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Reports from the US Department of Defense
Global Emerging Infections Surveillance Program

Supplement to November 2024

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On the Cover

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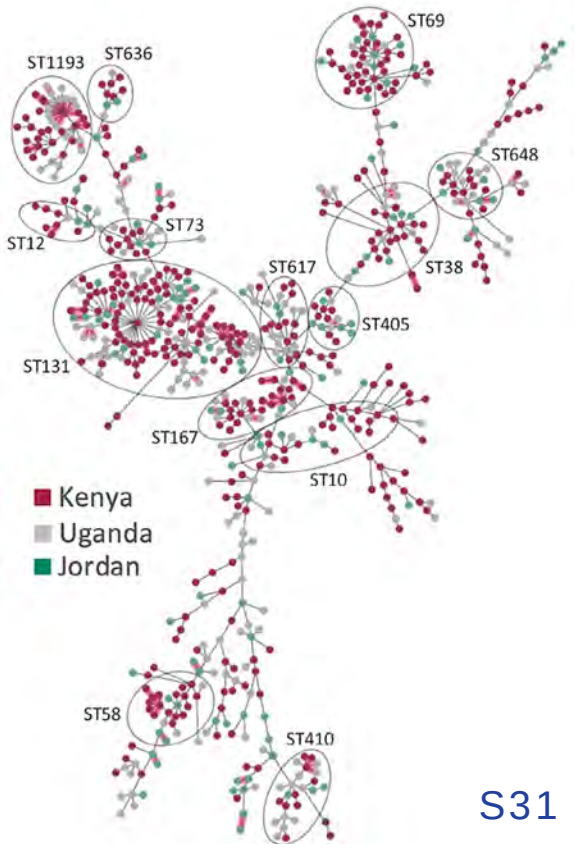

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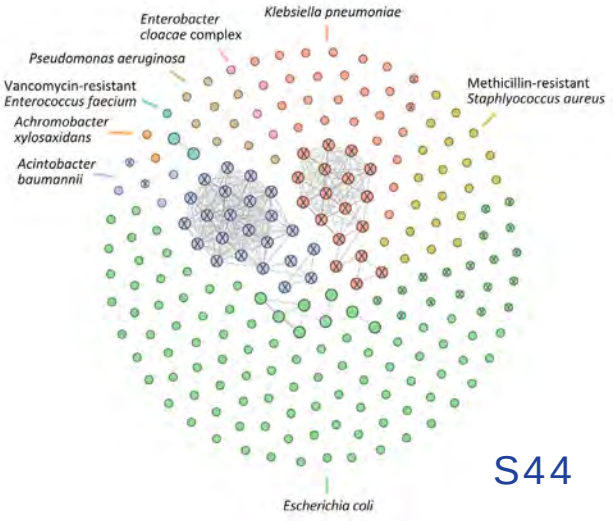

About the Cover

Redesigning Surveillance through the Global Emerging Infections Surveillance Program

David M. Brett-Major

This supplement contains new technical outputs and perspectives from the Global Emerging Infections Surveillance (GEIS) program within the Department of Defense (DoD). GEIS develops, invests in, disseminates, and integrates information from health surveillance activities conducted by military, academic, public health, and other partners. GEIS-funded researchers from around the world provide novel surveillance data that are used to update the military and civilian outputs from this long-standing initiative.

In this journal supplement, J. Early et al. provide an overview of the GEIS program's history and mission (*1*). Readers outside of the military health system might be unfamiliar with GEIS programming and outputs that have broader applications for public health considerations. The aspects of how this work occurred might also come as a surprise.

DoD emerging infectious disease investigators comprise a diverse group, and their collaborators are equally diverse. US taxpayers have been investing in networked surveillance and science through the DoD research and development enterprise for decades. That investment has kept pace with technological norms, including advanced sequencing techniques and bioinformatics. Work funded directly and indirectly by GEIS takes place in nearly every subregion of the globe and intersects with many normative US and international surveillance initiatives. Although GEIS often focuses on health protection needs of globally deployed military personnel, many nonmilitary stakeholders also benefit from its technical outputs. Such beneficiaries include those who are concerned for pandemic threats like SARS-CoV-2, threats posed to less well studied at-risk populations (e.g., rural persons exposed to ticks on the Eurasian steppes [*2*]), and the characterization of threats for which risks are not yet understood (e.g., bandavirus in Thailand [*3*]).

Three implications from this work are relevant to next steps in surveillance design and investment for the GEIS program and others who are not embedded in that enterprise. First, multidisciplinary efforts enable more contextualized findings that can be applied to use-case–based risk assessments and program improvements. Consider, for instance, the contrasting contexts in this supplement of the outbreak investigations into scrub typhus from trombiculid mites among military personnel in Australia (*4*) and the metagenomic sequencing of tickborne pathogens in Mongolia (*2*). In Australia, we see the end of a risk lifecycle and its human consequences; in Georgia, we see the beginning of risk and the enzootic findings of possible exposures not yet realized. Renewed interest in team science and multidisciplinary practice may enable funding and implementation approaches that integrate such assessments, in turn enabling more reliable interactions between clinical and public health actors, as well as entomologic, zoologic, social science, and other researchers.

Second, cross-cutting themes that matter in any setting matter in each setting. Several articles in the supplement address antimicrobial resistance (AMR) (*5*–*8*). Whether in travelers' diarrhea, infectious consequences of trauma, sexually transmitted infections, malaria, or its many other contexts, AMR forces incorporation of analytic approaches of interpathogen and interhost differences and environmental influences. Sponsors like GEIS and their implementers have an opportunity to advance pathogen-agnostic surveillance approaches, which can be done in part through continued innovation in AMR risk management and other broad challenges in ways that are applicable across a variety of pathogens.

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Third, novel technologies require validation based on the intended application (*9*). For instance, in the realm of genomic sequencing, the capability to generate a nucleic acid sequence is increasingly common, as is the ability to align and upload sequences to shared databases. Add to those capabilities other new online opportunities for structurally sound proteomic translation and we can quickly appreciate the vast data-verse enabled by such information. However, differences between hosts, host populations, vectors, and environments make over-extrapolation of any single sequencing result problematic. Assessing sequencing results to determine whether a primer for a nucleic acid test applies to a pathogen variant is entirely different than assessing sequencing results to examine mechanisms that cause immune escape from existing vaccines. In addition to progress in data integration and advanced analytics, use-case anchored question design also helps us navigate the complexities arising from examining diverse data sources and types.

Thank you for your interest this supplement. And many thanks to the programming and implementation efforts of the authors.

About the Author

Dr. Brett-Major is an infectious diseases physician and medical epidemiologist. His research interests include emerging infectious diseases risk towards attaining patient- and community-centered outcomes.

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The Central Role of the Global Emerging Infections Surveillance Program in Supporting Force Health Protection

June M. Early, Hunter J. Smith, Stephanie S. Cinkovich, M. Shayne Gallaway, Christan N. Stager, Matthew R. Kasper

The Global Emerging Infections Surveillance (GEIS) program is the only Department of Defense (DoD) organization that coordinates global surveillance for emerging infectious diseases that affect US military forces operating in the United States or foreign locations. Since 1997, the GEIS program has focused on surveilling pathogens likely to affect military operations and the health of service members. The foundation of the GEIS program is the long-standing, mutually beneficial relationships between the DoD overseas laboratories and their host-country partners and militaries. Through centralized programmatic support, the GEIS program provides the infrastructure needed for a rapid and scalable response to emerging threats. The GEIS program continues to enhance and evolve its initiatives to provide timely, reliable information to decision-makers in the DoD. The GEIS program has been and will continue to be a vital source of actionable biosurveillance information during infectious disease events of global public health concern.

In 1995, subject matter experts and policymakers in the US government convened to discuss the global threat of infectious diseases (*1*). With increasing urbanization, global interconnectedness, antimicrobial drug resistance, and climate change, experts called for increased capabilities and capacity to surveil for infectious diseases globally. To answer this call, in 1996, the Clinton Administration issued Presidential Decision Directive—National Science and Technology Council (NSTC)-7 on emerging infectious diseases. The NSTC-7 tasked the Department of Defense (DoD) to expand its mission to include support of global surveillance, training, research, and response to emerging infectious disease threats (*2*). NSTC-7 further charged the DoD to strengthen centralized coordination and epidemiologic capabilities to control and reduce disease. In response to that directive, the Assistant Secretary of Defense for Health Affairs established the DoD's Global Emerging Infections Surveillance (GEIS) program in 1997 with the primary mission of protecting US military forces from infectious disease threats at home and abroad (*3*). The renewed concern and interest in emerging pathogenic threats proved to be warranted, as the 21st century would bring multiple devastating emerging infectious disease events, including pandemic H1N1 (*4*), severe acute respiratory syndrome coronavirus (*5*), Middle East respiratory syndrome coronavirus (*6*), novel antimicrobial-resistant threats (*7*), Ebola virus (*8*), Zika virus (*9*), and more recently, SARS-CoV-2 (*10*). In this article, we provide context for how the GEIS program was initiated and has evolved, including strengths of the GEIS partner network and current priorities for infectious disease surveillance.

Upon establishment, the GEIS program was designated as the central hub for DoD infectious disease surveillance efforts. Over time, the program grew in line with US government funding for surveillance of emerging infectious disease threats, including multiple public health events of international concern (e.g., highly pathogenic avian influenza H5N1, pandemic H1N1, Ebola virus, etc.). The GEIS program has been part of the DoD's Armed Forces Health Surveillance Division since 2008 (*11*) and the

Author affiliations: Global Emerging Infections Surveillance (GEIS) Branch, Armed Forces Health Surveillance Division, Defense Health Agency, Silver Spring, Maryland, USA (J.M. Early, H.J. Smith, S.S. Cinkovich, M.S. Gallaway, C.N. Stager, M.R. Kasper); Cherokee Nation Businesses, Tulsa, Oklahoma, USA (S.S. Cinkovich)

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Defense Health Agency's (DHA) Public Health Directorate since 2015.

The GEIS program, through its network partners, conducts surveillance for military-relevant infectious diseases in service members or proxy populations where the US military operates; forges and maintains collaborative relationships with partner militaries and host nations to advance health diplomacy and strengthen global health security; sustains infrastructure, expertise, and technology needed to detect and characterize emerging or concerning infectious disease threats to promote readiness; and enables a coordinated network of laboratories in austere locations overseas and more technically advanced reach-back laboratories in the United States. Data and information outputs from those efforts are curated for the unique needs of diverse DoD decision-makers.

Historically, GEIS funding has been directed to all 3 branches of the armed forces: Army, Navy, and Air Force, and more recently, the Tri-Service DHA. However, GEIS has no direct command authority over the laboratories or organizations it funds. Most GEIS funding is distributed to the 6 DoD overseas laboratories: Walter Reed Army Institute of Research (WRAIR)-Armed Forces Research Institute of Medical Science (Bangkok, Thailand), Naval Medical Research Unit (NAMRU) EURAFCENT (headquartered in Sigonella, Italy, with detachments in Ghana and Egypt), NAMRU INDO PACIFIC (Singapore), NAMRU SOUTH (Lima, Peru), WRAIR-Africa (Nairobi, Kenya), and WRAIR-Europe and Middle East (Tbilisi, Republic of Georgia).

Those overseas laboratories, in close collaboration with their host-country partners, serve as forward sites for collection of specimens (e.g., human, animal, environmental); vectors (e.g., mosquitoes, ticks); isolates (e.g., bacterial, viral); and other relevant epidemiologic data for further advanced characterization (e.g., whole-genome sequencing) and analyses. In addition, funding is provided for surveillance programs across military installations and the Military Health System, in the United States and abroad, to monitor military-relevant infectious diseases, such as seasonal influenza and multidrug-resistant organisms. The GEIS program also routinely coordinates with other entities within the DoD, US government agencies, and international organizations to rapidly communicate information in response to infectious disease threats.

Scope and Value of the GEIS Network

The GEIS program's funded activities are organized into 3 focus areas: antimicrobial-resistant infections, febrile and vectorborne infections, and respiratory infections. All GEIS-funded surveillance activities across those focus areas center strictly around infectious diseases that are relevant to force health protection (i.e., relevant to military health in support of operational readiness). The antimicrobial resistant infections focus area surveils for multidrug-resistant organisms detected in nosocomial infections, community-acquired infections, and wound and trauma settings; sexually transmitted infections; and enteric infections. The febrile and vectorborne infections focus area provides surveillance and support for causes of undifferentiated acute febrile illness; vectorborne and zoonotic pathogens, vectors, and reservoir hosts of relevant infections; insecticide resistance; and the effectiveness of malaria countermeasures (e.g., antimalarials, rapid diagnostic tests). The respiratory infections focus area surveils for known and unknown respiratory pathogens and supports studies of vaccine effectiveness and potential shift and drift within influenza subtypes that might be associated with increased severity and transmission of respiratory infections.

The DoD overseas laboratories, many of which have been in existence for decades, maintain critical, long-standing relationships with allied militaries and partner nations, enabling collaborative detection and reporting of known, novel, and emerging infectious disease threats and bidirectional information exchange. To complement capabilities of the overseas laboratories, the GEIS program funds US-based reach-back laboratories for next-generation sequencing, bioinformatics, external quality assurance, species confirmation, and other supporting functions. The network connections among the US-based DoD laboratories, the overseas laboratories, and other partners contribute to the global footprint of the GEIS program (Figure) and ensure that the DoD can quickly detect, understand, and respond to emerging threats as they arise anywhere around the world where service members might be located. The geographic reach of the GEIS program enables an adaptable response to evolving threats wherever they may emerge worldwide, provides a robust source of biosurveillance data and information, and leads to more relevant insights for decision-makers, better protecting the health and readiness of our armed forces.

The GEIS program has formed and maintained flexible and scalable capabilities (e.g., personnel, equipment, and the supplies and reagents needed for sample collection and basic pathogen identification) for pandemic preparedness and response with guidance and coordination through its role as a central hub. An example of this centralized network

coordination is GEIS' Next Generation Sequencing and Bioinformatics Consortium (*12*), which is composed of subject matter experts from DoD laboratories around the world working to promote standardization and best practices, ultimately increasing the quality and utility of microbial genomic surveillance data. This high-quality and timely genomic surveillance data enables earlier detection and more rapid communication of novel and emerging infectious disease threats, enabling earlier interventions to support force health protection.

The long-standing regional partnerships between DoD laboratories and host-nation collaborators, combined with decades-long investment from the GEIS program, have advanced health diplomacy and global health security. Support from the GEIS program enables the surveillance of high-consequence pathogens and the collection of associated data, which are shared with local countries for public health decision-making. The partnerships between GEIS-supported DoD laboratories and local militaries and Ministries of Defense are also a unique tool for maintaining strong alliances with partner nations and strengthening global health security. Local surveillance capabilities and laboratory expertise have been built and sustained through GEIS-funded activities that are conducted by host-nation scientists and professionals in the partner countries where surveillance is conducted. This ensures expertise and infrastructure exist so that resources are in place to respond rapidly when an emerging threat is detected.

Data and downstream products routinely generated by the GEIS program are distributed to a multidisciplinary audience of decision-makers, clinicians, infection control preventionists, veterinarians, and other public health authorities. GEIS staff and subject matter experts ensure that data generated from surveillance activities are contextualized and capable of contributing to harmonized, military-relevant guidance. The infectious disease surveillance activities and subsequent reports and informational products result in data and information used to inform routine surveillance, countermeasure development, infection-control practices, force health protection posture, public health policy, outbreak detection and investigation, clinical practice guidelines, and more (Table).

