the clinic, we checked the possibility of laboratory contamination. The laboratory uses several controls to confirm sensitivity and specificity of assays; all controls consistently showed correct results. Swipe-tests did not reveal contaminated surfaces or equipment. All cases had relatively high cycle threshold (Ct) values (median Ct 35), as seen with prior *B. parapertussis* results from the laboratory (n = 17). In addition, 2 other laboratories confirmed *B. parapertussis* in original clinical samples and DNA eluates by targeting diverse regions of the IS1001 gene using an in-house PCR (1) or Real Accurate Quadruplex Bordetella PCR (PathoFinder, https://www.pathofinder.com). Confirmatory PCR tests also had high Ct values.

Finally, we tested unused ESwab 483CE nasopharyngeal swabs (Copan, https://www.copanusa.com), which included a flocked swab and 1 mL of liquid Amies medium in a plastic, screw cap tube. Liquid Amies media from 7 batches (1 sample per
batch) and flocked tips from 2 batches (2 tips per batch) were available for testing. All liquid Amies media were PCR-negative, but both batches of flocked swab tips were PCR-positive for IS1001. Moreover, 2 flocked tips were placed in 0.5 mL of Milli-Q water (Millipore, https://www.emdmillipore.com), a 4-fold higher concentration than for standard diagnostic tests. In the higher concentration, we saw lower $C_t$ values ($C_t$ ≈35) compared with regular diagnostic tests ($C_t$ ≥37).

We retested all 23 PCR-positive HCWs by using individually packaged 503CS01 flocked swabs (Copan) from a PCR-negative batch; 22 HCWs tested PCR-negative and 1 tested PCR-positive. Upon reexamination, we found that testing for the positive HCW case was not performed with an individually packaged swab provided by the laboratory but an ESswab from the suspect batch. Although unintentional, this case proved that the B. parapertussis could be traced to IS1001-positive nasopharyngeal swabs tips. No B. parapertussis could be cultured, which aligns with the notion that the swabs are gamma-irradiated after packaging. Gamma irradiation kills bacteria but does not affect DNA.

We alerted clinical and molecular microbiologists in the Netherlands, the supplier, and the Health Inspectorate regarding swabs contaminated with B. parapertussis IS1001-containing DNA. Subsequently, ≥6 laboratories in the Netherlands recognized and reported false-positive B. parapertussis to the National Institute of Public Health and the Environment. Contamination appeared to be associated with specific ESswab batch numbers (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-2097-App1.pdf), which explains why the pseudo-outbreak focused on 1 department in our hospital. The contamination was confirmed by the manufacturer, but the source was not disclosed.

B. parapertussis can cause pertussis-like symptoms, although symptoms usually are milder and occur less frequently than with B. pertussis (3). Each year, >6,400 B. pertussis cases are notified in the Netherlands based on culture, PCR, or serology, but only 26 B. parapertussis cases are notified (Appendix Figure). During 2020–2021, the COVID-19 pandemic and associated social distancing measures caused a large decrease in reported B. pertussis cases. Of note, B. parapertussis reports did not diminish during this period (Appendix), possibly because testing strategies changed, contaminated swabs were already circulating, or both.

Our report illustrates the importance of critically evaluating microbiological results lacking clinical and epidemiologic clues. We were confronted with a growing number of neonatal patients and HCWs with unexplained B. parapertussis–positive tests. Because these tests are requested infrequently, it took months before contamination with IS1001-like DNA in nasopharyngeal swabs became clear. Clinicians and public health agencies should be aware of the possibility of false-positive microbiology results and consider contaminated products when unexplainable results are found.

Acknowledgments
We thank our colleagues in the laboratory, the Department of Pediatrics, Infection Prevention, and the Safety and Public Health Service Gelderland Midden for their contributions to this work. We thank Canisius Wilhelmina Hospital (Nijmegen, the Netherlands) and the University Medical Center Utrecht (Utrecht, the Netherlands) for assistance in confirming contamination on the swabs and the Working Group on Molecular Diagnostics of Infectious Diseases for the national survey on contaminated swabs. We also thank Dimphey van Meijeren for collecting the data on the notifications to the Center for Infectious Disease Control.

About the Author
Dr. Flipse is a medical molecular microbiologist at the laboratory for Medical Microbiology and Immunology at Rijnstate Hospital. His primary interests are on molecular diagnostics and medical microbiology.

References

Address for correspondence: Jacky Flipse, Ziekenhuis Rijnstate Arnhem Laboratory for Medical Microbiology and Immunology, President Kennedylaan 100, Velp 6883AZ, the Netherlands; email: jflipse@rijnstate.nl
High Prevalence and Low Diversity of Rickettsia in Dermacentor reticulatus Ticks, Central Europe

Alena Balážová, Gábor Földvári, Branika Bilbija, Eva Nosková, Pavel Široký

Author affiliations: University of Veterinary Sciences Brno, Brno, Czech Republic (A. Balážová, B. Bilbija, P. Široký); Centre for Ecological Research, Budapest, Hungary (G. Földvári); Masaryk University, Brno (E. Nosková); Central European Institute of Technology, Brno (P. Široký)

DOI: https://doi.org/10.3201/eid2804.211267

We collected 1,671 Dermacentor reticulatus ticks from 17 locations in the Czech Republic, Slovakia, and Hungary. We found 47.9% overall prevalence of Rickettsia species in ticks over all locations. Sequence analysis confirmed that all tested samples belonged to R. raoultii, the causative agent of tick-borne lymphadenopathy.

The ornate dog tick, Dermacentor reticulatus, is a proven vector of pathogens of public health and veterinary importance, including tick-borne encephalitis virus, Omsk hemorrhagic fever virus, rickettsiae, Babesia spp., and several others (1). D. reticulatus ticks are now expanding into new areas of northern and central Europe (1), where a higher prevalence of associated diseases can be expected.

Although intensively studied during the past decade, bacteria of the genus Rickettsia have been overshadowed by other tick-borne pathogens of primary medical importance. Rickettsiae of the typhus group and spotted fever group (SFG) present the greatest health risks. The D. reticulatus tick is a vector for SFG rickettsiae. Among Rickettsia species, R. raoultii and R. slovaca are recognized as causative agents of rickettsioses with typical lymphadenopathies, called tick-borne lymphadenopathy or Dermacentor-borne necrosis erythema and lymphadenopathy (2), which are widespread in Eurasia (1). R. helvetica, which causes milder symptoms, was also reported from D. reticulatus ticks (1,3).

We analyzed 1,671 D. reticulatus ticks (851 female and 820 male) for prevalence, diversity, and distribution of SFG rickettsiae in the Czech Republic, Slovakia, and Hungary. Ticks were collected by flagging for previous studies conducted during 2009–2020 from 7 locations in the Czech Republic, 7 in Slovakia, and 5 in Hungary (Appendix, https://wwwnc.cdc.gov/EID/article/28/4/21-1267-App1.pdf). We selected places with a high abundance of D. reticulatus ticks for analyses, to promote high detection probability (Table). We used a duplex quantitative PCR method aiming for gltA gene fragments of Rickettsia (147 bp). We calculated prevalence (Sterne’s exact method if n <1,000, adjusted Wald method if n >1,000) and basic statistical comparisons in Quantitative Parasitology 3.0 (4). We also amplified fragments of 2 outer-membrane protein genes, ompA (590 bp) and ompB (475 bp), by conventional PCR and selected a subset of 5–10 positive samples from each location (144 total) for sequencing (Macrogen, https://www.macrogen.com) and identifying species (Appendix).

We identified all isolates as R. raoultii. Our ompA gene sequences were 99.83% identical to haplotypes from Italy (GenBank accession no. HM161792.1) and Denmark (accession no. MF166732.1). We used ompB gene sequences to create a phylogenetic tree (Figure; Table).
Appendix) in which both sequences were placed into a highly supported subclade formed by sequences of *R. raoultii*. We did not detect either *R. slovaca* or *R. helvetica* at the locations in the study, but the prevalence of these species in *D. reticulatus* ticks is generally low because the main vectors are *D. marginatus* ticks for *R. slovaca* and *Ixodes ricinus* ticks for *R. helvetica* (2,3).