As part of its coordinating role, the GEIS program also shares surveillance data generated from funded projects to inform DoD's medical research

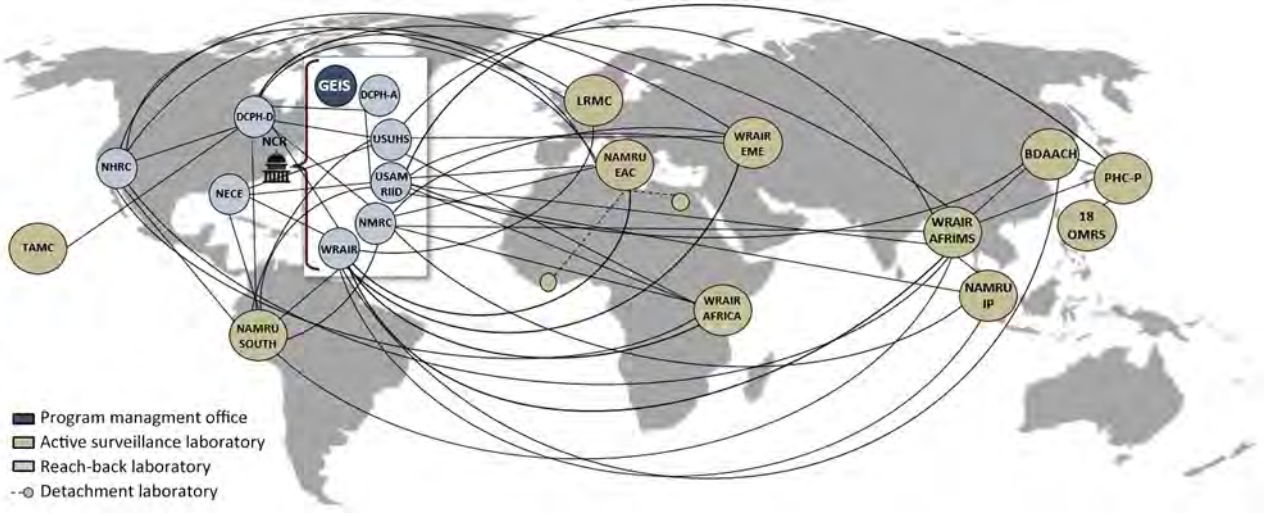

Figure. Interconnectivity of laboratories across the GEIS Program network. 18 OMRS, 18 Operational Medical Readiness Squadron (Okinawa, Japan); BDAACH, Brian D. Allgood Army Community Hospital (Pyeongtaek, South Korea); DCPH-A, Defense Center for Public Health—Aberdeen (Aberdeen, Maryland, USA); DCPH-D, Defense Center for Public Health—Dayton (Dayton, Ohio, USA); GEIS, Global Emerging Infections Surveillance (Silver Spring, Maryland, USA); LRMC, Landstuhl Regional Medical Center (Landstuhl, Germany); NAMRU EAC, Naval Medical Research Unit EURAFCENT (Sigonella, Italy); NAMRU IP, Naval Medical Research Unit Indo Pacific (Singapore); NAMRU SOUTH, Naval Medical Research Unit South (Lima, Peru); NCR, National Capital Region; NECE, Navy Entomology Center of Excellence (Jacksonville, Florida, USA); NHRC, Naval Health Research Center (San Diego, California, USA); NMRC, Naval Medical Research Command (Silver Spring, Maryland, USA); PHC-P, Public Health Command—Pacific (Okinawa, Japan); TAMC, Tripler Army Medical Center (Honolulu, Hawaii, USA); USAMRIID, US Army Medical Research Institute of Infectious Diseases (Fredrick, Maryland, USA); USUHS, Uniformed Services University of the Health Sciences (Bethesda, Maryland); WRAIR, Walter Reed Army Institute of Research (Silver Spring, Maryland, USA); WRAIR AFRICA, Walter Reed Army Institute of Research Africa (Kisumu, Kenya); WRAIR AFRIMS, Walter Reed Army Institute of Research—Armed Forces Research Institute of Medical Science (Bangkok, Thailand); WRAIR EME, Walter Reed Army Institute of Research Europe-Middle East (Tbilisi, Georgia).

Table. Select accomplishments of the Global Emerging Infections Surveillance Program in supporting force health protection, 1997–2024*

No.	Surveillance priority	Accomplishment
1	Influenza	Data on circulating influenza strains collected from GEIS-funded partners from ≈400 locations in >30 countries around the world directly informs the selection of strains in the annual influenza vaccination, leading to a safe and effective preventive countermeasure for service members and civilians alike.
2	Multidrug-resistant organisms	With funding support from the GEIS program, the WRAIR MRSN used genomic data from whole-genome sequencing to detect an outbreak of *Pseudomonas aeruginosa* in a military treatment facility, ultimately leading to the identification of an environmental reservoir as the source of transmission (W. Stribling et al., unpub. data, https://www.biorxiv.org/content/10.1101/2023.07.24.550326v2).
3	*Plasmodium falciparum* malaria	Data on *pfhrp2/3*-deleted *P. falciparum* parasites collected from GEIS-funded partners in Africa, Asia, and South America demonstrated that rapid diagnostic tests, like the DoD-authorized BinaxNOW, might not be suitable for accurately diagnosing malaria in countries where service members are or could be deployed, highlighting the need for the DoD to seek alternative diagnostics (*13*).
4	SARS-CoV-2 variants of concern	With funding support from the GEIS program, DoD laboratories were able to rapidly detect emerging SARS-CoV-2 variants of concern, including Alpha (N501Y) in Kenya (*14*), and Omicron (B.1.1.529) and Delta (B.1.617.2) in the United States.
5	Acute diarrhea	The GEIS program coordinated with DoD subject matter experts and decision-makers to develop clinical practice guidelines for management of acute diarrhea, a common medical condition with a significant operational impact, in the Middle East.

*DoD, Department of Defense; GEIS, Global Emerging Infections Surveillance; WRAIR MSRN, Walter Reed Army Institute of Research Multidrug-Resistant Organism Repository and Surveillance Network.

and development pipeline (e.g., therapeutics, vaccines, diagnostics, etc.). Although the GEIS program does not provide funding for conducting research for countermeasure development or testing and evaluating diagnostics, it does maintain active surveillance sites and laboratory testing capabilities through funding to DoD overseas laboratories and other DoD sites. Those sites generate data and pathogen isolates that are stored in repositories that can later be used to evaluate medical countermeasures and diagnostics. The GEIS program also coordinates closely with the Centers for Disease Control and Prevention and others through information sharing and programmatic reviews. Activities and portfolios across those programs are shared for visibility, opportunities for collaboration, and to reduce redundancy.

Current Strategic Vision and Looking Ahead

Recognizing that humans, animals, and our environment are all interconnected, the GEIS program conducts surveillance across One Health domains (*15*), leading to a more comprehensive understanding of how infectious diseases emerge and are transmitted. In addition, the GEIS network performs pathogen-agnostic sequencing (*16*) when the causative agent of a disease is unknown or when a genome has not been previously characterized. GEIS also conducts wastewater surveillance (*17*) as a tool to detect pathogens circulating in a given location. The GEIS program is seeking to leverage its One Health, pathogen-agnostic sequencing, and wastewater surveillance capabilities to support biodefense and biosecurity efforts aimed at preventing the next pandemic (*18*).

The GEIS program recognizes the growing importance of data stewardship and modernization and is invested in technology solutions to streamline management of surveillance and programmatic data. The GEIS program has begun an initiative to deploy a centralized health information system for collection, management, analysis, and reporting of standardized data from the DoD overseas laboratories. Data collected in the field and input into the health information system would then feed into a central, DHA-hosted data lake for additional access, curation, and analysis by DoD and GEIS personnel. Lowering the manual entry, analytic, and reporting burdens on GEIS partners will lead to a more efficient use of GEIS program funds and improve the transparency of the results of GEIS activities. In addition, to overcome the challenges of sharing, storing, and analyzing genomic data, the GEIS program is partnering with other organizations in the DoD to implement a secure, accessible, cloud-based solution for storing and analyzing genomic data submitted by partners across the globe. The GEIS program continues to refine its approach to data collection, storage, analysis, transfer, and reporting to improve communication and improve early warning of emerging threats for informed decision-making.

Conclusions

Since 1997, the GEIS program has been the only DoD organization coordinating global surveillance for emerging infectious disease threats, focusing on those likely to affect military operations and the health and readiness of service members. The emergence and spread of pandemic-level pathogens has illustrated the need for continued, well-resourced programs focused on detecting those threats and providing early warning for public health decision-making. The GEIS program has built, supported, and maintained a coordinated, responsive network of geographically diverse laboratory capabilities that can quickly pivot in

response to an emerging threat. The GEIS program continues to evolve and expand its approach to surveillance by implementing pathogen-agnostic sequencing, wastewater surveillance, and increasing the value of traditional disease surveillance activities. The success of the GEIS program is due in large part to the long-standing partnerships, relationships, and agreements between the DoD overseas laboratories and their host countries. Those continued long-term efforts enhance larger health diplomacy efforts between the United States and ally nations abroad by supporting collaborations on infectious pathogens of relevance for both the host nations and the US military. The expertise the GEIS program has cultivated has become a vital tool within the DoD and US government for expeditiously and comprehensively identifying, characterizing, and reporting data on military-relevant pathogens that threaten the health and readiness of US forces.

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About the Author

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Global Emerging Infections Surveillance Program Contributions to Pandemic Preparedness and Response

Kathleen E. Creppage, M. Shayne Gallaway, Dara A. Russell, June M. Early, Hunter J. Smith, Aileen C. Mooney, Ashley M. Hydrick, Matthew R. Kasper

Since its establishment in 1997, the US Department of Defense (DoD) Global Emerging Infections Surveillance (GEIS) program has provided support for infectious disease pandemic preparedness and response. The GEIS program has shown the value of having a central hub responsible for coordinating a global network of DoD laboratories that conduct surveillance for militarily relevant infectious disease threats. The program has supported the establishment and maintenance of capabilities for collecting, characterizing, and reporting on major infectious disease events, including the COVID-19 pandemic and mpox outbreak. The GEIS program enables the US government to mitigate infectious disease threats to DoD mission readiness and to effectively respond to pathogens worldwide. Continued investment in maintaining the GEIS program and its network is critical for timely detection and response to future emerging infectious disease threats in various populations within locations where gaps in US government or host-nation surveillance might exist.

The mission of the US Department of Defense (DoD) is to provide combat-credible military forces needed to deter war and protect the security of our nation and its allies (*1*). To this end, DoD supports pandemic preparedness and response through its unique global presence and ability to provide rapid response, deployment, and logistical capabilities, thereby complementing and enhancing the capabilities of its civilian counterparts (*1*). As the 2022 National Biodefense Strategy and Implementation Plan notes, infectious diseases ignore borders and can compromise the effectiveness of deployed forces and mission readiness, degrading operational capabilities and mission success (*2*). The COVID-19 pandemic highlighted the substantial burden that disease can place on military populations, which can include mission postponements or cancellations. For example, the aircraft carrier USS Theodore Roosevelt and the guided-missile destroyer USS Kidd both experienced mission interruptions because of onboard COVID-19 outbreaks (*3*,*4*), underscoring the need for proactive infectious disease surveillance and response.

The Global Emerging Infections Surveillance (GEIS) program was established in 1997 in response to the Presidential Decision Directive, National Science and Technology Council 7, on emerging infectious diseases (Figure) (*5*). The directive expanded the DoD's mission to include support of global surveillance, training, research, and response to emerging infectious disease threats through centralized coordination, improved preventive health programs and epidemiological capabilities, and enhanced involvement with military treatment facilities and United States and overseas laboratories (*5*). The GEIS program's purpose supports health protection for military forces and supports alignment with other federal agencies. The program's purpose ensures it is both unique and effective within the DoD because it informs US national biosurveillance and biodefense strategies by enhancing understanding and controlling the effects of emerging infectious diseases among US military service members. When the directive was issued, the DoD maintained 3 core laboratories within the United States and 6 overseas laboratories. GEIS now operates through a more expansive network of strategically positioned military service, Defense

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Health Agency, and public health partner laboratories across all 6 geographic combatant commands.

The GEIS program is the DoD's central coordinating hub for infectious disease surveillance and provides critical program management. The program management component encompasses foundational elements that contribute to rigorous scientific proposal review and thoughtful strategic selection of a surveillance portfolio to maximize efficiency and effectiveness of the GEIS network (GEIS-N) in threat surveillance. The GEIS program also leverages communication modalities to ensure timely information is shared with relevant parties and includes formal reports, peer-reviewed articles, and informal communications.

GEIS Pandemic Preparedness Timeline of Success

Since its inception, the GEIS program has shown the utility of funding and coordinating infectious disease surveillance. In 1998, the program published a 5-year plan titled Addressing Emerging Infectious Disease Threats: A Strategic Plan for the Department of Defense, a DoD strategy for protecting military forces and US citizens from relevant microbial threats (*6*). This initial plan paralleled the 1994 strategic plan of the US Centers for Disease Control and Prevention and its subsequent updates (*7*,*8*). After 1998, the GEIS program's baseline budget increased for overseas DoD laboratories; the program became the responsible management entity to ensure the execution and return on investment of those funds (*9*–*11*). The GEIS program has continued adapting its strategy for addressing potential threats, and the program and network have matured and evolved to better respond when public health emergencies have surfaced. DoD laboratories have consistently shown their ability to pivot to detect and characterize the next emerging pathogen. Through its baseline, supplemental COVID-19, and Biodefense Posture Review funds, GEIS has invested in activities that generate timely and actionable information regarding ongoing threats, including antimicrobial drug resistance, malaria countermeasure failures, and novel respiratory viruses, as well as supporting several concerning public health emergencies since 2010 (Figure).

In 2009, GEIS partner laboratories detected early cases of novel influenza A(H1N1) and coordinated additional diagnoses, advanced characterization through next-generation sequencing, and reporting of novel influenza cases in >10 countries worldwide (*11*). That same year, partner laboratories were able to detect a concerning number of clinically relevant drug-resistant and unexpected organisms in various parts of Southeast Asia, highlighting the flexibility of the network even in its earliest days. During the 2016 Zika virus infection outbreak, the GEIS program distributed nearly US $2 million to support enhanced Zika virus surveillance activities in 18 countries. Part of that comprehensive response was surveilling mosquito vectors and serum samples from US military personnel for traces of the virus (*12*,*13*). In addition, the GEIS program received and invested millions of dollars in support of SARS-CoV-2 surveillance, including assay development and distribution, laboratory training, diagnostics, sequencing, and phylogenetic analyses. The GEIS program's effective response to the SARS-CoV-2 pandemic emphasizes the rapid, comprehensive, and agile elements of the program and its network. Since 2018, the GEIS program has supported the detection of ≥27 novel pathogens by DoD laboratories.

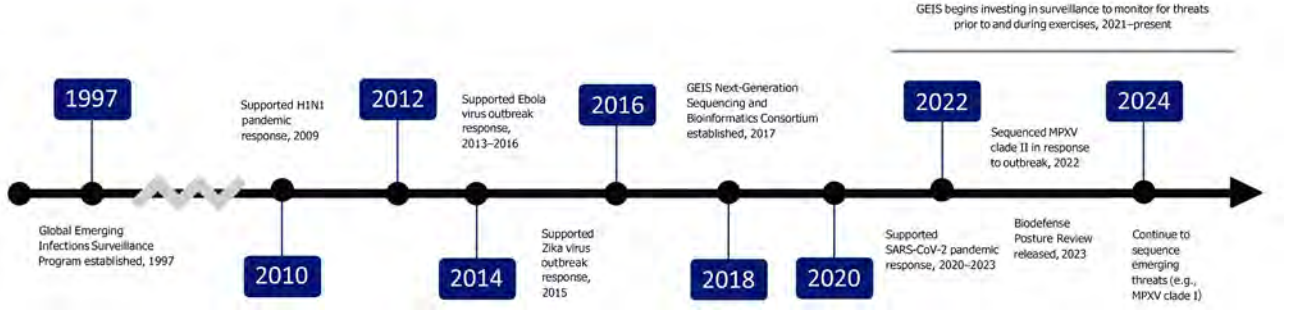

Figure. Timeline of key Department of Defense Global Emerging Infections Surveillance program events in support of pandemic preparedness and response, United States. Boxes and text indicate critical activities that occurred on or within specific time points. Time points were selected if multiple activities occurred within a several year period. Surveillance program was established in 1997 and has supported and continues to support multiple pandemic and outbreak responses, beginning with pandemic influenza. Key events in the GEIS program history are indicated where the program office or its partners provided support for infectious disease outbreak response or pandemic preparedness activities, including sequencing of samples to better characterize infectious disease threats as they emerged. GEIS, Global Emerging Infections Surveillance; MPXV, monkeypox virus.

Part of the success of this program and its support for pandemic preparedness and response is attributable to sustaining routine longitudinal regional surveillance, an often understated and underappreciated benefit of the network. GEIS-funded influenza surveillance is a model for how investments in routine activities, capabilities, and infrastructure built a foundation for pandemic preparedness and response. For example, the GEIS program provides funding for the DoD Global Respiratory Pathogen Surveillance Program, a network of sentinel surveillance sites that can respond to infectious disease threats. The pathogen surveillance program supplies the GEIS-N with a diverse, representative sample pipeline used for diagnostic methods and advanced characterization through next-generation sequencing of infectious respiratory pathogens. That network was integral to the DoD response to the COVID-19 pandemic, enabling an influx of samples from military installations across the world, including remote or inaccessible locations.

Microbial genomic sequencing technologies are leveraged across the GEIS-N to provide more comprehensive infectious disease information for pandemic preparedness and response. In 2017, the GEIS program established a consortium of next-generation sequencing and bioinformatics experts from across the DoD to focus on improving the translation of microbial sequence data into public health practice. That consortium, armed with resources, relationships, and expertise in virus characterization, provided support within the DoD at the onset of the COVID-19 pandemic. The GEIS program also coordinated vital statistics data collection and reporting efforts and, in 2021, developed and executed a plan for expanding SARS-CoV-2 genomic surveillance within the Military Health System (*13*). As a result, the GEIS program has maintained a repository of SARS-CoV-2 genomic surveillance data since 2020, with the knowledge that well-resourced, routine surveillance is the backbone of preparing for the next threat. Routine surveillance and timely advanced characterization data generated by GEIS-funded laboratories are also shared with partner nations and facilities that might otherwise lack access to those data. For example, as part of the Makerere University Walter Reed Project's antimicrobial resistance surveillance program, GEIS-funded partners characterized hypervirulent, multidrug-resistant *Klebsiella pneumoniae* in 4 tertiary healthcare facilities, providing evolution, convergence, and transmission data, which informed clinical decision making and infection control within those facilities (*14*). Much of that information is shared through publicly available databases, such as GISAID (https://www.gisaid.org) and GenBank, to enable analyses outside of the DoD. The GEIS program, along with additional partner laboratories, can rapidly scale-up sequencing capabilities in response to emerging threats by leveraging existing surveillance sites, personnel, and infrastructure.

Preparing for the Future of GEIS

The GEIS program is poised to support pandemic response efforts through continuous maintenance of a robust structure and through the flexible, scalable capabilities of its network. The infrastructure built around influenza and respiratory disease surveillance enabled a rapid pivot to support response efforts during the COVID-19 pandemic (*15*), which would not have been possible without an existing surveillance platform. As the COVID-19 pandemic declined, GEIS-N partners pivoted their sequencing capabilities to the next known disease of interest: mpox. Despite its rapid emergence and vastly different genome size and complexity compared with SARS-CoV2 (*16*), monkeypox virus samples were sequenced by ≥3 GEIS-funded partners, demonstrating the readiness and ability of GEIS-N partners to respond to diverse and complex challenges during the immediate post–COVID-19 pandemic era. The GEIS program also looks for opportunities to further invest in advancements in biosurveillance and pandemic preparedness, such as novel surveillance activities contributing to earlier detection and response; solutions for enhanced data collection, storage, and sharing; broad-based agreements that enhance partnerships within the GEIS-N; and partnerships with external agencies that can encourage a whole government approach to preventing, detecting, and responding to infectious disease threats. The GEIS program has also funded projects using wastewater surveillance methods for infectious disease detection and has explored opportunities to further coordinate and broaden expertise in this surveillance domain.

In summary, the success of the GEIS program and its partner network must be sustained; maintaining robust capabilities for future emerging threats is required for DoD's military readiness, as highlighted in the Biodefense Posture Review released in 2023 (*17*). Through the GEIS program and its network, DoD laboratories are monitoring for known and unknown pathogens (including those that might pose a novel threat), building an information baseline that is imperative for identifying potential threats to military service members and their operations. Continued investment in the GEIS program and its network is critical for timely detection of and response to future

emerging infectious diseases in various populations in locations where gaps in US government or host-nation surveillance might exist.

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Next-Generation Sequencing and Bioinformatics Consortium Approach to Genomic Surveillance

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Genomic surveillance programs benefit greatly from a network of committed, well-supported laboratories that conduct ongoing surveillance activities for pathogens of public health importance. The experiences of the Global Emerging Infections Surveillance program provide insights for building and maintaining genomic surveillance capabilities for public health and pandemic preparedness and response. To meet the needs of US Department of Defense and the Military Health System to use genomics to monitor pathogens of military and public health importance, Global Emerging Infections Surveillance convened a consortium of experts in genome sequencing, bioinformatics, and genomic epidemiology. The experts developed a 3-tiered framework for building and maintaining next-generation sequencing and bioinformatics capabilities for genomic surveillance within the Department of Defense. The consortium strategy was developed before the COVID-19 pandemic, leading to a network prepared to respond with existing resources and expand as new funding became available.

The Global Emerging Infections Surveillance (GEIS) program, established in 1997 under the US Department of Defense (DoD), is responsible for distributing funding and monitoring projects to support global surveillance for infectious diseases with pandemic potential and of importance to the US military health system (*1*). DoD medical research laboratories were among the earliest adopters of next-generation sequencing (NGS) technologies—genomic sequencing technologies developed after Sanger sequencing, such as 454 (*2*)—to enhance surveillance for infectious diseases of military and global health importance (*3*,*4*). NGS approaches can provide higher-throughput testing (*5*), identification of and creation of new taxa for novel or unexpected organisms (*6*), and advanced molecular characterization such as genetic investigation of emerging pathogens; for example, in Bennett et al. (*7*). Some early examples of GEIS-funded surveillance programs using sequencing for pathogen surveillance and outbreak investigations are the DoD Global Respiratory Surveillance Program (*8*) and the Multidrug-Resistant Organism Repository and Surveillance Network (*9*,*10*). Over time, GEIS funds were used to purchase and maintain sequencing platforms, bioinformatics software, and computational infrastructure for genomic data collection and analysis. As NGS technologies became more mature and commonly available, a growing portion of the GEIS portfolio contained sequencing and bioinformatics work, necessitating better coordination to set surveillance priorities and develop and implement the strategic direction of pathogen genomic sequencing efforts.

In 2017, GEIS created a consortium of NGS laboratories to better administer limited resources and coordinate NGS and bioinformatics activities funded by GEIS. The primary purpose of the newly established consortium was to develop a sustainable and reliable laboratory network capable of fully using sequencing technologies for infectious disease surveillance and epidemic response activities. In the first iteration of the GEIS NGSBC (Next-Generation Sequencing and Bioinformatics Consortium) Strategic Plan, the Consortium leadership made programmatic recommendations for building and maintaining pathogen genomic surveillance capabilities within the DoD. The recommendations included designated partners providing technical support and training. as well as

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close communication among participating laboratories and stakeholders to build demonstrable competencies in genomic surveillance and technical aspects of sequencing and bioinformatics. Because of prior global investments in metagenomics and pathogen discovery by biodefense initiatives, the initial interest from DoD leadership was in standardizing metagenomic sequencing workflows for clinical samples of unknown etiology (*11*). However, the surveillance portfolios and limited bioinformatics infrastructure at overseas DoD laboratories led to prioritizing high-quality viral genomes for pathogens with pandemic potential, such as influenza viruses and arboviruses.

Tiered Framework for Building and Maintaining Pathogen Sequencing and Bioinformatics Capabilities within the DoD

By 2019, the Consortium had developed a 3-tiered framework to support a series of layered assets and associated sequencing and bioinformatics capabilities within the DoD (12). Each sequencing laboratory has different needs for protocols, instrumentation, and personnel based on the physical location and the primary mission of the sequencing laboratory (e.g., clinical, medical research, or public health). Tier 1 represents the laboratories with the smallest footprint, flexibility, and most field-forward capabilities; tiers 2 and 3 scale up in laboratory complexity, size, and range of capabilities to account for operational needs and logistical needs (Figure). When designing the 3-tiered framework for sequencing laboratory capabilities, the Consortium assumed that companies and specific technologies change over time, so assets are typically grouped by sample and data throughput and laboratory space requirements (i.e., footprint). Today, the primary sequencers in use by GEIS partner laboratories include technologies from Illumina (https://www.illumina.com) and Oxford Nanopore Technologies (ONT; https://nanoporetech.com), which sell a range of devices that use different chemistries and vary in size, cost, and throughput (*3*,*13*,*14*). Genomic surveillance programs should be flexible enough to adopt rapidly improving sequencing technologies and bioinformatics software. However, differences in the speed of incorporation of newer technologies into existing sample sequencing workflows can cause substantial variation in methods among laboratories of the same tier.

Tier 1

Tier 1 laboratories are in closest proximity to where samples are often collected. They are usually field sites, austere or forward operating environments, and places where point-of-care testing is conducted. Tier 1 laboratories have a small physical and technological footprint and are located mostly outside of the continental United States. The laboratories can quickly provide a preliminary result, such as pathogen

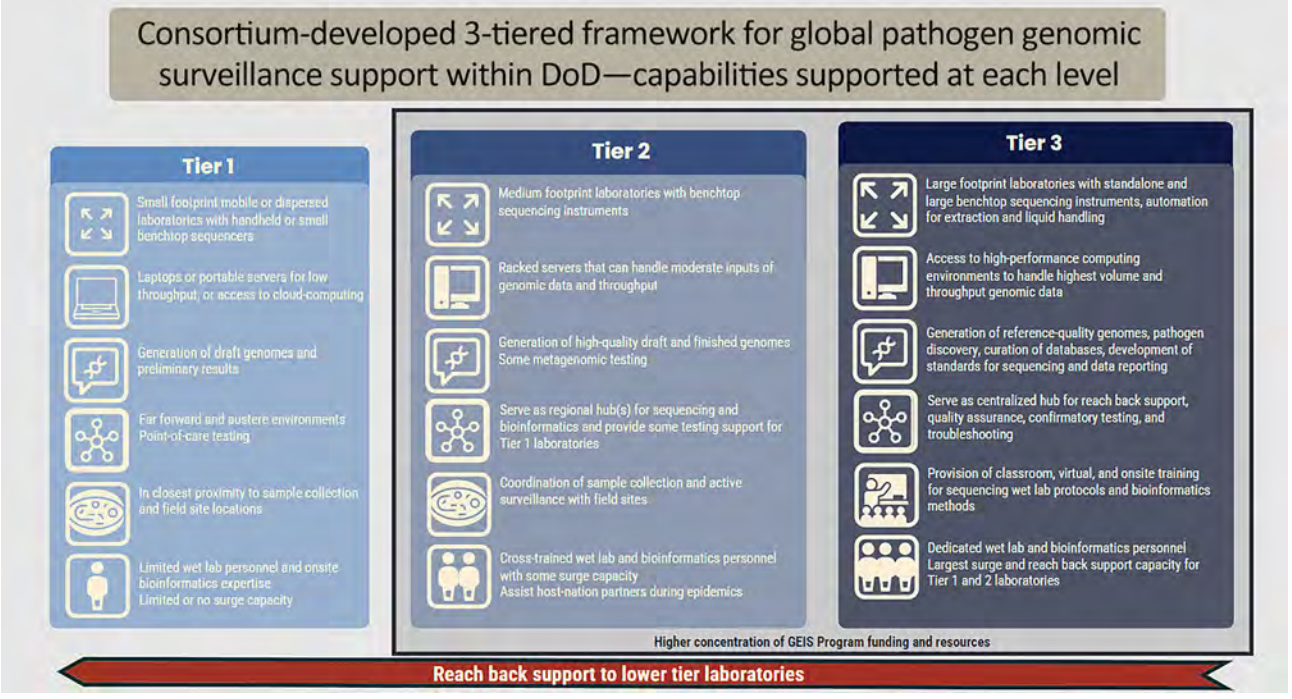

Figure. Tiered sequencing and bioinformatics capabilities for genomic surveillance within the US DoD. Modified from (*15*). DoD, Department of Defense; GEIS, Global Emerging Infections Surveillance.

identification, but typically will not perform deeper pathogen characterization. However, as sequencing methods and computational infrastructure become easier to implement in small or mobile laboratories, more characterization may occur at lower tiers. Tier 1 laboratories primarily use small hand-held or benchtop sequencers such as the ONT MinION or the Illumina MiniSeq and iSeq. Those platforms are most suitable for low-complexity samples, low throughput, and samples with previously suspected etiologic agents. In the laboratories, sequencing data are typically analyzed by using laptops and cloud-based bioinformatics. Regardless of throughput, tier 1 laboratories have lower sample transit time, enabling them to provide critical rapid responses to the GEIS network. Tier 1 partners also triage and select samples for additional analyses. Sequencing data may be produced at tier 1 and then after preliminary analyses sent back to tier 2 or 3 partners for further characterization. Historically, tier 1 laboratories have not been a GEIS NGSBC priority but are of high interest to the Armed Services, biodefense, and organizations with investments in mobile testing units.

Tier 2

Tier 2 laboratories have an intermediate footprint, conducting NGS and bioinformatics farther from sample collection than tier 1 while maintaining the capability to serve as regional training and sequencing support centers. Wherever possible, a train-the-trainer approach is taken, in which tier 2 laboratories receive training via tier 3 laboratories to provide regional training and reach back to tier 1 (*15*,*16*). In addition to pathogen identification, tier 2 laboratories can conduct strain-level identification for viruses, bacteria, and some eukaryotes, with some degree of genetic characterization, including identification of virulence or antimicrobial resistance determinants. Most laboratories can perform agnostic sequencing on samples with limited metadata. Tier 2 laboratories process larger batches of samples with higher depth and breadth of coverage than tier 1 laboratories, which enables sequencing and analysis of more complex, metagenomic samples. The increased throughput typically involves the mid-sized Illumina platforms such as Illumina MiSeq or NextSeq for short-read sequencing, and ONT devices such as MinION or GridION for long-read sequencing. To support bioinformatics, tier 2 laboratories usually have ≥1 racked servers in addition to standalone workstations, rather than relying solely on laptops, cloud-based analytics, or both. Personnel are often cross-trained between the wet laboratory (nucleic acid extraction and sequencing) and bioinformatics methods. The NGSBC has focused on addressing gaps in training or equipment and on strengthening support to tier 2 laboratories.

Tier 3

Tier 3 laboratories provide network-wide training and technical assistance while simultaneously running routine sequencing and bioinformatic analyses of samples collected throughout the GEIS network. The tier 3 laboratories located in the National Capital Region, the Naval Medical Research Command Biological Defense Research Directorate, the US Army Medical Research Institute of Infectious Diseases, and the Walter Reed Army Institute of Research collaboratively serve as core laboratories within NGSBC. The core laboratories assist the other NGSBC laboratories with gap assessments, development of curricula, training, coordination, and other forms of reach-back support (*15*). Tier 3 laboratories have the largest physical and technological footprints and have various combinations of short- and long-read sequencers, from ONT devices like GridION and PromethION or PacBio (Pacific Biosciences of California, https://www.pacb.com) to larger Illumina platforms such as NovaSeq. They also have a much larger repertoire of ancillary equipment, such as various machines for automated sample preparation, quality control assessments, or both. In tier 3 laboratories, high-performance computational resources are required to accompany the higher throughput of the sequencers in terms of depth of coverage of a given sample and the higher number of samples, the regular cadence of analyses, and the increased complexity inherent to advanced characterization. The level of throughput requires petabytes of data storage. In addition to strain-level identification, tier 3 laboratories routinely perform advanced genetic characterization including in silico genome closure, pathogen discovery, and phylogenetics. In tier 3 laboratories, personnel are typically dedicated to singular functions of wet laboratory or bioinformatic analysis. In comparison, personnel at tier 1 and tier 2 laboratories often perform laboratory and bioinformatics functions.

Preparedness for Pandemic Response

After SARS-CoV-2 emerged in late 2019, GEIS was quickly recognized as the central coordinator for DoD COVID-19 genomic surveillance activities, leading to supplemental funding for domestic and overseas response efforts (*17*). Within the DoD, NGSBC tier 3 laboratories were involved in the early development, evaluation, and dissemination of standardized testing and sequencing protocols, bioinformatics

workflows, and data reporting for COVID-19 (*18*). Outside the United States, several tier 2 laboratories with previous viral pathogen sequencing expertise were able to routinely sequence SARS-CoV-2 locally (*16*,*19*,*20*). GEIS also supported expanded sequencing capabilities and surge capacity in several laboratories in the Pacific (e.g., Japan, Hawaii, and the Philippines). As new SARS-CoV-2 variants emerged, focus shifted toward scaling up sequencing throughput and monitoring for changes potentially affecting medical countermeasures.

SARS-CoV-2 genomic surveillance data were used to directly inform public health mitigation efforts and deployment of medical countermeasures. GEIS partners developed and tested early sequencing protocols that can provide greater fidelity to answer questions such as whether persons were reinfected or their illness had recrudesced (*21*). Laboratories also developed novel bioinformatics methods to quickly scan for emerging intrahost SARS-CoV-2 variants from huge volumes of raw sequencing data (*22*). During the pandemic response, DoD public health and research groups demonstrated that genomics and phylogenetics could be used to understand the transmission of SARS-CoV-2 in military-specific settings, such as recruit or trainee depots and military installations (*23*,*24*), naval vessels (*25*), and overseas military locations (*26*–*28*). Genomic surveillance was also used to aid in naval outbreak investigations (*29*) and examine how well medical countermeasures and nonpharmaceutical interventions worked to slow or prevent SARS-CoV-2 spread in military populations (*23*,*30*).

Improving the Post-COVID Pandemic Pathogen Sequencing Landscape

The US DoD 2023 Biodefense Posture Review urges the DoD to improve readiness and coordination for biosurveillance and bioincident response and specifically mentions increasing sequencing capabilities within the DoD (*31*). GEIS supports a network of partner laboratories ready to perform sequencing and bioinformatics for routine public health surveillance while being prepared to respond to the next pandemic or emerging pathogen. The Consortium continues to fill a gap in genomic surveillance coordination throughout the DoD. The annual program support for routine data and sample collection, sequencing exercises, procurement of NGSBI equipment and reagents, training, and retention of highly skilled personnel is essential for maintaining readiness. Support for pathogen genomic surveillance was a GEIS priority for many years before the COVID-19 pandemic. Several strategies used before and during the pandemic provided many benefits (Table), probably leading to success and overall improvements in the pathogen-sequencing landscape for DoD public health.

As public attention has waned and the urgency of the COVID-19 pandemic has ended, so has funding for many public health response activities. In the absence of the extra funding, maintenance of genomic

Table. Global Emerging Infections Surveillance program strategic activities for developing and maintaining genomic surveillance capabilities

Strategy	Description	Benefits
Consortium	Forming a group of NGS and bioinformatics subject-matter experts and well-established DoD laboratory partners led by an experienced program office	Ensures that genomic surveillance remains a priority for DoD
Tiered framework	Development of a 3-tiered strategic framework for public health investments in sequencing and bioinformatics within DoD	Prioritizes limited resources to maintain (or expand) genomic surveillance capabilities
Coordination meetings	Regular meetings for GEIS program, DoD, and non-DoD stakeholders	Provides better coordination across a diverse set of stakeholders
Funding	Leveraging diverse DoD and US government funding streams from public health to biodefense, biosecurity, and pandemic response(s)	Maintains genomic surveillance capabilities
Routine assessments	Continuous assessment of NGS and bioinformatics capabilities through a variety of assessment tools (e.g., site visits, structured/unstructured surveys, and proficiency testing exercises)	Provides “ground truth” or validation of capabilities
Appropriate interventions	Deployment of interventions (e.g., equipment, protocols, training, and reach back testing) to address identified gaps	Maintains genomic surveillance capabilities and equipment/personnel readiness
Tracking products	Tracking of genomic surveillance products (e.g., genomes produced/shared, publications, presentations, protocols developed, and technical assistance provided)	Demonstrates return on investments in genomic surveillance and potential effects on public health
Communication with leadership	Providing senior leadership with regular updates on findings and impacts from genomic surveillance	Improves public health decision-making

*DoD, Department of Defense; GEIS, Global Emerging Infections Surveillance; NGS, next-generation sequencing.

surveillance readiness for infectious disease surveillance programs is in a precarious position. However, to maintain DoD genomic surveillance readiness for emerging biothreat response, funding for routine genomic surveillance programs such as seasonal respiratory pathogens or antimicrobial resistance is critical. In addition to funding, a reliable supply of samples for sequencing must be available, along with consistent, well-organized sample metadata that aid interpretation and analysis. The routine genomic surveillance activities that occur during the intermission between epidemics prevent erosion of capabilities and preserve existing infrastructure for the next pandemic response.