The mean prevalence of *Rickettsia* in *D. reticulatus* ticks was 47.9% (95% CI 45.5%–50.3%), without significant difference between sexes (p = 0.307 by χ² test). Remarkably, we observed the lowest prevalence (6.7%) in Dolov Dvor, Slovakia, ~3 km from Lándor, which had the highest prevalence (74.4%) (Table). Differences in the surrounding environments might account for this discrepancy: Dolov Dvor by an oxbow lake in the middle of arable land and Lándor in a forest along the river Váh. We assumed more abundant interconnected populations of host animals with unrestricted movement live in the forest environment. Data from Lednice, Czech Republic, situated in the middle of farmland, indicated ~20% prevalence, consistently lower than the ~60% in nearby areas of floodplain forests along the Morava River near Mikulčice. Comparing findings from the earlier and newer sample collections showed that the proportion of positive ticks remained consistent and variability over time was not significant. Specifically, we compared samples from Lednice (2009 and 2020; p = 0.574 by χ² test), Moravská Nová Ves (2009 and 2020; p = 0.178 by χ² test), and Mikulčice (2009) and Hodonín (2020), ~9 km apart (p = 0.739 by χ² test).

Distribution of the pathogen in *D. reticulatus* tick populations seems to be very uneven in Central Europe, which is also suggested by other studies (5). Our overall prevalence of 47.9% corresponds with similar data showing the prevalence of *R. raoultii* in *D. reticulatus* ticks to be 56.7% in Germany, 57.8% in Hungary, and 50.2% and 45.6% in 2 locations in Slovakia (5–7). On the other hand, researchers also found much lower prevalences of 10.8% in Slovakia (8) 15.6% in the Czech Republic (3) and 14.9% in Austria (9). Although significant seasonal differences in prevalence were reported (10), our data showed that

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype 1</td>
<td>DQ365797</td>
</tr>
<tr>
<td></td>
<td>HQ232262</td>
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<tr>
<td>Haplotype 2</td>
<td>KU961541</td>
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<td></td>
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<td>JQ792108</td>
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<td>EU036984</td>
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<td>CP010969</td>
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<tr>
<td></td>
<td>DQ365798</td>
</tr>
<tr>
<td>HQ335155</td>
<td>R. aesculimannii</td>
</tr>
<tr>
<td>DQ503428</td>
<td>R. massilae</td>
</tr>
<tr>
<td>AF123719</td>
<td>R. rhipicephali</td>
</tr>
<tr>
<td>CP003340</td>
<td>R. montanensis</td>
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<tr>
<td>HQ335130</td>
<td>R. africae</td>
</tr>
<tr>
<td>AF123721</td>
<td>R. conori</td>
</tr>
<tr>
<td>KY113111</td>
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</tr>
<tr>
<td>AF123722</td>
<td>R. sibirica</td>
</tr>
<tr>
<td>AF123711</td>
<td>R. honei</td>
</tr>
<tr>
<td>AY260451</td>
<td>R. helongjiangensis</td>
</tr>
<tr>
<td>AF123713</td>
<td>R. japonica</td>
</tr>
<tr>
<td>KU310591</td>
<td>R. helvetica</td>
</tr>
<tr>
<td>NC_006142</td>
<td>R. typhi</td>
</tr>
</tbody>
</table>
the high observed prevalence in the study locations remained consistent over a long time period.

Our data suggest an overall high prevalence of *R. raoultii* and its possible long-term stability in *D. reticulatus* tick populations in the studied region, highlighting the enduring high risk of acquiring this rickettsial infection. Besides veterinary consequences (*I*), this risk should be considered by medical personnel and public health authorities because the incidence of tick-borne lymphadenopathy might increase with the reported (*I*) expansion of the vector into new areas and its growing abundance in Central Europe.

**Acknowledgement**

We thank Michaela Kubelová, Markéta Rybářová, and Vojtech Baláž, who helped with collection and DNA isolations of part of the samples. Studied ticks originated from the collections of the authors.

This work was supported by project IGA 218/2020/FVHE UVPS Brno and by the grant In the light of evolution: theories and solutions (GINOP-2.3.2-15-2016-00057).

**About the Author**

Dr. Balážová is a junior scientist at Veterinary University in Brno, Czech Republic. Her research is aimed at vector-borne zoonotic diseases and the development of new molecular methods for pathogen detection.

**References**


**Address for correspondence:** Pavel Široký, Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Palackého 1946/1, 61242 Brno, Czech Republic; email: sirokyp@vfu.cz

**Spread of SARS-CoV-2 Variants on Réunion Island, France, 2021**

Alizé Mercier, David A. Wilkinson, Camille Lebarbenchon, Patrick Mavingui, Luce Yemadje-Menudier

Author affiliations: Santé Publique France, Saint-Denis, France (A. Mercier, L. Yemadje-Menudier); Université de La Réunion, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Institut de Recherche pour le Développement, Sainte-Citotilde, France (D.A. Wilkinson, C. Lebarbenchon, P. Mavingui); Plateforme Technologique du Cyclotron Réunion Océan Indien (CYROI), Sainte-Citotilde (D.A. Wilkinson)

DOI: https://doi.org/10.3201/eid2804.212243

In January 2021, after detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants, genomic surveillance was established on Réunion Island to track the introduction and spread of SARS-CoV-2 lineages and variants of concern. This system identified 22 SARS-CoV-2 lineages, 71% of which were attributed to the Beta variant.
Coronavirus disease (COVID-19) is a respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). On Réunion Island, an overseas department of France located in the Indian Ocean, the first cases of COVID-19 were detected on March 11, 2020, in a group of travelers (D.A. Wilkinson et al., unpub. data, https://doi.org/10.1101/2021.01.21.21249623). In response, a regional epidemiologic surveillance focusing on contact tracing and early detection of clusters was conducted. After several months of imported cases and sporadic autochthonous cases, a sharp increase in locally acquired infections was recorded in August 2020, after the return of many Réunion Island residents from travel abroad, primarily mainland France, where the incidence rate was high. The virus subsequently spread throughout the island.

In January 2021, after SARS-CoV-2 variants were detected, genomic surveillance was established to track the introduction and spread of SARS-CoV-2 lineages on the island. During January–June 2021, we generated a total of 1,528 genome sequences with >90% coverage using the ARTIC protocol (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html) and nanopore technology (MinION; Oxford Nanopore Technologies, https://nanoporetech.com). This collection represents 8.3% of all COVID-19 cases on Réunion Island during that period (n = 18,409). Sample selection was pseudo-random; a small proportion of cases was prioritized for sequencing because of atypical epidemiologic or clinical characteristics. Pangolin lineages were assigned to all genomes using Pangolin version 1.2.88 (https://github.com/cov-lineages/pango-designation/releases/tag/v1.2.88).

We present the main findings of genomic surveillance from weeks 1–22, 2021 (January 4–June 6, 2021). We focused on the evolution of the weekly proportions of the 8 most frequent SARS-CoV-2 variants and examined the correlation between the weekly number of confirmed cases and the proportion of sequences identified as Beta variant (B.1.351). We extracted lineage distributions in other islands of the Indian Ocean and South Africa from the GISAID database (http://www.gisaid.org) to investigate the origins of the Beta variant sublineages.

We identified 22 SARS-CoV-2 lineages, 71% of which were attributed to the Beta variant (sublineages B.1.351 and B.1.351.2) (Table). On the basis of available data in the GISAID database, lineage B.1.622 seems to be specific to Réunion Island; no other sequence had been reported elsewhere.