Conclusions

During the past decade, GEIS used medical research and biodefense investments in sequencing technologies to build and maintain genomic surveillance capabilities within the DoD (*32*,*33*). Sustained progress has been possible because of a dedicated program office and a consortium of global partner laboratories with genomics expertise. The Consortium implemented a tiered framework to support NGS and bioinformatics capabilities within the network based on level of laboratory operations, with technical assistance and support to lower tiers (*15*). During the COVID-19 pandemic, Consortium laboratories responded quickly by adopting new protocols and expanding sequencing capabilities in support of DoD public health, medical countermeasure development and monitoring, and pandemic response. The GEIS program prioritization of genomic surveillance has led to a DoD network more prepared to respond to future epidemics and emerging pathogens.

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Etiology and Epidemiology of Travelers' Diarrhea among US Military and Adult Travelers, 2018–2023

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Travelers' diarrhea has a high incidence rate among deployed US military personnel and can hinder operational readiness. The Global Travelers' Diarrhea study is a US Department of Defense–funded multisite surveillance effort to investigate the etiology and epidemiology of travelers' diarrhea. During 2018–2023, we enrolled 512 participants at partner institutions in 6 countries: Djibouti, Georgia, Egypt, Honduras, Nepal, and Peru. Harmonized laboratory methods conducted at each partner institution identified ≥1 pathogens, including *Escherichia coli* (67%–82%), norovirus (4%–29%), and *Campylobacter jejuni* (2%–20%), in 403 (79%) cases. Among cases, 79.7% were single infections, 19.6% were double infections, and 0.7% were triple infections. The most common enterotoxigenic *E. coli* colonization factors identified were CS3 (25%) and CS21 (25%), followed by CS2 (18%) and CS6 (15%). These data can inform best treatment practices for travelers' diarrhea and support US military health readiness.

Travelers' diarrhea (TD) is a gastrointestinal (GI) illness that affects millions of people each year, and infection rates range from 30% to 70% among travelers within 2 weeks of travel initiation, depending upon geographic region and seasonality of travel (*1*,*2*). Symptoms can range from mild cramps and loose stool to bloody diarrhea, fever, abdominal pain, and vomiting. Bacterial pathogens are the leading causative agents of TD, accounting for >80% of cases (*1*).

In March 2023, the US Military Infectious Disease Research Panel's Threat Prioritization Panel determined that bacterial diarrhea was the number 1 infectious disease threat to US military operations (*3*). In 2013, the Global Emerging Infections Surveillance branch, in collaboration with its worldwide network of partner laboratories and the Naval Health Research Center's Operational Infectious Diseases Directorate, launched the Global Travelers' Diarrhea (GTD) study to address issues posed to the US military by TD (*4*,*5*). This article describes the epidemiology of TD cases among US military populations and adult travelers during 2018–2023. In addition, this article characterizes coinfections, bacterial virulence factors, and pathogen factors relevant for medical countermeasure development.

Material and Methods

Partner Institutions and Enrollment Sites

Partner institutions included the Walter Reed Armed Forces Research Institute of Medical

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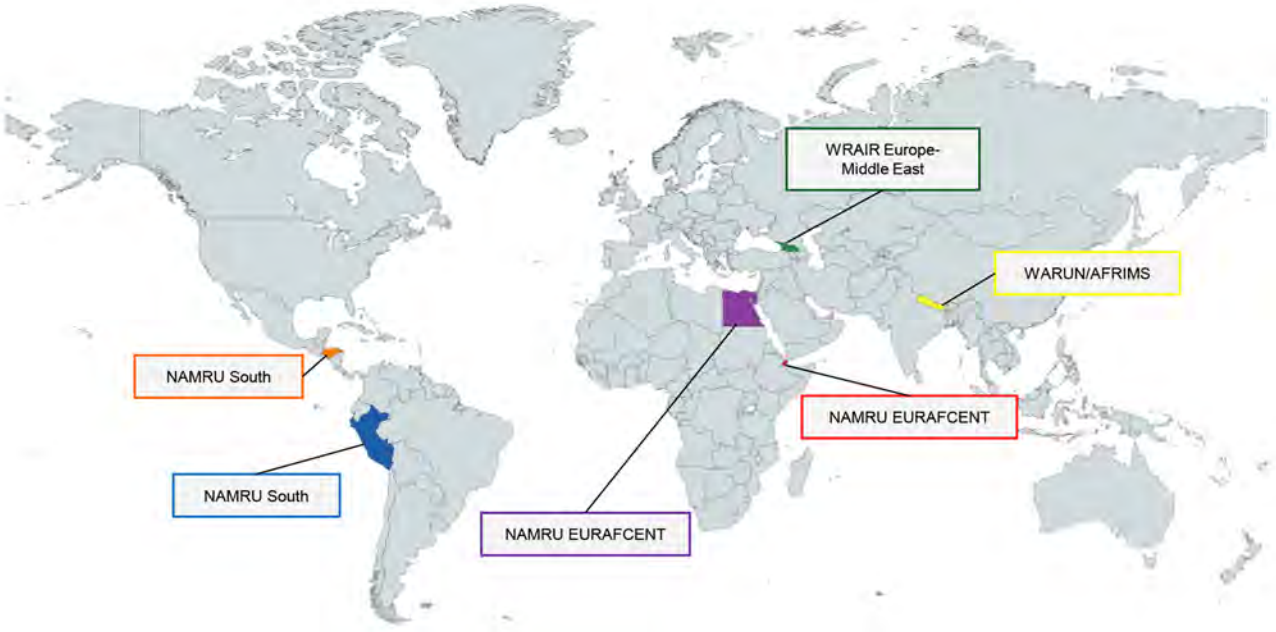
Sciences Research Unit Nepal located in Kathmandu, Nepal, which had enrollment sites in Kathmandu and Pokhara, Nepal; US Naval Medical Research Unit EURAFCENT detachment in Cairo, Egypt, which had enrollment sites in South Sinai Governorate, Egypt, and Djibouti City, Djibouti; US Naval Medical Research Unit SOUTH located in Lima, Peru, which had enrollment sites in Cusco, Peru, and Comayagua, Honduras; and Walter Reed Army Institute of Research Europe-Middle East located in Tbilisi, Georgia, which had enrollment sites in Tbilisi, Batumi, and Gardabani, Georgia (Figure 1).

Participant Enrollment

Each partner institution was required to have institutional review board approval before collecting GTD Study–associated samples. Upon determination of eligibility, participants signed an informed consent agreement, except at the US Naval Medical Research Unit EURAFCENT; its local institutional review board did not require informed consent after January



Partner institution	Enrollment Site	Location
NAMRU SOUTH	1) Joint Task Force Bravo-Soto	1) Comayagua, Honduras
	2) Amauta Spanish Language School	2) Cusco, Peru
NAMRU EURAFCENT	1) Multinational Force and Observers (MFO) South Camp	1) South Sinai Governorate, Egypt
	2) Camp Lemonnier	2) Djibouti City, Djibouti
WRAIR Europe-Middle East	1) MediClub Georgia	1) Tbilisi, Georgia
	2) Research Institute of Medical Parasitology and Tropical Medicine	2) Tbilisi, Georgia
	3) Batumi Salikh Abashidze Infectious Diseases, AIDS & Tuberculosis Regional Center	3) Batumi, Georgia
	4) Vaziani Military Base	4) Gardabani, Georgia
WARUN/AFRIMS	1) CIWEC Clinic	1) Kathmandu, Nepal
	2) CIWEC Clinic	2) Pokhara, Nepal

Figure 1. Partner institutions and enrollment site locations for a study of etiology and epidemiology of travelers' diarrhea among US military personnel and adult travelers, 2018–2023. The US Naval Medical Research Unit (NAMRU) south enrollment sites: orange, Comayagua, Honduras; blue, Lima, Peru. The US Naval Medical Research Unit EURAFCENT detachment enrollment sites: purple, Cairo, Egypt; red, Djibouti City, Djibouti. Walter Reed Army Institute of Research (WRAIR) Europe-Middle East enrollment sites: green, Tbilisi, Batumi, and Gardabani, Republic of Georgia. Walter Reed Armed Forces Research Institute of Medical Sciences Research Unit Nepal (WARUN/AFRIMS) enrollment sites: yellow, Kathmandu and Pokhara, Nepal.

2022. Study participation was voluntary. The study period was October 2018–April 2023.

Inclusion and Exclusion Criteria

The study inclusion criteria were adult travelers, ≥18 years of age, originating from the United States or any other upper-middle or high-income country (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0308-App1.pdf) (*6*), who had been in the enrollment country for <1 year, seeking healthcare services for acute GI illness. We defined illness as either acute diarrhea or acute gastroenteritis beginning ≥3 days after home departure. Adult travelers for this study included US military personnel, government employees, and citizens (e.g., nongovernmental organization workers, tourists, students, etc.). Patients were excluded from the study if they were experiencing chronic, persistent GI symptoms of ≥7 days before enrollment or noninfectious diarrhea or could not produce a fecal sample.

Case

The GTD study defined acute diarrhea as ≥3 loose/liquid feces, or ≥2 loose/liquid feces plus ≥2 additional GI symptoms. Case definitions for acute gastroenteritis were ≥3 vomiting episodes plus ≥1 additional GI symptom, or ≥2 vomiting episodes plus ≥2 additional GI symptoms occurring within the past 24 hours. Additional GI symptoms include diarrhea, vomiting, nausea, flatulence, cramping, muscle aches, headache, decreased urination, loss of appetite, bloating, abdominal pain, joint aches, malaise, fatigue, fever, or bloody feces.

Questionnaire

We assisted participants with completing a structured, standardized questionnaire. The questionnaire included questions about data variables describing demographics, symptoms, travel history, disposition, functional abilities, and treatment received.

Sample Receiving and Processing

Fecal specimens were stored as raw feces, in Cary-Blair (CB) medium, or feces in Cary-Blair with indicator (CBI) transport medium at 4°C for a maximum of 48 hours before transportation at 4°C to the GTD partner laboratory to perform testing. The partner laboratory assessed fecal specimens to verify appropriate temperature. Specimens that were frozen or room temperature were discarded and excluded from the study. Specimens received as raw feces were processed to a 20% weight/volume solution in phosphate-buffered saline before downstream testing, and specimens received in CB or CBI transport medium were directly used for downstream testing. Samples were either tested immediately upon arrival or frozen at –80°C and batch tested later.

Nucleic Acid Extraction and PCR

We extracted total nucleic acid from 20% fecal suspensions, fecal suspensions in CB or CBI, or boil prepped bacterial suspensions by using the QIAamp Viral RNA Mini kit (QIAGEN, https://www.qiagen.com). We used a total nucleic acid template for real-time PCR (rPCR) assays. We chose gene targets based on previously published literature (*7*–*10*).

We conducted real-time reverse transcription PCR (rRT-PCR) specific for norovirus genogroup I and II (GI and GII) by using the AgPath One Step RT-PCR kit (Ambion-Thermo Fisher Scientific, https://www.thermofisher.com) (*7*). We conducted rPCR specific for bacteria to detect the following organisms: *E. coli* pathotypes including enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), shiga-like toxin–producing *E. coli* (STEC), and enteroinvasive *E. coli* (EIEC)/*Shigella* (*8*,*9*); *Campylobacter jejuni*, including subspecies *jejuni* and *doylei* (*10*); and *Salmonella enterica* ssp. *enterica* (*10*). We conducted rPCR specific for ETEC colonization factors on total nucleic acid extracted from isolated colonies (*9*). We used the PerfeCTa qPCR Tough Mix Kit (Quantabio, https://www.quantabio.com) for DNA amplification in all bacterial PCR assays. Primer and probe sequences, PCR conditions, and multiplex assay details are available (Appendix Tables 2, 3). We conducted all PCR reactions by using the Applied Biosystems 7500 Fast Dx or 7500 Fast Real-Time PCR instruments (Thermo Fisher Scientific, https://www.thermofisher.com).

Bacteriology

We subcultured samples that were positive for ETEC by rPCR onto MacConkey agar and incubated them at 35–37°C for 18–24 hours (*11*). A laboratory testing schematic is available (Appendix Figure 1).

Coinfection Analysis and Data Management

We analyzed coinfections by 4 major pathogen groups: norovirus (GI and GII), *E. coli* (EAEC, ETEC, EPEC, STEC, and EIEC/*Shigella*), *Salmonella*, and *Campylobacter*. We then cleaned the deidentified questionnaire and laboratory testing data by using Excel (Microsoft, https://www.microsoft.com) or Tableau Desktop version 2023.3 (Tableau, https://www.tableau.com). We merged questionnaire data and laboratory data in Excel by using participant identification numbers as the linking identifier.

Table 1. Characteristics among acute diarrhea and acute gastroenteritis cases by geographic region and country, reported by participants in a study of etiology and epidemiology of travelers' diarrhea among US military personnel and adult travelers, 2018–2023*

	Geographic region and country						
	South and Central America		Northern Africa	Sub-Saharan Africa	Southern Asia	Eastern Europe	
Characteristics	Honduras	Peru	Egypt	Djibouti	Nepal	Georgia	Total
Enrollments	107 (21)	15 (3)	17 (3)	200 (39)	133 (26)	40 (8)	512 (100)
Average age, y (SD)	34 (9)	NA	33 (6)	35 (11)	33 (14)	38 (16)	34 (12)
Unknown age	13 (12)	15 (100)	0	132 (66)	0	2 (5)	162 (32)
Sex							
F	26 (24)	10 (67)	3 (18)	38 (19)	84 (63)	17 (43)	178 (35)
M	81 (76)	5 (33)	14 (82)	131 (66)	49 (37)	23 (58)	303 (59)
Unknown	0	0	0	31 (16)	0	0	31 (6)
Birth region							
East Asia	0	0	0	0	6 (5)	0	6 (1)
North America	100 (93)	5 (33)	17 (100)	64 (32)	37 (28)	6 (15)	229 (45)
Europe	2 (2)	10 (67)	0	2 (1)	71 (53)	26 (65)	111 (22)
Oceania	0	0	0	0	11 (8)	0	11 (2)
Middle East	0	0	0	0	1 (1)	1 (3)	2 (<1)
Unknown	5 (5)	0	0	134 (67)	7 (5)	7 (18)	153 (30)
Traveler type							
US military	105 (98)	0	17 (100)	200 (100)	0	7 (18)	299 (58)
Government, US or non-US	2 (2)	0	0	0	0	2 (5)	4 (1)
Nongovernmental organization or aid worker	0	0	0	0	9 (7)	2 (5)	11 (2)
Tourist	0	0	0	0	85 (64)	19 (48)	104 (20)
Student	0	15 (100)	0	0	10 (8)	1 (3)	26 (5)
Other	0	0	0	0	29 (22)	2 (5)	31 (8)
Unknown	0	0	0	0	0	7 (18)	37 (6)

*Values are no. (%), except as indicated. Percentages may not total 100% because of rounding.

Results

During October 2018–April 2023, a total of 512 participants who met the acute diarrhea or acute gastroenteritis case definitions were enrolled in the GTD study in Honduras (21%), Peru (3%), Egypt (3%), Djibouti (39%), Nepal (26%), and Georgia (8%) (Table 1). The average participant age was 34 (SD 12) years. Among participants, 35% were female, 59% male, and 6% unidentified sex. Participants were primarily born in North America (45%) or Europe (22%); however, that did not necessarily imply country of origin before travel and enrollment in the study. Most participants were US military service members (58%) or tourists (20%) (Table 1).

Across all sites, 403 (79%) of 512 samples tested positive for ≥1 pathogens, identifying a total of 867 pathogens (Table 2). Of the 403 positive samples, 79.7% were single infections, 19.6% were double infections, and 0.7% were triple infections. No samples were positive for all 4 pathogen groups (Table 2).

E. coli was the most common pathogen identified in Peru (67%), Nepal (77%), Georgia (75%), Honduras (69%), Egypt (82%), and Djibouti (70%), whereas *Salmonella* was the least identified in all countries except Egypt (6%) and Djibouti (6%) (Figure 2). Coinfection analysis identified *E. coli* in a higher number of coinfections than any of the

Table 2. Positivity and pathogen co-infections recovered from participants, by geographic region and country, in a study of etiology and epidemiology of travelers' diarrhea among US military personnel and adult travelers, 2018–2023*

	Geographic region and country						
	South and Central America		Northern Africa	Sub-Saharan Africa	Southern Asia	Eastern Europe	
Sample positivity	Honduras, n = 107	Peru, n = 15	Egypt, n = 17	Djibouti, n = 200	Nepal, n = 133	Georgia, n = 40	Total, n = 512
Positive samples†	80 (75)	12 (80)	15 (88)	146 (73)	119 (89)	31 (78)	403 (79)
Single infections	71 (89)	7 (58)	15 (100)	130 (89)	75 (63)	23 (74)	321 (80)
Double infections	8 (10)	5 (42)	0	15 (10)	43 (36)	8 (26)	79 (20)
Triple infections	1 (1)	0	0	1 (1)	1 (1)	0	3 (1)
Total no. pathogens‡	178	26	28	288	276	71	867

*Values are no. (%), except as indicated. Percentages are of total positive samples, not samples tested. Samples cannot be positive for both Shiga-like toxin–producing *Escherichia coli* and enteropathogenic *E. coli* because the *eae* gene used to identify enteropathogenic *E. coli* can be found in Shiga-like toxin–producing *E. coli*; therefore, samples that tested positive for *stx*1/2 alone or in combination with *eae* were resulted as Shiga-like toxin–producing *E. coli*, and samples that tested positive for *eae* without *stx*1/2 were resulted as enteropathogenic *E. coli*. All other *E. coli* pathotype combinations were possible to identify.

†Samples positive for ≥1 of the following pathogens: norovirus genogroup I, norovirus genogroup II, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, Shiga-like toxin–producing *E. coli*, enteroinvasive *E. coli*/*Shigella*, *Salmonella*, and *Campylobacter*.

‡Only of pathogens tested for in this study.

other 3 pathogen groups across all 6 countries (Appendix Figure 2).

ETEC colonization factors were identified from ETEC isolates recovered from samples collected in 3 countries: Honduras, Djibouti, and Nepal (Table 3). In total, 106 isolates were tested. The most identified ETEC colonization factors were CS3 (25%) and CS21 (25%), as well as CS2 (18%) and CS6 (15%) (Table 3). The least identified ETEC colonization factors were CS17/19 (1%) and CS14 (2%). Colonization factors CS5 and CS7 were not identified from any country (Table 3).

Conclusions

This study describes the etiology and epidemiology of TD among US military and adult civilian travelers across South and Central America, Northern and sub-Saharan Africa, Southern Asia, and Eastern Europe. We found *E. coli* was the leading (67%–82%) etiology of TD across global surveillance sites (Figure 2). ETEC was the most identified *E. coli* pathotype in 5 of 6 countries (Figure 2). Our investigation also identified *Campylobacter*, *Salmonella*, and norovirus as TD etiologies, although with lower proportions than observed for *E. coli* (Figure 2). Those data on TD disease etiology are consistent with the literature discussing both military and civilian populations throughout the globe, indicating bacterial pathogens are the leading causative agents of disease (*4*,*12*–*15*). Our study data suggest *E. coli*, specifically pathotypes ETEC, EAEC, and EPEC, are the leading causes of TD in Southern and Central Asia, Northern Africa, the Middle East, sub-Saharan Africa, and Central and South America (Figure 2), which is consistent with the GTD study data for all 6 surveillance countries (*12*).

The highest rates of *Campylobacter* and *Salmonella* associated with TD are found in Southeast and East Asia, and high rates are also found in Southern and Central Asia (*12*). The GTD study did not include a surveillance site in Southeast or East Asia, but among included countries, we identified the highest rates of *Campylobacter* in Peru (20%) and Nepal (16%), and the highest rates of *Salmonella* in Egypt (6%) and Djibouti (6%) (Figure 2).

Previous work by the GTD study found *E. coli* (including all pathotypes tested for) in 42% of the cases enrolled during 2013–2018 (n = 410) (*4*), which is lower than the cases enrolled during 2018–2023 (n = 512; 72%) (Figure 2). That increase may represent improved laboratory diagnostic methods for *E. coli* pathotypes; samples collected during 2013–2018 were analyzed by conventional PCR, whereas samples collected during 2018–2023 were analyzed by rPCR. Of note, case numbers of *Salmonella* and *Campylobacter* were similar across study periods despite updates to laboratory protocols (Figure 2) (*4*).

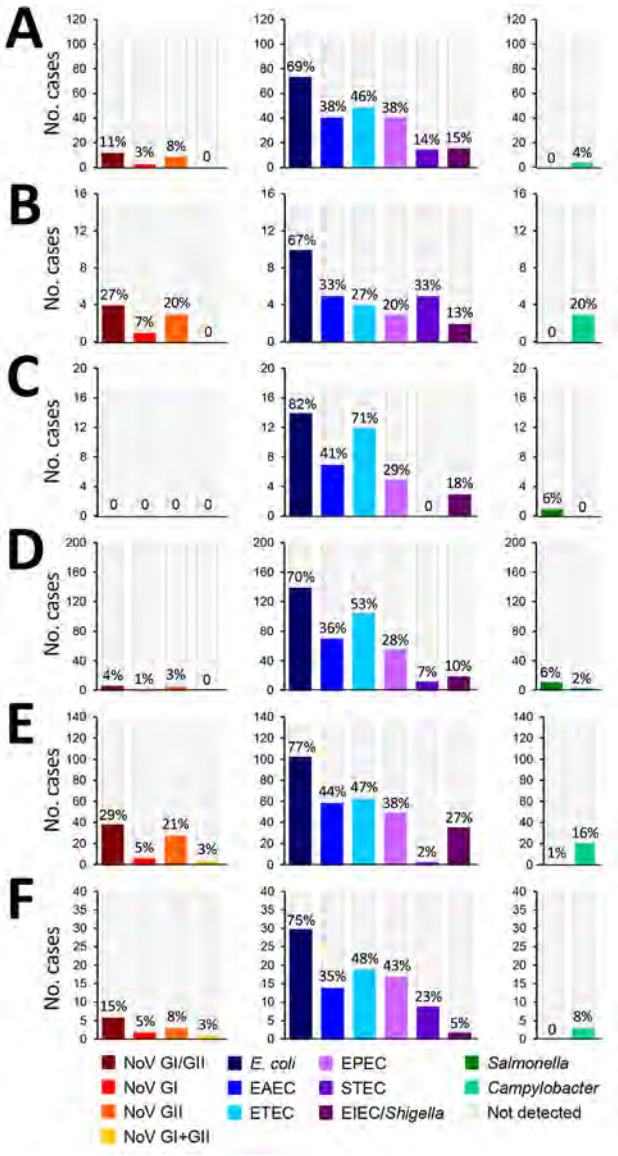


Figure 2. Enteric pathogens detected in a study of etiology and epidemiology of travelers' diarrhea among US military personnel and adult travelers, 2018–2023. The study included travelers from the following countries: A) Honduras (n = 107); B) Peru (n = 15); C) Egypt (n = 17); D) Djibouti (n = 200); E) Nepal (n = 133); F) Georgia (n = 40). We consider the proportions from Peru, Egypt, and Georgia to be unstable (n ≤ 40) and the results should be interpreted with caution. The y-axis and bars represent the number of times each pathogen was detected for each country. The percent positivity for each pathogen is listed above the corresponding bar in each graph. Percent positivity does not add up to 100% for each country because of the occurrence of coinfections. EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*/*Shigella*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; g, genogroup; NoV, norovirus; STEC, Shiga-like toxin–producing *E. coli*.

Table 3. Enterotoxigenic *Escherichia coli* colonization factors identified in pathogens recovered from participants of a study of etiology and epidemiology of travelers' diarrhea among US military personnel and adult travelers, by geographic region and country, 2018–2023*

	Geographic region and country			
	South/Central America	Sub-Saharan Africa	Southern Asia	
Factors	Honduras, n = 32	Djibouti, n = 37	Nepal, n = 37	Total, n = 106
CFA/I	3 (9)	0	0	3 (3)
CS4	0	2 (5)	2 (5)	4 (4)
CS6	3 (9)	7 (19)	6 (16)	16 (15)
CS14	0	1 (3)	1 (3)	2 (2)
CS1/PCF071	2 (6)	0	6 (16)	8 (8)
CS2	4 (13)	10 (27)	5 (14)	19 (18)
CS17/19	1 (3)	0	0	1 (1)
CS21	9 (28)	11 (30)	7 (19)	27 (25)
CS3	10 (31)	6 (16)	10 (27)	26 (25)
CS5	0	0	0	0
CS7	0	0	0	0

*Values are no. (%) enterotoxigenic *E. coli* samples from each country.

Vaccines used prophylactically to prevent TD have the potential to reduce disease incidence and severity; however, no vaccines for *E. coli*, *Campylobacter*, or *Shigella* are currently licensed by the US Food and Drug Administration. ETEC vaccine candidates currently under investigation are based on antitoxin or anticolonization factor immunity. Approximately 50%–80% of all colonization factor-positive clinical ETEC isolates found within the general population encode colonization factors A/I, CS3, CS5, and CS6 (*16*), making them potential vaccine targets (*17*,*18*). In this study, CS6, CS3, CS2, and CS21 were the most identified colonization factors across the geographic regions tested; however, colonization factor A/I was only identified in 3% of isolates and CS5 was not identified in any isolate (Table 3). Our results combined with the efforts of the Global Emerging Infections Surveillance network of global surveillance laboratories, including maintaining repositories of clinical samples collected throughout the world, may help guide future vaccine development and therapeutics for TD and other diseases of interest to the US military and global health.

One limitation of this study is that we considered the disease etiology proportions from Egypt (n = 17), Peru (n = 15), and Georgia (n = 40) unstable because of low enrollment; we recommend interpreting the results with caution. In addition, selection bias may have influenced results because of site accessibility and the potential that travelers with mild TD are less likely to seek care than are travelers with severe TD, which might have affected the pathogens observed.

Moving forward, we recommend the GTD study expand to include antimicrobial resistance (AMR) characterization of bacterial pathogens identified from TD cases by using antimicrobial susceptibility testing and next-generation sequencing technologies to identify genetic markers of AMR and virulence factors of enteric bacterial pathogens. Those combined efforts could provide insight on the effects of AMR across unique global regions, enhance antimicrobial stewardship to limit changes in drug resistance patterns in enteric pathogens, and improve military health readiness through targeted prevention and treatment interventions for TD.

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Respiratory Disease Surveillance in the Middle East and Latin America during the COVID-19 Pandemic, 2020–2022

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Characterizing the epidemiology of circulating respiratory pathogens during the COVID-19 pandemic could clarify the burden of acute respiratory infections and monitor outbreaks of public health and military relevance. The US Department of Defense supported 2 regions for influenza-like illness and severe acute respiratory infections surveillance, one in the Middle East through US Naval Medical Research Unit EURAFCENT, and another in Latin America through US Naval Medical Research Unit SOUTH. During 2020–2022, coinciding with the COVID-19 pandemic, we collected a total of 16,146 nasopharyngeal and oropharyngeal swab samples from sentinel sites in Jordan (n = 11,305) and Latin America (n = 4,841). Samples were tested for SARS-CoV-2, influenza, and other respiratory pathogens. SARS-CoV-2 was the most frequently detected pathogen during 2020; other respiratory pathogens had distinct temporal and frequency distributions according to geographic location. Our findings support the need for continued sentinel surveillance as a vital tool for assessing the burden of respiratory diseases globally.

Acute respiratory infection (ARI) surveillance is a tool to monitor shifts in the occurrence and burden of respiratory infections in a population. Since the 2009 influenza pandemic, the World Health Organization (WHO) has recommended implementation of 2 sentinel surveillance programs: the influenza-like illness (ILI) and the severe acute respiratory infection (SARI) programs (*1*). Such programs allow countries and public health authorities to monitor circulating respiratory pathogens, and to understand the seasonality and trends of pathogens, especially those of pandemic potential, such as influenza and coronaviruses (*2*).

Since 2020, the COVID-19 pandemic and public health implementation of preventive measures have had broad effects on persons, communities, and governments. Given that preventive measures were designed

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to mitigate respiratory virus transmission, notable disruptions to the typical seasonal circulation patterns of common respiratory virus infections, including infections caused by influenza viruses, respiratory syncytial virus (RSV), and others, have been reported globally (*3*).

We describe the changing temporal and geographic pattern of respiratory pathogens in 2 regions using respiratory surveillance programs through the US Naval Medical Research Unit (NAMRU EURAFCENT) in the Middle East and US Naval Medical Research Unit SOUTH (NAMRU SOUTH) in Latin America. Jordan was selected as a representative of the Middle East region because it was the only country among the region that was able to sustain its SARI and ILI surveillance during the pandemic despite all the challenges the healthcare system faced during the study period.

The institutional review board of the US Naval Medical Research Command approved protocol nos. NAMRU3.2007.0003, N3 703, and NAMRU3.PJT.20.0001. The institutional review board of NAMRU SOUTH approved protocol nos. NMRCD.2010.0010, NAMRU6.2011.0012, NAMRU6.2012.0011, NAMRU6.2012.0012, and NAMRU6.2018.0003, in compliance with all applicable federal regulations governing the protection of human subjects.

Materials and Methods

Surveillance Sites

The Jordanian Ministry of Health (JMoH) determined ILI and SARI surveillance programs in Jordan as national public health surveillance activities under JMoH responsibility. NAMRU EURAFCENT initiated a collaboration with the JMoH in ILI surveillance activities in 2006 and SARI surveillance in 2010. JMoH supervised all sentinel sites, including SARI (n = 4) and ILI (n = 4) sites. Enrollment was limited to civilian populations who met ILI or SARI case definitions. Surveillance sites in Latin America were in Panama (n = 1), Guatemala (n = 1), Honduras (n = 1), Colombia (n = 7), Peru (n = 22), and Paraguay (*1*) (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0303-App1.pdf). For the 6 countries, ILI and SARI surveillance activities were conducted in hospitals and clinics where local and US military and civilian populations received medical care.

In Jordan, after the first COVID-19 case was reported in March 2020, SARI surveillance continued to enroll hospitalized cases as hospitals continued to provide medical care. ILI activities were paused because primary health centers closed during the country lockdown; activities resumed in October 2020. All NAMRU SOUTH surveillance activities in Latin America stopped in March 2020 because of the lockdowns and were resumed progressively, beginning in Honduras in June 2020 and the remaining countries in November 2020.

Case Definition and Specimen Collection

Respiratory specimens were collected from patients of all ages (except patients <31 days of age in Jordan) who met the ILI or SARI case definition at any of the surveillance sites. SARI and ILI shared the same clinical manifestation; for SARI cases, the need for hospitalization was an additional indicator for severity. JMoH, in collaboration with NAMRU EURAFCENT, used the WHO ILI and SARI case definitions (*4*). NAMRU SOUTH in Latin America used modified WHO ILI and SARI case definitions and a specific case definition for COVID-19 that did not consider fever as a requirement for specimen collection (*5*) (Appendix Table 2). The 2 regions had similar enrollment procedures in which each eligible case was issued a unique study code that was used to link the clinical and epidemiologic information with laboratory data.

Respiratory samples were collected in viral transport medium using nasopharyngeal and oropharyngeal swabs (*4*,*6*). In Jordan, samples were shipped once a week to the Central Public Health Laboratory (Amman, Jordan). In Latin America, specimens were tested at each field site and then shipped to NAMRU SOUTH headquarters (Lima, Peru) for further molecular analysis.

Laboratory Testing

In Latin America, NAMRU SOUTH tested all samples at field sites using the BioFire FilmArray System Respiratory Panel 2.1 (RP 2.1; bioMérieux, https://www.biomerieux.com), which detects 22 respiratory pathogens at 97.1% sensitivity and 99.3% specificity, including SARS-CoV-2. In Jordan, JMoH and NAMRU EURAFCENT used the Fast Track Diagnostics Respiratory Pathogens 33 (FTD-33; Siemens Healthineers, https://www.siemens-healthineers.com) assay, which has 76% sensitivity and 97% specificity (*7*). JMoH tested ILI cases only for influenza and SARS-CoV-2 during October 2020–January 2021. JCPHL tested influenza using singleplex CDC methods (*8*), then the FTD-33 assay. SARS-CoV-2 was tested using TaqPath COVID-19 CE-IVD RT-PCR Kit (Applied Biosystems, https://www.thermofisher.com). In Jordan and Latin America, results were reported daily or weekly to local ministry of health offices, public health authorities, and NAMRU SOUTH or NAMRU EURAFCENT.

Data Analysis

We conducted a descriptive analysis for which we calculated 95% CIs using a binomial distribution.

We used Stata version 16 software (StataCorp LLC, https://www.stata.com) for statistical analyses. We assessed frequency distribution of pathogens as number of specific pathogens over total number of common pathogens between FTD-33 and BioFire FilmArray RP 2.1 tests. We described trends of seasonality for influenza, SARS-CoV-2, RSV, and rhinovirus/enterovirus (RV/EV) as the monthly proportion of positive samples among total samples tested.

Results

Patient Demographics

A total of 11,305 samples were collected in Jordan (2,782 from ILI cases and 8,523 from SARI cases) and 4,841 in Latin America (4,154 from ILI cases and 687 from SARI cases). Most (50.1% in Jordan and 51.7% in Latin America) samples were collected during 2021 (Table 1). ILI cases were concentrated in the 16–50 year age group (58.7%) in both regions, in contrast to SARI cases, which were reported more (68.9%) in younger persons (<16-years age group) in Latin America, and more (44.7%) in older persons (>50-year age group) in Jordan (Tables 2, 3).

Circulating Pathogens

Of 11,305 tested samples from Jordan, 46% (5,204) were positive for ≥1 respiratory pathogen; of the 4,841 samples from Latin America, 75.1% (3,637) were positive. Pathogen positivity was higher in Latin America for both SARI (83.1%) and ILI surveillance (73.8%) than in Jordan (SARI 47.8% and ILI 40.5%). The rate of monodetection, the detection of a single pathogen, in SARI was similar in both regions, 65.3% in Latin America and 67.4% in Jordan; in contrast, monodetections among ILI cases were significantly higher (85.1%) in Latin America than in Jordan (64.0%) (Table 1). In addition, a total of 4,428 pathogens were identified in Latin America and 7,994 in Jordan, of which 3,738 pathogens were common targets identified by the tests performed. Frequency distribution of those pathogens tested in both regions showed that SARS-CoV-2 was the most recurrently detected respiratory pathogen in both regions, 43.7% in Jordan and 27.7% in Latin America; RV/EV was next most detected at 17.5% in Jordan and 24.7% in Latin America (Figure 1).