### Table. Observed lineages of severe acute respiratory syndrome coronavirus 2, Réunion, France, 2021

<table>
<thead>
<tr>
<th>Pangolin lineage</th>
<th>No. genomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.1.351.2 (Beta, sub-lineage 2)</td>
<td>716</td>
</tr>
<tr>
<td>B.1.351 (Beta, sub-lineage 0)</td>
<td>361</td>
</tr>
<tr>
<td>B.1.177</td>
<td>154</td>
</tr>
<tr>
<td>B.1.622</td>
<td>71</td>
</tr>
<tr>
<td>B.1.177 (Alpha)</td>
<td>65</td>
</tr>
<tr>
<td>B.1.160</td>
<td>55</td>
</tr>
<tr>
<td>B.1.160.18</td>
<td>36</td>
</tr>
<tr>
<td>B.1.1.353</td>
<td>18</td>
</tr>
<tr>
<td>B.1.617.2 (Delta)</td>
<td>14</td>
</tr>
<tr>
<td>B.1.438.2</td>
<td>10</td>
</tr>
<tr>
<td>B.1.525 (Eta)</td>
<td>8</td>
</tr>
<tr>
<td>B.1.416.1</td>
<td>5</td>
</tr>
<tr>
<td>B.1.177.24</td>
<td>3</td>
</tr>
<tr>
<td>B.1</td>
<td>3</td>
</tr>
<tr>
<td>B.1.177.37</td>
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<tr>
<td>B.1.1</td>
<td>1</td>
</tr>
<tr>
<td>B.1.1.241</td>
<td>1</td>
</tr>
<tr>
<td>B.1.160.27</td>
<td>1</td>
</tr>
<tr>
<td>B.1.177.81</td>
<td>1</td>
</tr>
<tr>
<td>B.1.221</td>
<td>1</td>
</tr>
<tr>
<td>B.1.428.2</td>
<td>1</td>
</tr>
<tr>
<td>P.2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>1,528</td>
</tr>
</tbody>
</table>

The Beta variant was first detected on Réunion Island during the first week of January 2021, although it may have been introduced before its detection by full-genome sequencing. During the first 6 weeks of 2021, lineages known to have high levels of circulation in Europe (e.g., B.1.160, B.1.177) represented most sequenced genomes (Figure). This finding highlights the strong effect of air travel on COVID-19 dynamics on an island such as Réunion (I; D.A. Wilkinson et al., unpub. data).

Since mid-February 2021 (week 7 of 2021), the Beta variant has become dominant on Réunion Island, despite low-level circulation of the Alpha variant, another variant of concern that was dominant in mainland France and other countries in Europe at that time. We detected a correlation (Spearman \( \rho = 8.4 \times 10^{-4}; p<0.001 \)) between the number of COVID-19 cases in January–February 2021 and the number of sequences attributed to the Beta variant, which has been shown to have increased transmissibility (C.A. Pearson et al., unpub. data, https://cmid.github.io/topics/covid19/sa-novel-variant.html). Several additional factors could explain the dominance of Beta variant; genetic and epidemiologic factors may have contributed to a founder effect, a higher frequency of virus introductions resulting from holiday travels, possible superspreading events, and local and regional contexts (2). Indeed, geographic proximity and population movements with Mayotte, another overseas department of France, and Comoros link Réunion Island to South Africa, where Beta variant was first reported (3).
We detected 2 sublineages of Beta variant, B.1.351 and B.1.351.2. Sublineage B.1.351.2 accounted for 3-fold more cases than B.1.351. It was detected concurrently in Mayotte, Comoros, and Réunion Island. This finding, coupled with information from GISAID, suggests that lineage B.1.351.2 was imported to Comoros and Mayotte from South Africa and could have been introduced to Réunion Island from Mayotte (4) (Appendix 1, https://wwwnc.cdc.gov/EID/article/28/4/21-2243-App1.pdf). This possible introduction from Mayotte is supported by the flow of travelers between the 2 departments and the notable peak in COVID-19 cases that occurred in Mayotte during weeks 1–11, mainly caused by the Beta variant (5). However, analysis of the origin of lineages is strongly affected by each location’s capacity to sequence and report genomes in GISAID, which renders comparison between different locations difficult (4).

Our study provides valuable insights into the interactions between SARS-CoV-2 lineages on Réunion Island, which represents a closed system with controlled entries, especially when travel restrictions are in place. Additional research on genomic epidemiology and the effect of air travel can further improve understanding of why some variants become dominant over others, particularly in insular contexts. The future of genomic surveillance on Réunion Island will focus on mutation screening to increase reactivity, combined with real-time sequencing, as a robust approach to track the spread of emerging SARS-CoV-2 variants of concern and to inform public health actions (6,7).

Acknowledgments
We thank all the partners involved in the surveillance of COVID-19 in the Regional Health Agency and the Regional Health Insurance Fund. We acknowledge the key role of the network of sampling laboratories on Réunion Island (CHU, CHOR, Laboratoire de Saint-Benoit, Bioaustrial, Réunilab, Cerballiance). We also acknowledge and thank the members of the EMERGEN consortium, as well as Laetitia Ali Oicheih, Elsa Balleydier, Adeline Feri, Javier Castro Alvarez, Sibylle Bernard-Stoecklin, Anna Maisa, Justine Schaeffer, Bruno Coignard, Guillaume Spaccaferri, Bruno Lina, Marie-Alice Simbi, Magali Turpin, Christian Mériaux, and Maya Cesari. We are also grateful to the originating and submitting laboratories of the sequences from GISAID database (Appendix 2, https://wwwnc.cdc.gov/EID/article/28/4/21-2243-App1.xlsx). All data submitters may be contacted via the GISAID website (https://www.gisaid.org).
Community Transmission of SARS-CoV-2 Omicron Variant, South Korea, 2021


Author affiliations: Honam Regional Center for Disease Control and Prevention, Gwangju, South Korea (E.-Y. Kim, H. Jeong, J.-H. Chung, J. Yu); Korea University Anam Hospital, Seoul, South Korea (Y.J. Choe); Korea Disease Control and Prevention Agency, Cheongju, South Korea (H. Park, M. Yu, J. Kim, H.R. Lee, E.J. Jang, J.J. Lee, H.Y. Lee, J.M. Kim, J.H. Choi, S.E. Lee, I.-H. Kim, A.K. Park, J.E. Rhee, E.-J. Kim, S. Lee, Y.-J. Park); Gwangju Metropolitan Government, Gwangju (H.-P. Ko); Jeollabuk-do Government, Jeonju, South Korea (H.J. Ahn, M.-Y. Go); Jeollabuk-do Center for Infectious Disease Control and Prevention, Jeonju, South Korea (J.-H. Lee); Jeollanam-do Government, Muan, South Korea (W.I. Kim, B.S. Lee); Jeollanam-do Communicable Disease Management Support Team, Muan (S. Kim)

DOI: https://doi.org/10.3201/eid2804.220006

In South Korea, a November 2021 outbreak caused by severe acute respiratory syndrome coronavirus 2 Omicron variant originated from 1 person with an imported case and spread to households, kindergartens, workplaces, restaurants, and hospitals, resulting in 11 clusters within 3 weeks. An epidemiologic curve indicated rapid community transmission of the Omicron variant.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) B.1.1.529 (Omicron) variant of concern has been suggested to be more transmissible than previous variants of concern (1). We describe an outbreak caused by the Omicron variant that originated from 1 person with an imported case and rapidly spread within 3 weeks to the community in South Korea.

Details of the surveillance and quarantine system in South Korea have been described (2). Public health officers interviewed case-patients, and to identify links between clusters, we created epidemic curves

1These authors contributed equally to this article.
and transmission chains according to circumstances and dates of exposure.

On November 25, 2021, an asymptomatic 32-year-old man who had arrived at Incheon Airport, Seoul, South Korea, from Tehran, Iran, was quarantined in a relative’s house; 9 days later (December 5), he tested positive for SARS-CoV-2 (Figure). After contact tracing, household members were confirmed to have SARS-CoV-2 infection, and further transmission to the kindergarten, workplaces, and restaurants was identified (Figure).