Patterns of Seasonality

During the surveillance period, 4 peaks of SARS-CoV-2 were reported in Jordan (September 2020, March 2021, February 2022, and August 2022) and 3 peaks in Latin America (March 2021, February 2022, and July 2022). Temporal pathogen distribution by region showed that influenza virus was not detected until October 2021 in Jordan and November 2021 in Latin America. During the influenza silent period, March 2020–November 2021, pathogens, including SARS-CoV-2, RSV, and RV/EV continued to circulate (Figures 2, 3).

Discussion

Globally, circulation of respiratory viruses was disrupted during the COVID-19 pandemic; the magnitude, timing, and duration of the disruption varied across viruses and geographic locations. We found that even though Jordan used an extended panel that detected 33 pathogens in addition to PCR for influenza and SARS-CoV-2, the positivity rates were much higher for both SARI (83% vs. 48%) and ILI (74% vs. 41%) in Latin

Table 1. Characteristics of samples collected as part of respiratory disease surveillance in the Middle East and Latin America during the COVID-19 pandemic, 2020–2022*

Characteristics	Jordan		Latin America	
	SARI, n = 8,523	ILI, n = 2,782	SARI, n = 687	ILI, n = 4,154
Year of enrollment				
2020	909 (11)	137 (5)	3 (0.4)	44 (1.1)
2021	4,408 (52)	1,251 (45)	252 (37)	2,250 (54)
2022	3,206 (38)	1,394 (50)	432 (63)	1,860 (45)
Total samples tested				
Influenza	8,523 (100)	2,782 (100)	687 (100)	4,154 (100)
SARS-CoV-2	8,144 (96)	2,782 (100)	687 (100)	4,154 (100)
Other respiratory pathogens	8,147 (96)	2,467 (89)	687 (100)	4,154 (100)
Detections				
No detections	4,446 (52.2)	1,655 (59.5)	116 (16.9)	1,088 (26.2)
Positive, % of positive samples	4,077 (47.8)	1,127 (40.5)	571 (83.1)	3,066 (73.8)
Monodetections	2,661 (65.3)	721 (64.0)	385 (67.4)	2,609 (85.1)
Codetections	1,416 (34.7)	406 (36.0)	186 (32.6)	457 (14.9)
2 pathogens	888 (21.8)	255 (22.6)	139 (24.3)	388 (12.7)
3 pathogens	341 (8.4)	104 (9.2)	36 (6.3)	56 (1.8)
>3 pathogens	187 (4.6)	47 (4.2)	11 (1.9)	13 (0.4)

*Samples were tested by Fast-Track Diagnostics 33 (FTD-33; Siemens Healthineers, https://www.siemens-healthineers.com) panel in Jordan and by BioFire FilmArray Respiratory Panel 2.1 (RP 2.1; bioMérieux, https://www.biomerieux.com) in Latin America. Values are no. (%). ILI, influenza-like illness; SARI, severe acute respiratory illness.

Table 2. Characteristics of ILI case-patients detected as part of respiratory disease surveillance in the Middle East and Latin America during the COVID-19 pandemic, 2020–2022*

Characteristic	No. case-patients (% [95% CI])	
	Jordan	Latin America
Total ILI cases	2,782	4,154
Sex		
M	1,281 (46 [43.3–48.7])	2,144 (51.6 [49.5–53.2])
F	1,501 (54 [51.3–56.7])	2,010 (48.4 [46.3–50.5])
Age group, y		
<16	715 (25.7 [22.5–28.9])	1,088 (26.2 [23.6–28.8])
16–50	1,634 (58.7 [56.3–61.1])	2,437 (58.7 [56.8–60.7])
>50	433 (15.6 [12.2–19.0])	629 (15.1 [12.3–17.9])
Positive test result		
Influenza	113 (4.1 [3.4–4.8])	410 (9.9 [7.0–12.8])
SARS-CoV-2	273 (9.8 [8.7–10.9])	1,146 (27.6 [25.0–30.2])
RSV, n = 2,467	34 (1.4 [0.9–0.19])	304 (7.3 [4.4–10.2])
Other respiratory pathogens, n = 2,467	1,326 (53.7 [51.7–55.7])	1,747 (42.1 [39.8–44.4])

*ILI, influenza-like illness; RSV, respiratory syncytial virus.

America, which used FilmArray RP 2.1 to detect 22 pathogens including SARS-CoV-2 and influenza. Those findings could be explained by the case definitions used in each region. In Jordan, WHO ILI and SARI case definitions require fever and cough within the past 10 days in addition to hospitalization for SARI cases. In contrast, the modified WHO case definition used in Latin America included sore throat or rhinorrhea but limited fever onset to within the past 48 hours.

Influenza virus was not detected until October 2021 in Jordan and November 2021 in Latin America, as reported (*9*,*10*). In Jordan, influenza peak was detected during its usual pattern in December–January. In Latin America, however, influenza began to peak in November 2021, which was out of season for nontropical Latin America, where it typically peaks during June–August (*11*), and Central America, where it usually is present year-round (*12*). Influenza detection decreased globally but other noninfluenza pathogens, including SARS-CoV-2, were detected despite strict physical preventive measures and reductions in travel; that trend suggests different or more efficient transmission mechanisms or pathogen survival on surfaces (*13*).

Unlike influenza, SARS-CoV-2 peaks were observed in January–February 2022 in both regions, which could have been from the combination of the emergence of the Omicron variant and the global relaxing of preventive measures (*14*). In addition, ILI surveillance in Latin America revealed that during periods of decreased detection of SARS-CoV-2, the number of non–SARS-CoV-2–positive cases increased, suggesting a depletion of susceptible populations for SARS-CoV-2 and opportunities for non–SARS-CoV-2 pathogens to circulate. In Jordan, SARI data showed that during periods of intensive SARS-CoV-2 prevention measures, detection of other respiratory pathogens decreased (Figure 3). Like influenza virus and RSV, circulation of other respiratory pathogens, including seasonal human coronavirus, human parainfluenza virus, and human metapneumovirus, was notably lower at the onset of the COVID-19 pandemic when preventive measures were in place.

In Jordan during the 4 peaks of COVID-19, positivity of the other respiratory pathogens in SARI cases decreased (Figure 3), most likely because of the measures implemented in response to the increase in

Table 3. Characteristics of SARI case-patients detected as part of respiratory disease surveillance in the Middle East and Latin America during the COVID-19 pandemic, 2020–2022*

Characteristic	No. case-patients (% [95% CI])	
	Jordan	Latin America
Total SARI cases	8,523	687
Sex		
M	4,593 (53.9 [52.5–55.3])	368 (53.6 [48.5–58.7])
F	3,930 (46.1 [44.7–47.6])	319 (46.4 [41.5–51.8])
Age group		
<16	2,931 (34.4 [32.7–36.1])	473 (68.9 [64.7–73.0])
16–50	1,780 (20.9 [19.0–22.8])	123 (17.9 [11.1–24.7])
>50	3,812 (44.7 [43.1–46.3])	91 (13.2 [6.3–20.1])
Positive test result		
Influenza, n = 8,523	219 (2.6 [2.3–2.9])	52 (7.6 [4.0–14.8])
SARS-CoV-2, n = 8,144	1,361 (16.7 [15.9–17.5])	79 (11.5 [4.5–18.5])
RSV, n = 8,147	448 (5.5 [5.0–6.0])	134 (19.5 [12.8–26.2])
Other respiratory pathogens, n = 8,147	4,255 (52.2 [51.1–53.3])	556 (80.9 [77.6–84.2])

*ILI, influenza-like illness; RSV, respiratory syncytial virus; SARI, severe acute respiratory infection.

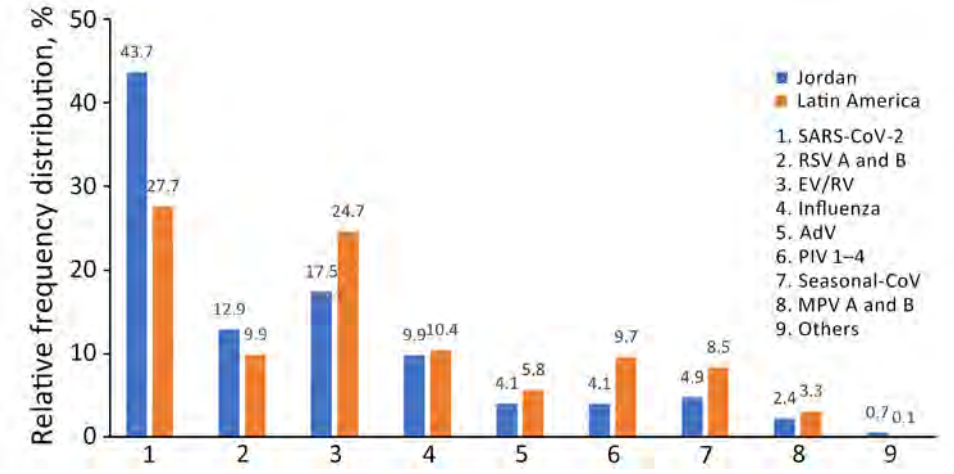

Figure 1. Overall relative frequency distribution of respiratory pathogens commonly tested as part of respiratory disease surveillance in the Middle East (Jordan) and Latin America during the COVID-19 pandemic, 2020–2022. Percentages were calculated relative to the total number of common targets tested by both Fast-Track Diagnostics 33 (FTD-33; Siemens Healthineers, https://www.siemens-healthineers.com) and BioFire FilmArray Respiratory Panel 2.1 (RP 2.1; bioMérieux, https://www.biomerieux.com) assays. Samples include 3,738 from Jordan and 4,4,28 from Latin America. Others includes *Chlamydia pneumoniae* (0.44% in Jordan and 0.07% in Latin America), *Bordetella pertussis* (0.1% in Jordan and 0.05% in Latin America), and *Mycoplasma pneumoniae* (0.17% in Jordan and 0.02% in Latin America). AdV, adenovirus; MPV, metapneumovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV/EV, rhinovirus/enterovirus.

COVID-19 cases. Our observation (data not shown) is consistent with other studies conducted in different regions of the world showing a significant decline in respiratory virus circulation during the COVID-19 pandemic as increased preventive measures were undertaken (Figures 2, 3).

For RSV, sporadic cases were detected in Jordan during July and December 2020, and in May and

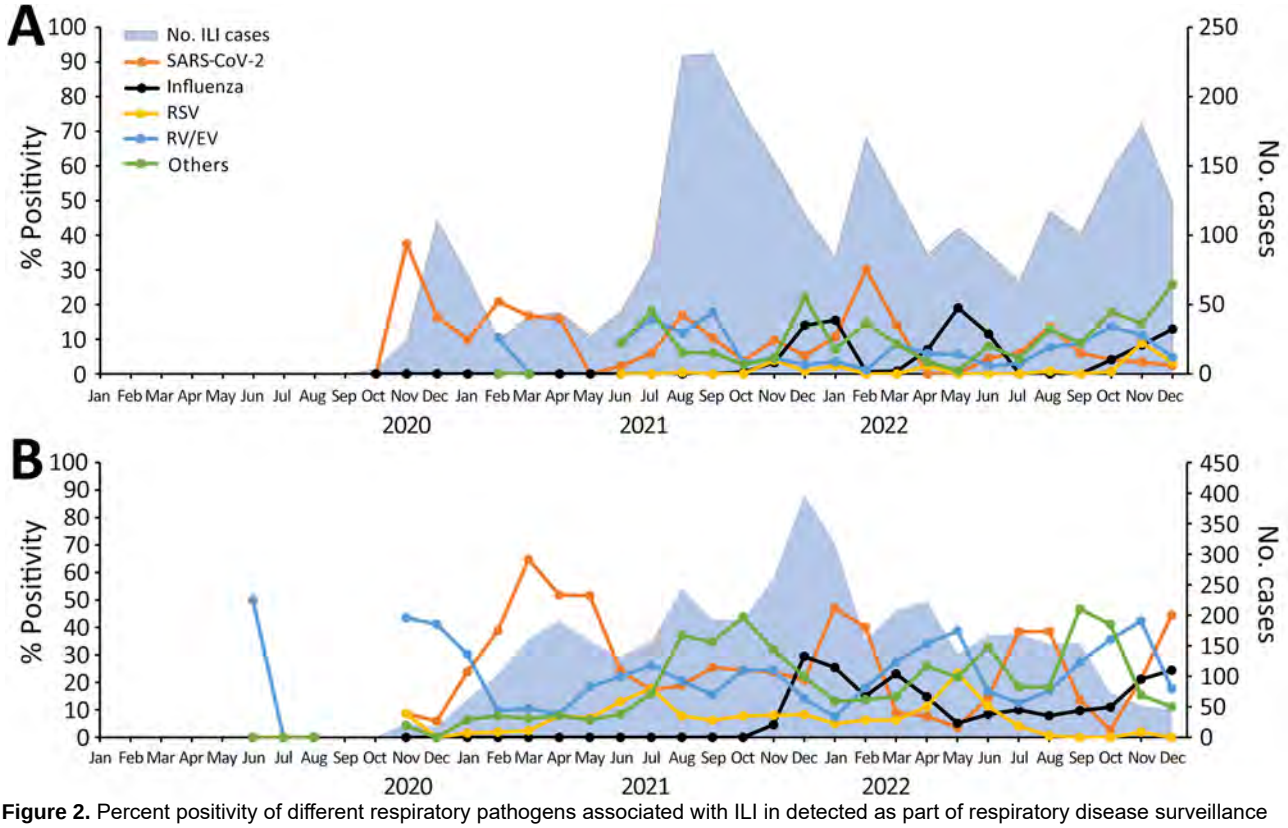

Figure 2. Percent positivity of different respiratory pathogens associated with ILI in detected as part of respiratory disease surveillance in the Middle East (Jordan, A) and Latin America (B) during the COVID-19 pandemic, 2020–2022. In Jordan, testing ILI cases with Fast-Track Diagnostics 33 (FTD-33; Siemens Healthineers, https://www.siemens-healthineers.com) started in February 2021. During October 2020–January 2021, ILI cases were tested only for influenza and SARS- CoV-2. Others includes *Chlamydia pneumoniae*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*. ILI, influenza-like illness; RSV, respiratory syncytial virus; RV/EV, rhinovirus/enterovirus.

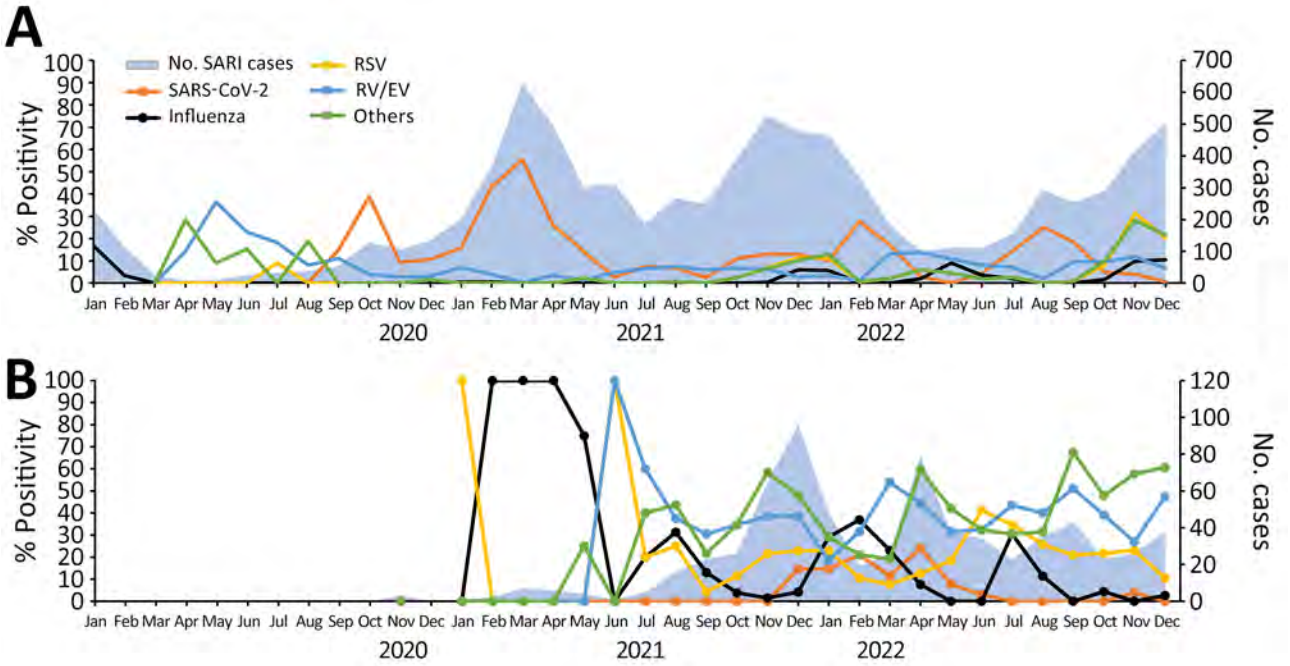

Figure 3. Percent positivity of different respiratory pathogens associated with SARI detected as part of respiratory disease surveillance in the Middle East (Jordan, A) and Latin America (B) during the COVID-19 pandemic, 2020–2022. Others includes *Chlamydia pneumoniae*, *Bordetella pertussis*, and *Mycoplasma pneumoniae*. ILI, influenza-like illness; RSV, respiratory syncytial virus; RV/EV, rhinovirus/enterovirus; SARI, severe acute respiratory infection.

August 2021, which is different than the winter season (December–February) peak typically seen in Jordan (*15*). Increased activity of RSV in Latin America was reported in June–July 2021 and May–June 2022; similar increases were reported in the United States later in October–November 2022 (*16*).

Although Latin America and Jordan surveillance sites were in different hemispheres, we observed a similar temporal pattern of SARS-CoV-2. Those similarities may have been driven by the introduction of new SARS-CoV-2 variants rather than seasonal factors (*17*).

We describe the results of surveillance systems in 2 different geographic locations that continued to function during the COVID-19 pandemic, despite the public health challenges during the global emergency. Moreover, expanding testing beyond SARS-CoV-2 and influenza viruses highlighted the role of other respiratory pathogens that add to the burden of the ILI and SARI and potential interactions that affect the epidemiology of respiratory infections.

Because we relied on passive surveillance, our study population was limited to patients seeking medical care at health centers, which may have changed during the pandemic. It is possible that, during COVID-19 waves, hospitalization criteria required for SARI changed. Those variables could explain why fewer other respiratory viruses were detected during COVID-19 waves. In addition, Latin America surveillance sites are predominantly for ILI surveillance; in Jordan, SARI cases were higher priority. Finally, our study did not address how differences in laboratory techniques and case definitions affected detection of respiratory pathogens.

In conclusion, our study showed that respiratory pathogens other than SARS-CoV-2 were circulating during the COVID-19 pandemic in 2020–2022. The expanded use of multiplex molecular respiratory virus assays and the increased awareness of the public health burden of respiratory pathogens have renewed interest in characterizing the epidemiology of respiratory viruses. As demonstrated during the COVID-19 pandemic, continued sentinel surveillance is vital for assessing the burden of respiratory diseases globally.

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Author contributions: M.S., Y.T., T.S.O., and B.H. conceived this manuscript. M.S., Y.T., T.S.O., and S.J.A. contributed to defining the research methodology. M.S., Y.T., T.S.O., and B.H. acquired and curated the data. T.S.O. conducted the formal analysis. M.S. and Y.T. wrote the first draft. J.S.A. and T.S.O. edited and reviewed the manuscript. M.S., Y.T., T.S.O., and W.D.G. supervised the research project. All authors approved the final version.

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Genomic Epidemiology of Multidrug-Resistant *Escherichia coli* and *Klebsiella pneumoniae* in Kenya, Uganda, and Jordan

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Surveillance of antimicrobial resistance in Kenya, Uganda, and Jordan identified multidrug-resistant high-risk bacterial clones: *Escherichia coli* sequence types 131, 1193, 69, 167, 10, 648, 410, 405 and *Klebsiella pneumoniae* sequence types 14, 147, 307, 258. Clones emerging in those countries exhibited high resistance mechanism diversity, highlighting a serious threat for multidrug resistance.

Global transmission of high-risk pandemic clones of gram-negative bacteria presents a serious threat to human health and complicates bacterial disease management, resulting in high illness and death rates and an enormous economic burden on healthcare systems (*1*). The pathogens are characterized by resistance to multiple classes of antimicrobial drugs, carriage of virulence genes, transmissibility to humans and animals, and global distribution. The negative effects of antimicrobial-resistant infections in terms of gross domestic product and disease burden will be disproportionally borne by low- and middle-income countries (*2*,*3*).

Global high-risk clones are of particular concern because they are multidrug resistant, can persist in hosts, are highly pathogenic, can have a fitness advantage, and can transfer easily between hosts (*4*,*5*). Using an antimicrobial resistance (AMR) surveillance program spanning 10 years (2012–2022), we describe the population structure and features of high-risk multidrug-resistant (MDR) *Escherichia coli* and *Klebsiella pneumoniae* in Kenya, Uganda, and Jordan.

Methods

We examined the population structure of MDR isolates (defined as resistance to ≥3 classes of antimicrobial drugs) (*6*) from Kenya, Uganda, and Jordan (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0370-App1.pdf) during 2012–2022, collected through the US Armed Forces Health Surveillance Division, Global Emerging Infections Surveillance program. Our study followed an active surveillance approach (with additional passive isolates in Kenya only), and according to the Centers for Disease Control and Prevention definition, infections were either healthcare-associated or community-acquired (Table) (*7*).

During 2012–2019, in Jordan, the Naval Medical Research Unit EURAFCENT, together with the

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Jordan Ministry of Health, collected 148 *E. coli* and 212 *K. pneumoniae* isolates from 9 hospitals (Appendix). During 2012–2022, in Kenya, the Walter Reed Army Institute of Research-Africa and the Kenya Ministries of Health and Defense collected 430 *E.coli* and 97 *K. pneumoniae* isolates from 12 hospitals. Also during 2012–2022, in Uganda, Makerere University Walter Reed Project, together with the Uganda Ministry of Health and Ministry of Defense, collected 207 *E.coli* and 69 *K. pneumoniae* isolates from 4 hospitals. Together, those collections resulted in a total of 785 *E. coli* and 378 *K. pneumoniae* MDR clinical isolates analyzed in our study (Appendix). The isolates were collected from patients 0.1–104 years of age and from different sources, including wounds (n = 323), urine (n = 411), blood (n = 79), pus (n = 134), respiratory tract (n = 195), and others (Table). To identify MDR strains for further characterization through whole-genome sequencing, we tested susceptibility to a panel of different classes of antimicrobials by using disk diffusion and the VITEK2 system (bioMérieux, https://www.biomerieux.com) in accordance with Clinical and Laboratory Standards Institute guidelines (*8*).

We subjected all MDR *E.coli* and *K. pneumoniae* isolates to whole-genome sequencing and de novo assemblies as previously described (*9*) and deposited the data in GenBank (BioProject accession nos. PRJNA955428, PRJNA1015582, PRJNA1076681, PRJNA1076682, PRJNA1078230, PRJNA1078534, PRJNA1078535). We assessed the population structure by using core-genome multilocus sequence typing and species-specific minimum spanning trees as previously described (*9*).

Results

The 785 *E. coli* genomes represented 124 sequence types (STs), of which 20 (16.1%) were shared between

Table. Demographic and clinical characteristics of patients from whom isolates were collected in study of genomic epidemiology of multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* in Kenya, Uganda, and Jordan

	Escherichia coli, no. (%)			*Klebsiella pneumoniae*, no. (%)		
Variable	Kenya, n = 430	Uganda, n = 207	Jordan, n = 148	Kenya, n = 97	Uganda, n = 69	Jordan, n = 212
Age groups, y						
0–4	7.2 (31)	1.9 (4)	19.6 (29)	7.2 (7)	7.2 (5)	26.9 (57)
5–9	1.9 (8)	0	5.4 (8)	0	1.4 (1)	4.7 (10)
10–17	2.1 (9)	3.4 (7)	4.1 (6)	3.1 (3)	0	4.2 (9)
18–49	61.4 (264)	67.6 (140)	22.3 (33)	60.8 (59)	58 (40)	22.6 (48)
≥50	27.4 (118)	24.2 (50)	45.9 (68)	28.9 (28)	27.5 (19)	41.5 (88)
Not available	0	2.9 (6)	2.7 (4)	0	5.8 (4)	0
Sex						
M	47.4 (204)	37.7 (78)	59.5 (88)	60.8 (59)	56.5 (39)	75.0 (159)
F	51.9 (223)	62.3 (129)	40.5 (60)	39.2 (38)	42.0 (29)	25.0 (53)
Not available	0.7 (3)		0	0	1.4 (1)	0
Infection type						
CAI	81.4 (350)	48.8 (101)	48.0 (71)	68.0 (66)	33.33 (23)	14.2 (30)
HAI	15.8 (68)	42.5 (88)	52.0 (77)	28.9 (28)	56.52 (39)	85.8 (182)
Not available	2.8 (12)	8.7 (18)	0	3.1 (3)	10.14 (7)	0
Year of isolation						
2011	0	0	0	0	0	0.5 (1)
2012	0	0	6.8 (10)	0	0	9.0 (19)
2013	0	1.0 (2)	16.2 (24)	0	0	14.2 (30)
2014	0	0	18.2 (27)	0	0	7.1 (15)
2015	7.7 (33)	10.1 (21)	26.4 (39)	4.1 (4)	7.2 (5)	34.0 (72)
2016	4.2 (18)	9.7 (20)	21.6 (32)	3.1 (3)	21.7 (15)	21.2 (45)
2017	7.4 (32)	7.7 (16)	3.4 (5)	10.3 (10)	10.1 (7)	10.8 (23)
2018	25.6 (110)	5.8 (12)	4.1 (6)	29.9 (29)	5.8 (4)	0.9 (2)
2019	19.8 (85)	4.3 (9)	3.4 (5)	17.5 (17)	5.8 (4)	2.4 (5)
2020	6.7 (29)	13.5 (28)	0	5.2 (5)	7.2 (5)	0
2021	15.3 (66)	22.2 (46)	0	21.6 (21)	30.4 (21)	0
2022	13.3 (57)	25.6 (53)	0	8.2 (8)	11.6 (8)	0
Sample type						
Wound/skin	49.1 (211)	10.6 (22)	11.5 (17)	71.1 (69)	2.9 (2)	0.9 (2)
Urine	39.3 (169)	57.5 (119)	34.5 (51)	20.6 (20)	39.1 (27)	11.8 (25)
Blood	0.2 (1)	1.0 (2)	20.3 (30)	0.0	2.9 (2)	20.8 (44)
Pus	8.4 (36)	29.5 (61)	0	7.2 (7)	43.5 (30)	0
Throat	0.5 (2)	0	0	0	0	0
Respiratory	0.0 (2)	0	33.8 (50)	0	2.9 (2)	66.5 (141)
Other	2.1 (9)	1.4 (3)	0	1.0 (1)	8.7 (6)	0
Not available	0.5 (2)	10.6 (22)	0	0	0	0

*CAI, community-acquired infection; HAI, healthcare-associated infection.

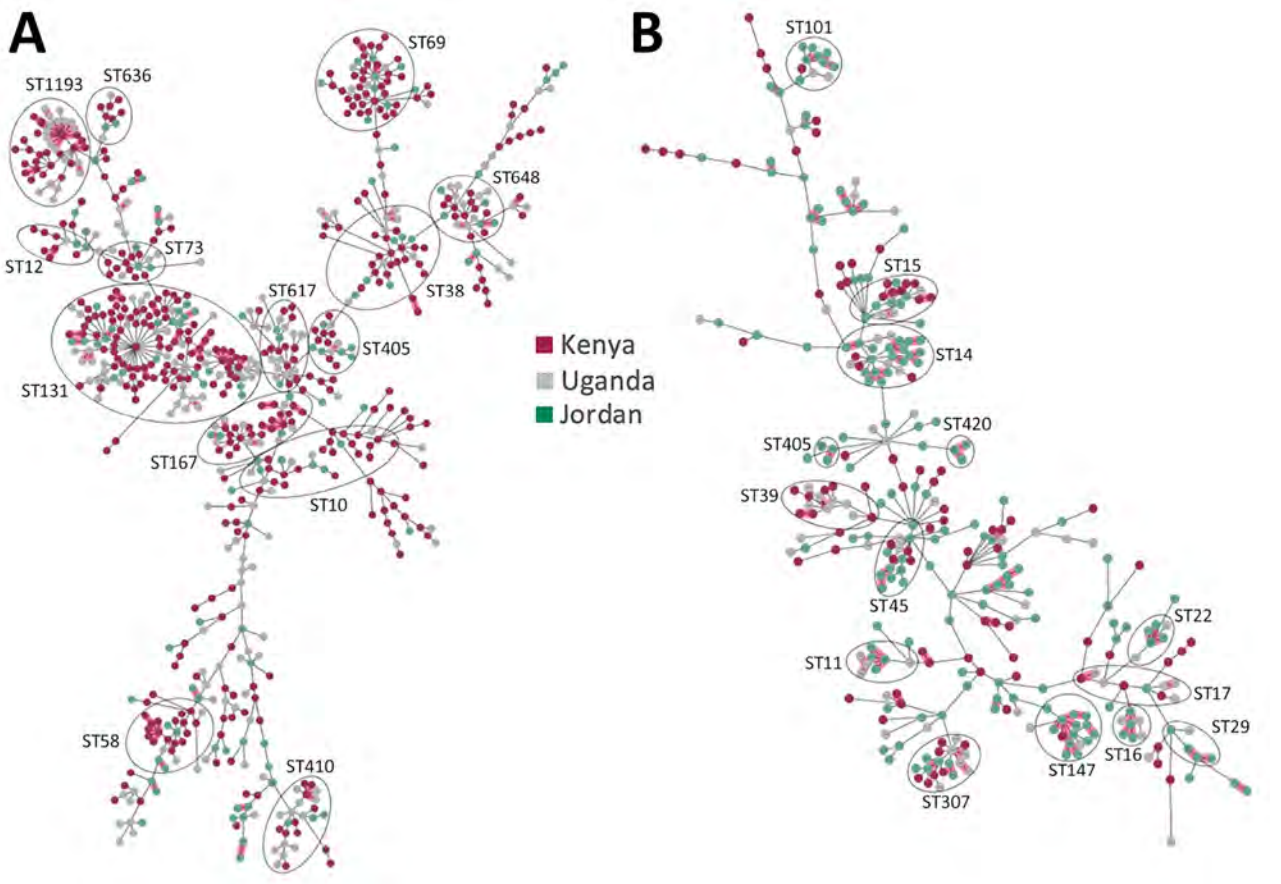

Figure 1. Population structure and diversity of high-risk *Escherichia coli* and *Klebsiella pneumoniae* sequence types across Kenya, Uganda, and Jordan. Minimum-spanning trees of *E. coli* (n = 785) and *K. pneumoniae* (n = 378) isolates are based on core-genome multilocus sequence typing. Each node represents an isolate; dominant STs are indicated in circled clusters. Branch length between nodes is proportional to the allelic differences between nodes. Purple indicates isolates from Kenya, gray from Uganda, and green from Jordan. ST, sequence type.

countries (Figure 1). For *E. coli*, the dominant ST was ST131 (Figure 1) in all 3 countries (Kenya 21.6%, n = 93; Uganda 21.3%, n = 44; and Jordan 16.9%, n = 25), collectively representing 20.6% (n = 162). The global high-risk clones (STs 131, 1193, 167, 69, 38, 10, 648, 410, 405, 73, 12, 117, 127, 95, and 393) constituted 62.4% (490/785) of all isolates. Evolution of the high-risk strains over the years was noted; in 2020, ST1193 became dominant in Kenya and Uganda, and no isolates were available from Jordan after 2020 (Appendix Figure 2). ST131 isolates decreased dramatically in Kenya in 2020 and in Uganda in 2018 and 2019; ST10 peaked in Jordan in 2012, in Kenya in 2018–2020, and in Uganda in 2020, after which it declined. ST648 sporadically appeared annually across all countries. The dominant *E. coli* phylogroups in all countries were B2, A, D, and B1, which comprised 90% of the isolates; B2 was the most dominant at 39.5%.

Similarly, genetic diversity of *K. pneumoniae* was high. There were 123 distinct STs, and only 11 (8.9%) STs were shared across the 3 countries (Figure 1). Jordan and Uganda had 75 distinct STs each, and Kenya had 37 STs. No clear evolutionary patterns of STs were observed over the years; STs appeared sporadically in different years except for ST420, which emerged in Uganda from 2020 to become a dominant ST, and ST14, which was the dominant strain in Jordan during 2013–2015. The high-risk clonal groups (CGs; 14, 15, 16, 101, 147, 307, 23, 65, 231, 258, 86) were detected and represented in 29.1% of the isolates. The high-risk CG14 (ST14) and CG147 (ST147) were very dominant in Jordan; CG15 and CG55 were exclusive to Kenya, and the global high-risk clone CG258 (ST258) was only in Jordan.