We confirmed all SARS-CoV-2 cases by using reverse transcription PCR of nasopharyngeal swab specimens. We extracted RNA from the specimens by using a QIAamp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com), then amplified the receptor-binding domain of the SARS-CoV-2 spike gene by using a One-Step RT-PCR (QIAGEN) with 2 primers selected from ARTIC nCoV-2019 V3 sequencing primer set (https://artic.network/ncov-2019; nCoV-2019_76_LEFT: 5’-AGGGCAAACTGGAAAGATTGCT-3’, nCoV-2019_76_RIGHT 5’-ACACCTGTGCCTGTTAACCAT-3’). Sequencing of the amplified 417-bp fragments of PCR products (420–543 residues of spike protein) confirmed that the specimens were the Omicron variant. We selected 15 specimens for whole-genome sequencing (WGS) with a QIAGEN QIAseq SARS-CoV-2 Primer Panel and a QIAseq FX DNA Library Kit UDI 1–4 and used NextSeq 1000/2000 P2 Reagents Kit version 3 (Illumina, https://www.illumina.com) for sequencing. For phylogenetic analysis, we aligned SARS-CoV-2 sequences with MAFFT version 7 (3) and inferred maximum-likelihood phylogenetic trees with IQtree version 2.1.3 (4).

We identified 586 contacts from 29 household clusters, 9 restaurant clusters, 4 workplace clusters, 2 kindergarten clusters, 2 sauna clusters, 2 long-term care facility clusters, 1 karaoke cluster, and 1 church cluster (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/4/22-0006-App1.pdf). A total of 182 of these contacts were verified as case-patients (Table). Community transmission started in the kindergarten and then spread to the workplace, restaurants, sauna/karaoke, long-term care facility, and church (Appendix Figure 1). The secondary attack rates for each cluster were as follows: family gatherings, 83.3%; church, 80%; households, 58.9%; restaurants, 46.8%; kindergarten 1, 39.2%; and kindergarten 2, 24.0% (Appendix Figure 1). As of January 3, 2022, no case-patient was classified as having critical illness or died (Table). WGS showed that virus from 15 household and kindergarten case-patients were closely related to each other and grouped into the same genetic cluster (Table; Appendix Figure 2).

This outbreak, which was caused by a single-case importation of SARS-CoV-2 Omicron variant to South Korea, started with household transmission to kindergarten and led to 182 cases within 3 weeks, despite high rates of vaccination coverage among adults. As of January 3, 2022, the rate of vaccine coverage in all populations was 83.0% (5). Emerging evidence suggests that transmissibility of SARS-CoV-2 Omicron is higher than that for other variants of concern (1,6). Unlike the previous introduction of the SARS-CoV-2 original strain and variants, Omicron affected children attending kindergarten during its early phase, which partly reflects the immune gap in children. Moreover, the early clusters include family gatherings, restaurants, karaoke events, and saunas, where the universal all-time mask policy may not be feasible, as highlighted in previous studies (7). Multifaceted preventive strategies, including vaccination,
increasing ventilation, quarantine, and isolation, need to be strengthened to mitigate transmission of the SARS-CoV-2 Omicron variant.

This study is limited because WGS confirmation of the Omicron variant was conducted for selected clusters only and identification of other major clusters was based on field epidemiologic investigations. However, given the thorough contact tracing of the exposed case-patients, all clusters are deemed epidemiologically linked to the Omicron outbreak.

This outbreak demonstrates that despite high vaccination coverage, transmission of the SARS-CoV-2 Omicron variant via symptomatic and asymptomatic persons was rapid, causing community transmission from 1 person with an imported case. As the Omicron variant continues to spread, we suggest vigilant monitoring of childcare facilities and vaccinating of elderly persons with booster doses.

Acknowledgments

We thank the relevant ministries, including the Ministry of Interior and Safety, Si/Do and Si/Gun/Gu, medical staff in health centers, and medical facilities for their efforts in responding to the COVID-19 pandemic.

The isolated Omicron variant of concern strains are deposited at the National Culture Collection for Pathogens (http://nccp.kdca.go.kr).

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Korea Disease Control and Prevention Agency or the institutions with which the authors are affiliated. This study was conducted as a legally mandated public health investigation under the authority of the Korean Infectious Diseases Control and Prevention Act (no. 12444 and No. 13392).

About the Author

Dr. E.Y. Kim is a public health officer at Honam Regional Center for Disease Control and Prevention, with a main research interest in epidemiologic investigation and surveillance of infectious diseases. Dr. Cho is a clinical assistant professor of pediatrics at Korea University Anam Hospital. His main research addresses quantification and understanding of the mechanisms of the effects of immunization programs on public health.

References


Address for correspondence: Young-Joon Park, Director of Epidemiologic Investigation, (28159) Korea Disease Control and Prevention Agency, Oson Health Technology Administration Complex, 187, Osongsaengmyeong 2-ro, Oson-eup, Heungdeo-gu, Cheongju-si, Chungcheongbuk-do, South Korea; email: pahmun@korea.kr

Table. Epidemiologic characteristics of 182 severe acute respiratory syndrome coronavirus 2 Omicron variant cluster case-patients, South Korea, November 26–December 26, 2021

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group, y</td>
<td></td>
</tr>
<tr>
<td>0–17</td>
<td>32 (17.6)</td>
</tr>
<tr>
<td>18–39</td>
<td>52 (28.6)</td>
</tr>
<tr>
<td>40–64</td>
<td>70 (38.5)</td>
</tr>
<tr>
<td>≥65</td>
<td>29 (15.4)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>97 (53.3)</td>
</tr>
<tr>
<td>M</td>
<td>85 (46.7)</td>
</tr>
<tr>
<td>Transmission site*</td>
<td></td>
</tr>
<tr>
<td>Household</td>
<td>61 (33.7)</td>
</tr>
<tr>
<td>Sauna/karaoke</td>
<td>39 (21.5)</td>
</tr>
<tr>
<td>Restaurant</td>
<td>30 (16.6)</td>
</tr>
<tr>
<td>Kindergarten</td>
<td>19 (10.5)</td>
</tr>
<tr>
<td>Workplace</td>
<td>12 (6.6)</td>
</tr>
<tr>
<td>Church</td>
<td>8 (4.4)</td>
</tr>
<tr>
<td>Long-term care facility</td>
<td>7 (3.9)</td>
</tr>
<tr>
<td>Family gathering</td>
<td>5 (2.8)</td>
</tr>
<tr>
<td>Vaccination status</td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>3 (20.9)</td>
</tr>
<tr>
<td>Partially vaccinated</td>
<td>3 (1.6)</td>
</tr>
<tr>
<td>Fully vaccinated</td>
<td>141 (77.5)</td>
</tr>
<tr>
<td>Outcome†</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>39 (21.4)</td>
</tr>
<tr>
<td>Critical illness</td>
<td>0</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
</tr>
</tbody>
</table>

*Excluding index case-patient.
†As of January 3, 2022.

Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 28, No. 4, April 2022
Time from Exposure to Diagnosis among Quarantined Close Contacts of SARS-CoV-2 Omicron Variant Index Case-Patients, South Korea

Hye Ryeon Lee,1 Young June Choe,1 Eun Jung Jang, Jia Kim, Ji Joo Lee, Hye Young Lee, Hanul Park, Sang Eun Lee, Moonsu Kim, Seonggon Kim, Hanna Yoo, Ju-Hyung Lee, Hyun Jeong Ahn, Mi-Young Go, Won Ick Kim, Bu Sim Lee, Hwa-Pyeong Ko, Jeonghee Yu, Eun-Young Kim, Hyoseon Jeong, Jae-Hwa Chung, Jin Su Song, Jihee Lee, Mi Young Kim, Young-Joon Park

Author affiliations: Korea Disease Control and Prevention Agency, Cheongju, South Korea (H.R. Lee, E.J. Jang, J. Kim, J.J. Lee, H.Y. Lee, H. Park, S.E. Lee, Y.-J. Park); Korea University Anam Hospital, Seoul, South Korea (Y.J. Choe); Incheon Metropolitan City, Incheon, South Korea (M. Kim, S. Kim, H. Yoo); Jeollabuk-do Center for Infectious Disease Control and Prevention, Jeonju, South Korea (J.-H. Lee); Jeollabuk-do Government, Jeonju (H.J. Ahn, M.-Y. Go); Jeollanam-do Government, Muan, South Korea (W.I. Kim, B.S. Lee); Gwangju Metropolitan Government, Gwangju, South Korea (H.-P. Ko); Honam Regional Center for Disease Control and Prevention, Gwangju (J. Yu, E.-Y. Kim, H. Jeong, J.-H. Chung); Capital Regional Center for Disease Control and Prevention, Seoul (J.S. Song, J. Lee, M.Y. Kim)

DOI: https://doi.org/10.3201/eid2804.220153

To determine optimal quarantine duration, we evaluated time from exposure to diagnosis for 107 close contacts of severe acute respiratory syndrome coronavirus 2 Omicron variant case-patients. Average time from exposure to diagnosis was 3.7 days; 70% of diagnoses were made on day 5 and 99.1% by day 10, suggesting 10-day quarantine.

Since its identification in November 2021, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Omicron variant of concern has rapidly spread across the globe (1). In South Korea, the first case-patient infected with the Omicron variant was identified on November 24, 2021, among inbound international travelers (2). In response, all close contacts of Omicron case-patients were required to quarantine for 14 days, regardless of vaccination and symptom status (3). Prolonged quarantine of close contacts of persons infected with newly emerging pathogens may pose a substantial burden on society. To determine optimal quarantine duration, we quantified time from exposure to diagnosis among close contacts of SARS-CoV-2 Omicron index case-patients. The study was conducted as a legally mandated public health investigation under the authority of government activity.

The study population consisted of close contacts of 2 clusters of SARS-CoV-2 Omicron case-patients detected on November 24 and 25, 2021. All quarantined close contacts were actively monitored by public health officers and were tested with reverse transcription PCR on days 1, 9, and 13 (4). We retrieved demographic information from the epidemiologic investigation form. Coronavirus disease (COVID-19) vaccination status was verified through documentation. Presence of fever or worsening or onset of symptoms was assessed daily, and persons were tested when deemed necessary. We calculated the time from exposure to the index case-patient and diagnosis of SARS-CoV-2 for close contacts in days (mean, median, interquartile range). We used the inverse Kaplan-Meier curve to visualize the percentage of diagnoses in a given interval.

We identified 107 close contacts of SARS-CoV-2 Omicron index case-patients, and the average time from exposure to diagnosis (± SD) was 3.7 (± 2.6) days (Table; Figure). We calculated the cumulative cases by time from exposure to diagnosis for total cases according to age group, according to vaccination status, and according to symptom status. Among all case-patients, diagnoses were made for 50% on day 3, and for 70% on day 5 (Figure, panel A). By day 10, almost all (106 [99.1%]) diagnoses had been made; 1 diagnosis was made on day 13 for an unvaccinated child with a previous negative test result. For all age groups (Figure, panel B), diagnoses were made for 50% on day 3; among those ≥20 years of age, for 70% on day 4; and among those <20 years of age, on day 5 (p = 0.051). Among close contacts who were symptomatic on the day of encounter, diagnoses were made for 50% on day 3 and 70% on day 5. Among asymptomatic close contacts, diagnoses were made for 50% on day 5 and 70% on day 8 (p = 0.001) (Figure, panel D).

We found that most (99.1%) diagnoses for the close contacts of the Omicron index case-patients were made within 10 days of quarantine; mean interval varied according to symptom status. Supported by this finding, the quarantine duration in South Korea was shortened from 14 to 10 days for all case-patients and even shorter (7 days) if quarantine facilities were at capacity because of a surge of cases (3).

Among the limitations of our study, individual public health officers decided to test on days 2–8 and 10–12; therefore, the chance of being tested on any day

1These authors contributed equally to this article.
may have differed for each close contact. Nonetheless, early testing may have been driven by the presence of symptoms and influenced by an individual close contact’s desire to get tested, which may have affected the decision of public health officers. Moreover, because tests were not performed after day 13, we do not have information on long incubation periods. Given that the study population was from 2 distinctive clusters, incubation periods may differ according to characteristics of illness in the index case-patients, which may affect study results. Despite these limitations, we were able to quantify the time from exposure to diagnosis and estimate the optimal duration of quarantine for persons exposed to Omicron.

Table. Time from close contact exposure to index case-patients infected with severe acute respiratory syndrome coronavirus 2 Omicron variant of concern to diagnosis of infection, South Korea*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Close contacts, no. (%)</th>
<th>Time from exposure to diagnosis, d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average (± SD)</td>
</tr>
<tr>
<td>All close contacts</td>
<td>107</td>
<td>3.7 (± 2.6)</td>
</tr>
<tr>
<td>Age group, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>37 (34.6)</td>
<td>4.4 (± 3.0)</td>
</tr>
<tr>
<td>20–59</td>
<td>64 (59.8)</td>
<td>3.3 (± 2.5)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>6 (5.6)</td>
<td>3.7 (± 2.0)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>56 (52.3)</td>
<td>3.5 (± 2.5)</td>
</tr>
<tr>
<td>M</td>
<td>51 (47.7)</td>
<td>4.0 (± 2.8)</td>
</tr>
<tr>
<td>Symptom status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomatic</td>
<td>85 (79.4)</td>
<td>3.3 (± 2.1)</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>22 (20.6)</td>
<td>5.2 (± 3.8)</td>
</tr>
<tr>
<td>Vaccination status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not vaccinated</td>
<td>58 (54.7)</td>
<td>4.0 (± 2.7)</td>
</tr>
<tr>
<td>Partially vaccinated</td>
<td>6 (5.7)</td>
<td>3.0 (± 2.1)</td>
</tr>
<tr>
<td>Fully vaccinated</td>
<td>42 (39.6)</td>
<td>3.4 (± 2.7)</td>
</tr>
</tbody>
</table>

*IQR, interquartile range.

Figure. Cumulative events by time from exposure to index case-patients infected with severe acute respiratory syndrome coronavirus 2 Omicron variant of concern to diagnosis of infection, South Korea. A) Total cases; B) by age group; C) by vaccination status; and D) by symptom status.
Implementing quarantine early in a pandemic is crucial for slowing the spread of a novel pathogen (5). Previous studies have suggested that the incubation period for Omicron could be shorter than that for the SARS-CoV-2 Delta variant (J. Chen, unpub. data, https://arxiv.org/abs/2112.01318). Estimating the duration of infectiousness is more challenging than measuring incubation periods; one study that measured viral load of Omicron suggested that viral load had diminished by days 10–13, which is in line with our findings (6).

To mitigate spread of highly contagious pathogens, the most effective public health measures are isolation and quarantine; however, these measures inevitably lead to personal and socioeconomic costs, necessitating evidence-based guidance from policy makers. A 10-day quarantine period may encompass most persons exposed to Omicron; however, quarantine duration may become shorter after balancing societal cost with public health benefit.

Acknowledgments
We thank the relevant ministries, including the Ministry of Interior and Safety, Si/Do and Si/Gun/Gu, medical staff in health centers, and medical facilities for their efforts in responding to the COVID-19 pandemic.

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About the Author
Mrs. H.R. Lee is a public health officer at Korea Disease Control and Prevention Agency. Her main research interest is epidemiologic investigation and surveillance measures of infectious diseases. Dr. Choe is a clinical assistant professor of pediatrics at Korea University Anam Hospital. His main research addresses quantification of and understanding the mechanisms of the effects of immunization programs on public health.

References

Address for correspondence: Young-Joon Park, Director of Epidemiologic Investigation, (28159) Korea Disease Control and Prevention Agency, Osong Health Technology Administration Complex, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Republic of Korea; email: pahmun@korea.kr

Legionella pneumophila Subspecies fraseri Infection after Allogeneic Hematopoietic Stem Cell Transplant, China

Xiaojuan Wang,1 Yifan Guo,1 Yawei Zhang, Qi Wang, Shuo Yang, Hua Yang, Tianyi Wang, Hui Wang

Author affiliations: Peking University People’s Hospital, Beijing, China (X. Wang, Y. Guo, Y. Zhang, Q. Wang, S. Yang, H. Wang); Peking University Health Science Center, Beijing (Y. Guo, T. Wang); Zhongshan Torch Development Zone Hospital, Zhongshan, China (H. Yang) DOI: https://doi.org/10.3201/eid2804.211433

DOI: https://doi.org/10.3201/eid2804.211433

1These authors contributed equally to this article.
We describe an immunosuppressed patient with bacteremia and pneumonia caused by *Legionella pneumophila* subspecies *fraseri* in China. We confirmed this diagnosis by using nanopore sequencing of positive blood cultures and subsequent recovery from buffered-charcoal yeast extract culture. Nanopore sequencing is an effective tool for early diagnosis of atypical infections.

*Legionella pneumophila* is an opportunistic atypical pathogen of community-acquired or hospital-acquired pneumonia (1–3). Underestimates of its prevalence are likely because the *Legionella* urinary antigen testing and buffered-charcoal yeast extract (BCYE) culture routinely used for diagnosis are sometimes available only in a few tertiary hospitals that specialize in respiratory diseases. Therefore, early diagnosis and prompt therapy for *L. pneumophila* infection are crucial. We report a case of *L. pneumophila* subspecies *fraseri* bloodstream infection in a patient in China after allogeneic hematopoietic stem cell transplant (aHSCT). We confirmed this diagnosis by using nanopore sequencing of positive blood cultures and by recovering *L. pneumophila* using BCYE medium. The study was approved by the Institutional Review Board of the Peking University People’s Hospital (approval no. 2019PHB134–01).

A 55-year-old man with acute T/myeloid mixed-cell leukemia was hospitalized because of nasal bleeding on day 62 after aHSCT. After treatment for thrombocytopenia, he experienced mild diarrhea on day 101. Three days later (day 104), his temperature was 38.8°C (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/4/21-1433-App1.pdf), but he had no accompanying symptoms. One aerobic blood culture vial, 1 anaerobic blood culture vial, and 1 Myco/F blood culture vial (BD, https://www.bd.com) per puncture point (2 puncture points) were collected for culture. Chest radiography and CT displayed multiple solid nodules in both lungs; the largest was in the right lower lobe, which suggested infection (Figure). The patient then experienced a 3-day continuous high fever with a maximum temperature of 39.5°C, a small amount of white sputum, and a blood oxygen saturation of 90% on day 107; the same number of blood culture vials were collected. CT showed a large, solid, fuzzy shadow with a unclear boundary, uneven internal density, and low-density plaques in the lower lobe of the right lung (Figure). The patient was administered linezolid (0.6 g every 12 h) and imipenem (0.5 g every 8 h) to control the infection. The patient’s temperature decreased to a normal level, but the C-reactive protein values did not decrease to reference range (Appendix Figure 2).

After ≈10 days (256 h) of incubation, we observed that the blood cultures in the Myco/F vial collected on day 104 after aHSCT, which are commonly used to culture *Mycobacterium* spp. and fungus, contained the only growing gram-negative bacilli. We then transferred the positive blood cultures to blood nutrition plates for further culture in 5% carbon dioxide, anaerobic, and microaerobic environments, but this culture failed. Simultaneously, we directly used a serum separator-gel tube and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (4) to identify positive blood cultures but were unsuccessful. Subsequently, we extracted DNA from the positive blood cultures for nanopore sequencing (5,6); *L. pneumophila* subsp. *fraseri* was detected after 1 hour (Appendix; BioProject Short Read Archive accession no. "904 Emer..."
The coverage length and depth were 37.67% and 2.04 with 1,337 raw reads. After timely communication of the sequencing results to the clinical department, the patient was administered azithromycin (0.5 g 1×/d for 1 d), followed by moxifloxacin (0.4 g 1×/d for 9 days) to ease symptoms of discomfort caused by azithromycin. The infection was finally controlled, and diarrhea symptoms improved 3 days after appropriate moxifloxacin therapy was initiated.

Afterward, we analyzed serum collected on May 24 (day 115) and May 27 (day 118) and detected L. pneumophila IgM by using an ELISA kit (Euroimmun, https://www.euroimmun.com). Legionella urinary antigen testing was not performed. Meanwhile, 100 μL of the positive blood cultures was inoculated on the in-house BCYE agar supplied with Legionella BCYE growth supplement medium (OXOID; Thermo Fisher Scientific, https://www.thermofisher.com). Two days later, we successfully isolated L. pneumophila and displayed wet blue-purple luster colonies (Appendix Figure 3); L. pneumophila was finally identified using MALDI-TOF mass spectrometry. During the course of infection, no more sputum specimens were available because the patient had no obvious cough or expectoration.

The fatality rate of Legionnaires’ disease is between 5% and 30% (7). Risk factors for L. pneumophila infection include age >50 years, solid tumors or hematologic malignancies, solid organ transplant, and immunosuppression (8,9). Hence, shortening the turnaround time to identify microorganisms is crucial for timely diagnosis and appropriate therapy, which influence death rates. In this case, the diagnosis of L. pneumophila pneumonia was not possible on the basis of the atypical radiologic evidence, high fever (>38°C), elevated C-reactive protein, and diarrhea, although such manifestations are the most common symptoms of L. pneumophila infections (10). Furthermore, administration of corticosteroids and immunosuppressive drugs likely obscured the respiratory symptoms. Rapid nanopore sequencing with a short turnaround time has the potential to effectively expedite the detection of L. pneumophila infection, guaranteeing the appropriate antibiotic therapy, especially for immunosuppressed patients with atypical symptoms.

Acknowledgments
We thank Zhenzhong Li and Hongbin Chen for bioinformatics analysis, Ying Shang and Zhengwu Yang for serologic testing for L. pneumophila IgM, Jingzhi Wang for clinical communication and consultation, and Feifei Zhang for preparing of BYCE culture medium.

This work was supported by the National Key Research and Development Program of China (2018YFE0102100 and 2018YFC1200102).

About the Author
Dr. Wang is a researcher in the Department of Clinical Laboratory, Peking University People’s Hospital, Beijing, China, focusing on the detection and study of resistance mechanisms of antimicrobial agents. Mr. Yifan Guo is a PhD candidate under the supervision of Dr. Wang in the Institute of Medical Technology, Peking University Health Science Center and the Department of Clinical Laboratory, Peking University People’s Hospital, focusing on metagenomic sequencing and bioinformatics analysis.

References

Address for correspondence: Hui Wang, Department of Clinical Laboratory, Peking University People’s Hospital, Xicheng District, Beijing, 100044, China; email: wanghui@pkuph.edu.cn
Measles is a highly contagious, vaccine-preventable, systemic viral disease caused by measles virus (MV), an enveloped, single-stranded, negative-sense RNA virus in the genus Morbillivirus, family Paramyxoviridae. MV may cause persistent central nervous system (CNS) infections that result in fatal neurologic diseases, such as subacute sclerosing panencephalitis and measles inclusion body encephalitis. Live-attenuated MV-containing vaccines, such as measles-mumps-rubella (MMR), are administered in a 2-dose series and are estimated to be >95% effective in preventing clinical measles (1,2). Serious adverse events are relatively uncommon after MV vaccination; rare reports have documented measles-like illness, predominantly in immunocompromised children (2–5). To our knowledge, before the case we report, only 1 sequence-confirmed, postvaccination MV CNS infection had been reported (6).

A previously healthy infant received dose 1 of the MMR ProQuad vaccine (Merck, https://www.merck.com) at her 1-year well-child visit. Over the following week, the patient experienced fevers, and acute myeloid leukemia was diagnosed. During induction chemotherapy, a diffuse morbilliform rash developed. A nasopharyngeal swab sample was positive for MV RNA by a laboratory-developed multiplex quantitative reverse transcription PCR (7). We detected all 3 genomic targets: the nucleoprotein, hemagglutinin, and large protein genes. In addition, the carboxyl-terminal nucleoprotein typing sequence was identical to the MV component of the ProQuad vaccine, the Edmonston-Enders (Moraten) strain (8). The patient received intravenous immunoglobulin and vitamin A. The rash resolved after ≈8 days.

Four months after her initial acute myeloid leukemia diagnosis, the patient received a paternal haploidentical stem cell transplant. One month after the transplant, she experienced altered mental status; magnetic resonance imaging showed abnormal signals, and positron emission tomography showed hyperperfusion involving the insula and thalamus (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/28/4/21-2357-App1.pdf). The patient experienced respiratory decompensation of suspected neurologic origin associated with brain lesion progression involving the right mesial temporal lobe, which was biopsied. We cut 5-µm sections of formalin-fixed, paraffin-embedded (FFPE) brain tissue and mounted them on glass slides. We prepared hematoxylin and eosin–stained sections and glial fibrillary acidic protein immunoperoxidase-stained sections (GFAP Clone GA5; Leica Biosystems, https://www.leicabiosystems.com) by standard methods for diagnostic neuropathology evaluation. Histology showed deep gray and white matter with astrogliosis (Appendix Figure 2) but without notable inflammation, neoplastic cells, or definite inclusions.

To evaluate for CNS MV, we extracted total nucleic acids from FFPE brain tissue scrolls using a Quick-RNA FFPE kit (Zymo Research, https://www.zymoresearch.com) according to manufacturer instructions. MV quantitative reverse transcription PCR detected all 3 genomic targets. We then performed metagenomic RNA sequencing as described elsewhere (9). In brief, we converted 5 µL of extracted total nucleic acids to complementary DNA and prepared sequencing libraries using the NEBNext RNA ULTRA II kit (New England Biolabs, https://www.neb.com) according to manufacturer instructions. We sequenced samples on the NovaSeq 6000 system (Illumina, https://www.illumina.com) using 150-nt paired-end sequencing and aligned them to the MV vaccine complete genome (GenBank accession no. FJ211583) using the bwasw module in Burrows-Wheeler Aligner version 0.7.9a-r786 (http://bio-bwa.sourceforge.net) with match score 2 and mismatch penalty −3 (options -a2 -b3). We used a custom python script to call mutations from the sequence alignment file. We discarded mutations <5% as noise. The consensus whole-genome sequence (Genbank OL473966) was generated from the sequence alignment file using SAMtools version 0.1.19 mpileup
Metagenomic RNA sequencing of brain tissue acquired 50.7 million reads, 190,303 of which aligned to Edmonston-Enders (Moraten) MV. We obtained 500× coverage over 98.2% of the whole MV genome with mean coverage of 1,540× reads. We identified no known hyperfusogenic fusion gene mutations (10). However, 38.0% (68/179) of uracil-containing codons in the matrix (M) gene contained ≥1 uracil-to-cytosine mutation at levels ≥5% (Figure 1). Despite treatment with intravenous ribavirin, intrathecal interferon-α, and inosine pranobex, the patient experienced persistent dysautonomia, respiratory failure, and myoclonus; without conceivable neurologic recovery, she was extubated and died shortly thereafter. The family declined autopsy.

Unique to this case was identification of biased M-gene hypermutation. In the previously reported case of sequence-confirmed, postvaccination MV CNS infection, M-gene hypermutation was not observed, likely because of limitations of Sanger sequencing for detecting minority variants (6). However, biased M-gene hypermutation is a characteristic of prolonged CNS replication in subacute sclerosing panencephalitis and measles inclusion body encephalitis. Because neurons lack SLAM/CD150 (signaling lymphocytic activation molecule/cluster of differentiation 150) and nectin4, the known receptors for wild-type MV, reduced M-gene expression may contribute to CNS persistence and disease progression by MV cell-to-cell transmission (10). The biased M-gene hypermutation we observed in this fatal case of postvaccine MV encephalitis confirmed viral CNS persistence. However, because Edmonston vaccine strains also use the ubiquitous CD46 receptor, the role of these genomic changes in neuropathogenesis remains to be determined.

The concurrence of live-attenuated MV vaccination and impending diagnosis of hematologic malignancy in this case was an unfortunate but uncommon circumstance. Nevertheless, clinicians should be vigilant for signs and symptoms of immunocompromise before administering live-attenuated vaccines.

**About the Author**

Dr. Costales is a global health diagnostics fellow in the Department of Pathology at the Stanford University School of Medicine, Stanford, California, USA. Her research interests include global health and infectious disease pathology.

**References**


Address for correspondence: Benjamin A. Pinsky, Stanford University School of Medicine, 3375 Hillview Ave, Rm 2913, Palo Alto, CA, 94304, USA; email: bpinsky@stanford.edu

etymologia revisited

Coronavirus

The first coronavirus, avian infectious bronchitis virus, was discovered in 1937 by Fred Beaudette and Charles Hudson. In 1967, June Almeida and David Tyrrell performed electron microscopy on specimens from cultures of viruses known to cause colds in humans and identified particles that resembled avian infectious bronchitis virus. Almeida coined the term “coronavirus,” from the Latin corona (“crown”), because the glycoprotein spikes of these viruses created an image similar to a solar corona. Strains that infect humans generally cause mild symptoms. However, more recently, animal coronaviruses have caused outbreaks of severe respiratory disease in humans, including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and 2019 novel coronavirus disease (COVID-19).

Sources:

https://wwwnc.cdc.gov/eid/article/26/5/et-2605_article
When American artist John August Swanson died in September 2021, he left an extensive body of artwork focusing on social justice issues, religious themes, and everyday activities. The TheoArts Gallery, Boston University School of Theology, notes, “His art reflects the strong heritage of storytelling he inherited from his Mexican mother and Swedish father. John August Swanson’s narrative is direct and easily understood. He addresses human values, cultural roots, and a quest for self-discovery through visual images.” His artwork is housed in collections at the Smithsonian's National Museum of American History, National American Art Museum, and National Air and Space Museum; the Art Institute of Chicago; Tate Gallery (UK); the Vatican Museums’ Collection of Modern Religious Art; and Emory University’s Candler School of Theology.

Swanson painted with oils, watercolors, and acrylics; created lithographs and etchings; and made colorful, detailed serigraphs—an artists’ term for silk-screen painting. According to Emory University’s Pitts Theology Library, “These serigraphs necessitate
an advanced level of technical acumen and typically feature 30 to 60 separate colors, each of which requires a separate stencil drawn by the artist. Swanson’s elaborate serigraph process results in pieces that have unique textures and colors that are characteristic of his mastery of this medium.”

Swanson’s The Carousel, this month’s cover image, is a vibrant serigraph depicting the popular amusement ride—sometimes also called a merry-go-round—and illustrating a certain association and interaction between humans and animals. It is a dizzying, complex spectacle imbued with 29 distinct colors and intricate patterns that may be missed without scrutiny. Many animals, including the elephant, camel, lion, and zebra, are rendered in colors similar to their natural tones, but Swanson’s blue tiger near the center breaks that convention. Detailed textures and whirling shapes enliven fur, scales, shells, and feathers, rich brocades that contrast with the smooth-skinned elephant and white and black zebra.

Swanson decorates the inner wall of the carousel—which hides its engine—with more images of animals and iconography of religion. The floor is partitioned by distinct brightly colored wedges and repeating shapes that continue around the platform. More repeating patterns decorate the carousel’s upper and lower edges.

The people depicted in the serigraph, save for their age differences, are largely uniform. Riders and spectators share skin tone, facial feature shape, dark eyes, and hair color. Their clothing, featuring more patterns, swirls, and colors, distinguishes one from another. Except for the trio at the ticket booth, most people and animals are facing counterclockwise, the direction in which carousels typically rotate in North America. The background whorls of unnaturally verdant grass merging into a panoply of trees, capped by glimpses of pale blue sky, and a small cluster of buildings in the upper left all suggest that the location is a city park.

Swanson’s insouciant image, including domesticated and wild animals from around the world, also works as a metaphor for humankind’s relationships with and connections to other animals through the lens of zoonotic disease. An observer watching such a carousel would see the figures emerge and reemerge, not unlike disease detectives tracking zoonotic diseases. Zoonoses, an unavoidable consequence of the interactions among humans and animals, are caused by harmful germs such as viruses, bacteria, parasites, and fungi. The Centers for Disease Control and Prevention notes that more than 6 out of every 10 known infectious diseases in people come from animals and that 3 out of every 4 new or emerging infectious diseases in people come from animals. Knowing which animals could have zoonotic diseases proves challenging because both domesticated animals and wildlife may appear well, act healthy, yet carry lethal pathogens.

Like Swanson’s cycling carousel, emerging and reemerging zoonotic diseases—which are transmitted from animals to humans—and the pathogens that cause them, also occur in cycles as they propagate among different ecosystems. The sylvatic cycle occurs in natural settings among wild animals and vectors; the synanthropic cycle occurs where domestic and companion animals live in close association with humans. Other cycles of transmission are human-to-human transmission and human-to-animal transmission. Many interrelated factors, including climate change, modern agricultural practices, destruction of natural habitats, urbanization, and seasonality of infectious diseases, contribute to the complex and dizzying array of zoonoses that pose a challenge to public health.

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- Risk for Asymptomatic Household Transmission of *Clostridioides difficile* Infection Associated with Recently Hospitalized Family Members
- Crimean–Congo Hemorrhagic Fever Virus in Ticks Collected from Wild and Domestic Animals, Corsica, France
- Highly Pathogenic Avian Influenza A(H5N8) Clade 2.3.4.4b Viruses in Satellite-Tracking Wild Ducks, Ningxia, China, 2020
- Epidemiologic and Genomic Analysis of SARS-CoV-2 Delta Variant Superspreading Event in a Nightclub, Amsterdam, the Netherlands, June 2021
- Imported Monkeypox from International Traveler, Maryland, USA, 2021
- Disparities in First Dose COVID-19 Vaccination Coverage among Children 5–11 Years of Age, United States
- Pathogens that Cause Illness Clinically Indistinguishable from Lassa Fever, Nigeria, 2018
- Rapid Replacement of SARS-CoV-2 Variants by Delta and Subsequent Arrival of Omicron, Uganda, 2021
- Assessing Human Population Immunity for an Influenza Pandemic Risk Assessment
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- Novel Hendra Virus Variant Circulating in Black Flying Foxes (*Pteropus alecto*) and Grey-Headed Flying Foxes (*Pteropus poliocephalus*)
- Breast Milk as Route of Tickborne Encephalitis Virus Transmission from Mother to Infant
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- Usefulness of Domestic Dogs as Sentinels for West Nile Virus but not *Aedes*-borne Flaviviruses, Mexico
- Increased COVID-19 Severity among Pregnant Patients Infected with SARS-CoV-2 Delta Variant, France
- Rare Case of Rickettsiosis Caused by *Rickettsia monacensis*, Portugal, 2021
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- Emergence of atpE Mutation in *Mycobacterium tuberculosis* and Potential for Bedaquiline Treatment Failure
- Emerging Novel Reassortant Influenza A(H5N6) Viruses in Poultry and Humans, China, 2021
- High-Dose Convalescent Plasma for Treatment of Severe COVID-19

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Article Title
Shewanella spp. Bloodstream Infections in Queensland, Australia

CME Questions

1. You are advising a large public health department regarding the potential incidence of Shewanella species bloodstream infections (BSI). On the basis of findings of the population-based surveillance study in Queensland, Australia, during 2000–2019 by Laupland and colleagues, which one of the following statements about the incidence and epidemiology of Shewanella spp. BSI is correct?
   A. During more than 86 million person-years of surveillance, there were 86 Shewanella species BSI, for an incidence of 1.0 per million Queensland residents per year
   B. Most BSI were caused by Shewanella putrefaciens
   C. Occurrence of Shewanella species BSI peaked in the drier, cooler months
   D. Highest rates of Shewanella species BSI were in the western outback regions

2. According to findings of the population-based surveillance study in Queensland, Australia, during 2000–2019 by Laupland and colleagues, which one of the following statements about risk factors for the development of Shewanella spp. BSI and clinical course is correct?
   A. Males had twice the risk for Shewanella species BSI versus females
   B. Comorbidity was uncommon (median Charlson Comorbidity Index [CCI], 0), and 30-day mortality was 5%
   C. The most common foci of infection were soft tissue (41%), unidentified (38%), abdominal (8%), lower respiratory (7%), and endovascular (2%)
   D. Most infections were polymicrobial, with resistance to gentamicin and ciprofloxacin

3. On the basis of findings of the population-based surveillance study in Queensland, Australia, during 2000–2019 by Laupland and colleagues, which one of the following statements about clinical and public health implications of the incidence and epidemiology of and risk factors for development of Shewanella spp. BSI is correct?
   A. Shewanella species BSI cases are unlikely to increase in coming years
   B. Given the rarity of Shewanella a species BSI, ongoing surveillance is unnecessary
   C. The study confirms that Shewanella species BSI are unlikely to be healthcare-associated
   D. The true rate of disease attributable to Shewanella species in the study population is likely higher than detected
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Article Title

Unique Clinical, Immune, and Genetic Signature in Patients with Borrelial Meningoradiculoneuritis

CME Questions

1. Your patient is a 47-year-old man with suspected meningoradiculoneuritis. On the basis of the clinical and genetic study by Ogrinc and colleagues, which one of the following statements about clinical features of specific clinical presentations of Lyme neuroborreliosis (LNB) is correct?

   A. Approximately half of patients had meningoradiculoneuritis

   B. Patients with meningoradiculoneuritis were less likely to have an erythema migrans (EM) skin lesion during the course of disease than patients with LNB without radicular pain

   C. Patients with meningoradiculoneuritis were more likely to have more constitutional symptoms, particularly sleep disturbance, headache, and fatigue, than other patients with LNB

   D. Patients with meningoradiculoneuritis had shorter symptom duration than patients with LNB without radiculitis or those with suspected LNB

2. According to the clinical and genetic study by Ogrinc and colleagues, which one of the following statements about immune and genetic features, including the TLR1-1805GG single nucleotide polymorphism (SNP), of specific clinical presentations of LNB is correct?

   A. Patients with meningoradiculoneuritis had decreased levels of B cell chemoattractants CXCL12 and CXCL13 compared with those without radicular pain

   B. Patients with meningoradiculoneuritis had markedly elevated levels of T cell-associated mediators CXCL9, CXCL10, and IL-17 compared with those without radicular pain

   C. Patients with meningoradiculoneuritis had lower frequency of TLR1-1805GG polymorphism than those without radicular pain

   D. In all LNB subgroups, most mediators associated with innate and adaptive immune responses were concentrated in serum

3. On the basis of the clinical and genetic study by Ogrinc and colleagues, which one of the following statements about clinical implications of the role of innate and adaptive immune responses and the TLR1-1805GG SNP in specific clinical presentations of LNB is correct?

   A. The findings suggest that immune and genetic pathophysiology of meningoradiculoneuritis is similar to that of other patients with LNB

   B. The study identified the functional link between the TLR1-1805GG SNP and excessive site-specific T and B cell immune responses in CSF

   C. Greater immune responses in meningoradiculoneuritis are likely related simply to the longer disease duration

   D. Immune mediators in CSF that are affected in meningoradiculoneuritis offer attractive targets as biomarkers for diagnosis, as well as markers of disease activity and resolution
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