We analyzed whole-genome sequences for resistance determinants by using AMRFinderPlus (*10*) and ARIBA (*11*) and iTOL software version 6.8.1 (https://itol.embl.de) for visualization (*12*), as previously described (*9*). *E. coli* had 145 (Figure 2) and *K. pneumoniae* had 200 (Figure 3) diverse resistance determinants for

various classes of antimicrobial drugs. Among the resistance determinants of concern were the acquired extended-spectrum β-lactamases (ESBLs), mainly because of carriage of the $bla_{\text{CTX-M-15}}$ gene, identified in 50.8% of *E. coli* isolates and 68.8% of *K. pneumoniae* isolates, distributed in different STs (Figure 3). For *E. coli,* most (28.5%) ESBLs were in ST131, and the $bla_{\text{CTX-M-27}}$ allele was detected in 15% of the isolates. Carbapenem resistance was detected more in *K. pneumoniae* than in *E. coli*. In *K. pneumoniae*, carbapenemase genes were detected in 47 (12%) isolates, 43 of which were from Jordan; some isolates were co-harboring multiple carbapenemases, other resistance determinants, or both, including carbapenem resistance genes. ESBL genes in Jordan included $bla_{\text{NDM-1}}$ (11.3%), $bla_{\text{OXA-48}}$ (7.3%), $bla_{\text{OXA-181}}$ (0.9%), $bla_{\text{NDM-5}}$ (0.9%), and $bla_{\text{VIM-4}}$ (0.3%) (Figure 3). Isolates from Uganda carried $bla_{\text{OXA-181}}$ (2.9%) and $bla_{\text{NDM-5}}$ (1.4%). $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-5}}$ were detected in isolates from Kenya, both at 2.1%. Four isolates, all from Jordan, belonged to lineage ST147 and were of

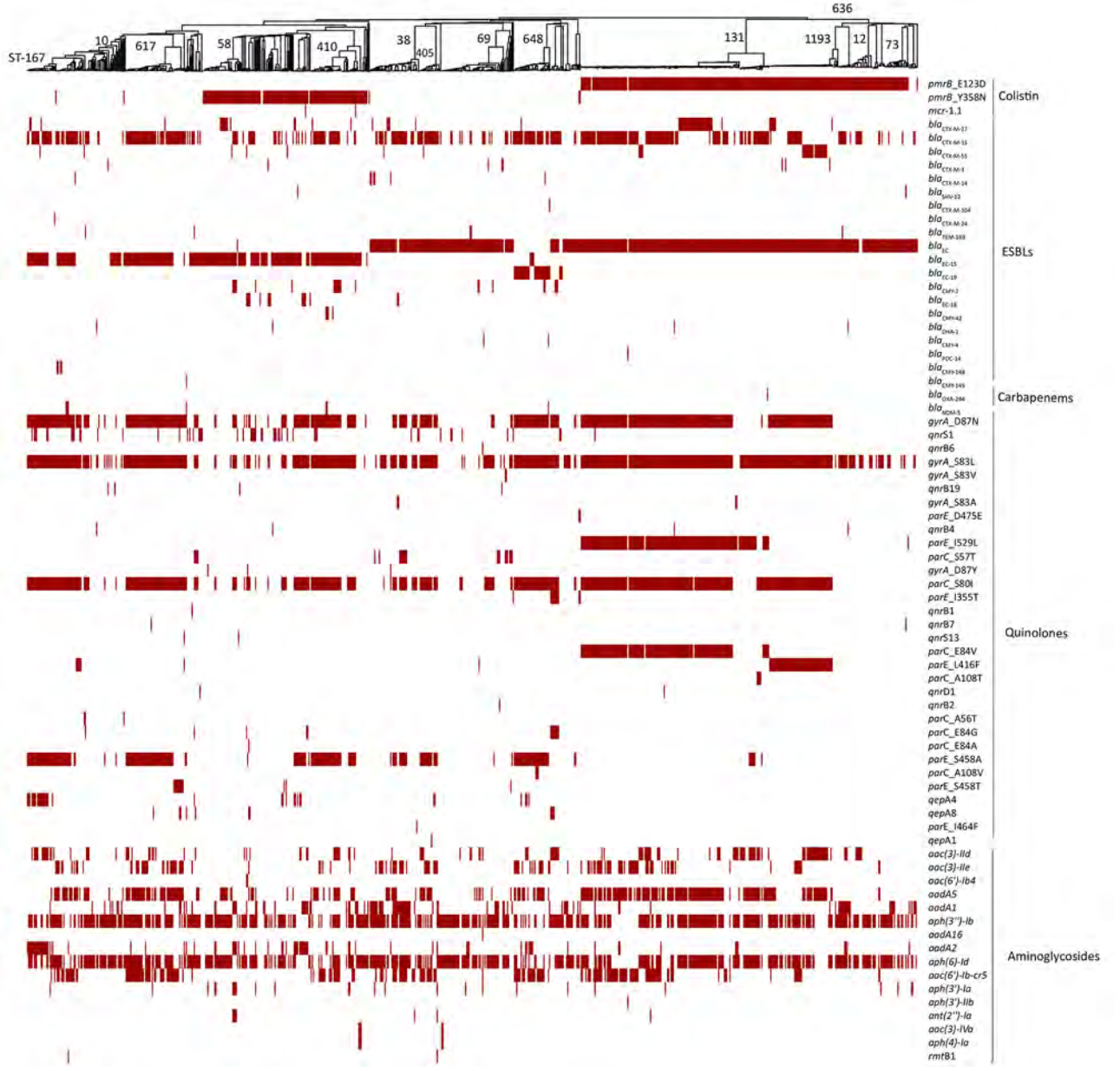

Figure 2. Comprehensive distribution of antimicrobial-resistance genes in 785 *Escherichia coli* isolates from Kenya, Uganda, and Jordan. Antimicrobial-resistance genes associated with nonsusceptibility to various antibiotic classes (polymyxins, third- and fourth-generation cephalosporins, carbapenems, phenicols and quinolones, and aminoglycosides) for each isolate are labeled for presence (red) or absence (white). The presence or absence of gene(s) is mapped onto a neighbor-joining tree curated from its minimum-spanning tree. The major high-risk STs are labeled on the neighbor-joining tree. ST, sequence type.

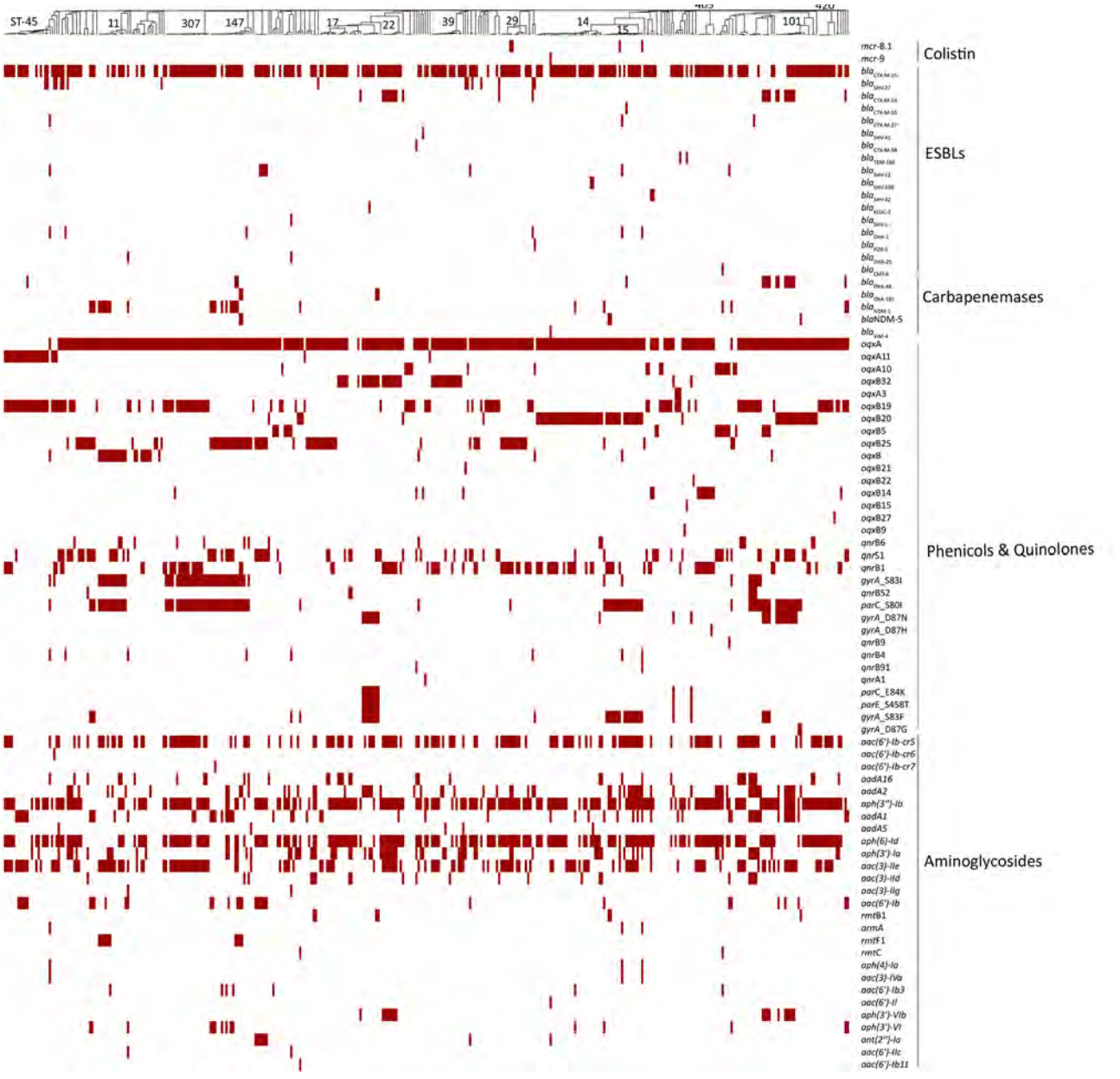

Figure 3. Comprehensive distribution of antimicrobial-resistance genes in 378 *Klebsiella pneumoniae* isolates from Kenya, Uganda, and Jordan. Antimicrobial-resistance genes associated with nonsusceptibility to various antibiotic classes (polymyxins, third- and fourth-generation cephalosporins, carbapenems, phenicols and quinolones, and aminoglycosides) for each isolate are labeled for presence (red) or absence (white). The presence or absence of gene(s) is mapped onto a neighbor-joining tree curated from its minimum-spanning tree. The most prevalent STs are labeled on the neighbor-joining tree. ESBLs, extended-spectrum β-lactamases; ST, sequence type.

serotype K64:O2a that co-carried bla_{NDM-1} and bla_{OXA-48} (n = 2) or bla_{NDM-5} and $bla_{OXA-181}$ genes (n = 2); 1 isolate from ST23, of serotype K1:01, also carried bla_{NDM-1} and bla_{OXA-48}. In *E.coli*, carbapenemase genes were detected in 8 isolates: bla_{NDM-5} (n = 7) and $bla_{OXA-244}$ (n = 1) (Figure 2). Four isolates carrying bla_{NDM-5} co-carried $bla_{CTX-M-15}$, belonging to lineages ST167 (n = 3) and ST648 (n = 1). The remaining isolates that did not co-carry $bla_{CTX-M-15}$ belonged to ST410 (n = 2) and ST361 (n = 1).

The plasmid-encoded mobile colistin resistance *mcr-1.1* genes for colistin resistance were detected in only 2 (0.3%) of the *E. coli* isolates; 5 isolates of *K. pneumoniae* carried *mcr-8.1* in 3 isolates and *mcr-9* in 1 isolate distributed among ST15, ST14, ST29, and ST16. One *K. pneumoniae* isolate carried *mcr-9* and bla_{VIM-4}. Several other resistance determinants were detected (Figures 2, 3), many of which were carried on plasmid replicons (i.e., IncFIB [77.7%], IncFIA_1 (59.5%], and

IncFIB(K)_1 [59.9%] for *E. coli* and IncFIB(K)_1 [59.9%] and IncFII(pKP91)1 [56.6%] for *K. pneumoniae*). Of note, most *K. pneumoniae* isolates harboring carbapenemase-resistance genes had multiplasmid replicons ranging from 2 to 9 replicons per isolate, especially the self-transmissible IncFII-IncFIB plasmid carrying the bla_{NDM-1} gene. Variability in the surveillance strategies and clinical characteristics of patients between countries could have skewed the between-country isolate genomic characteristics and numbers of *E. coli* and *K. pneumoniae* isolates in the different populations.

Discussion

The increasing spread of high-risk clones of *E. coli* and *K. pneumoniae* constitutes a serious threat for managing infections caused by those bacteria (*5*) to civilian and military populations, which often operate in harsh environments that increase their exposure to MDR pathogens. The population structure revealed high genetic diversity of STs and resistance determinants in the different countries. The *E. coli* population was dominated by ST131 in all 3 countries, consistent with its global dominance regardless of source (*13*), and was followed by ST1193, a high-risk clone that recently diverged from ST131.

Emerging *E. coli* ST1193 in Uganda and Kenya are frequently associated with extra-intestinal community-acquired urinary tract (*14*) and bloodstream infections, often with quinolone resistance-determining region mutations, ESBL bla_{CTX-M} genes, and IncF plasmids (*15*). Of note, potential zoonotic STs (ST10, ST95, and ST117) were detected, some of which are common in food animals (*16*–*19*) and known to carry an abundance of virulence factors and pathogenic potential that enable them to transmit, persist, and adapt to different hosts and environments (*17*).

K. pneumoniae isolates ST39 and ST17 were found mainly in East Africa countries and have previously been described in Kenya and Uganda (*20*,*21*). ST17 has been associated with regional outbreaks in Tanzania and Kenya and is prone to causing hospital outbreaks, making it an ST to monitor closely (*22*,*23*). In Jordan, high-risk CG14 (ST14) and CG147 (ST147) were dominant compared with East Africa countries, which could be associated with Jordan's surveillance being focused on nosocomial infections (*24*), as well as the MDR CG258, which indicate the unique threats in Jordan. ST25, identified in MDR isolates from Kenya and Jordan, is concerning because of its reported hypermucoviscous phenotype and virulence-AMR convergence, resulting in poor clinical outcomes, although we did not detect that convergence in our study (*25*,*26*).

We identified a high diversity of resistance mechanisms; about half of the isolates carried an ESBL gene, mainly because of the extensively distributed $bla_{CTX-M-15}$ gene, which was more prevalent among *K. pneumoniae* than among *E. coli*. Our study also detected several carbapenemase genes, primarily in *K. pneumoniae* isolates. Jordan reported more carbapenemase-resistance isolates than did the East Africa countries, similar to previous reports of high carbapenemase-resistance levels in Jordan (*24*) and India, which reported 30%–35% and co-expression of NDM and OXA-48 in 15.3% of carbapenemase-resistance isolates (*27*).

The increased resistance to last-line antimicrobial drugs (i.e., carbapenems and third- and fourth-generation cephalosporins) is concerning amid the increased excess, access, and misuse of antimicrobial drugs. The increase in mobile genetic elements that mobilize and spread resistance determinants further enhances spread. IncF and Col plasmids were the most common plasmid replicons among the MDR isolates; IncF plasmids are considered the more relevant contributors to the spread of AMR (*28*,*29*).

Overall, our study highlights the emergence and threat of genetically diverse high-risk MDR clones of 2 of the most critical groups of MDR bacteria causing severe infections with limited treatment options. The abundance of global high-risk STs bearing resistance genes indicates their effective dissemination, the potential for intraspecies and interspecies transmission of resistance genes, and emergence of new high-risk clones. To curtail the threat, continuous surveillance to monitor spread and emergence of dangerous clones is critical for supporting effective preventive measures and tailored therapies to match the regional and global risk to public and military health.

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Scrub Typhus Outbreak among Soldiers in Coastal Training Area, Australia, 2022

Rebecca Suhr, Samantha Belonogoff, Fiona McCallum, James Smith, G. Dennis Shanks

A scrub typhus outbreak occurred among 24 soldiers from 2 Australian Defence Force infantry units following separate training events conducted in the same coastal location in tropical North Queensland, Australia, in June 2022. Seven soldiers visited a hospital, 5 requiring admission. Outbreak recognition was hampered by the geographic dispersion of soldiers after the exercise and delayed case identification resulting from such factors as prolonged incubation, cross-reactive serologic responses to other pathogens, the nonspecific symptoms of scrub typhus, and the illness's nonnotifiable status in the state of Queensland. Our investigation focused on personal protective measures in a subanalysis of 41 soldiers, revealing an association between scrub typhus infection and the use of doxycycline chemoprophylaxis and permethrin uniform dipping.

Scrub typhus is a bacterial infection caused by *Orientia tsutsugamushi* of the *Rickettsia* family, transmitted to humans by the bite of an infected *Leptotrombidium* species chigger (larval trombiculid mite) (*1*). After an incubation period of 6–21 days, scrub typhus can cause such symptoms as fever, headache, rash, myalgia, gastrointestinal upset, lymphadenopathy, and occasionally a characteristic eschar or skin ulcer (*2*). Reports have estimated the median mortality rate of uncomplicated, treated scrub typhus to be 1.45%. However, if untreated, severe complications can develop, and mortality rates can be as high as 24% (for patients with associated multiorgan failure) and 14% (with patients with associated meningoencephalitis) (*2*–*4*). Early diagnosis is often difficult and delayed, given the nonspecific symptomatology and cross-reactive serology associated with the illness, which can be consistent with various other pathogens endemic to areas such as tropical North Queensland in Queensland, Australia (*5*).

Scrub typhus is a neglected tropical disease and a serious public health problem, and ≈1 million cases are estimated to occur annually (*2*). Globally, scrub typhus is considered a rural disease endemic to dense, vegetative areas within (and sometimes beyond) (*6*) a geographic triangle that is specific to the Indo-Pacific region but can extend to include Japan, Afghanistan, and northern Australia. In Australia, accurate disease surveillance is lacking because scrub typhus is only notifiable in Western Australia. The disease is likely underdiagnosed in the North Queensland regional areas (*7*), where case reporting is predominantly limited to military outbreaks at Cowley Beach Training Area (CBTA) (*8*–*10*) (Figure 1).

Scrub typhus emerged as a significant disease for Australian and allied militaries deployed to southeast Asia and southwest Pacific regions during World War II (*11*), and, in the absence of antibiotic treatment options, scrub typhus infections greatly affected military capabilities, resulting in attrition as high as 9% (*12*). Despite the development and introduction of chloramphenicol and chemical treatment for uniforms after the war, scrub typhus remains a serious vectorborne disease that continues to threaten soldiers training in jungle and coastal environments (*13*–*15*). Our report of a recent outbreak of this illness highlights this threat.

In July 2022, the Australian Defence Force Malaria and Infectious Disease Institute assisted in the investigation of an emerging cluster of febrile soldiers in 2 infantry units from Brisbane and Townsville, Queensland, Australia (designated Brisbane-based and Townsville-based), that had recently returned from different exercises at the CBTA. Three soldiers received diagnoses of laboratory-confirmed scrub typhus in the early phase of the outbreak, but positive serologic results prompted physicians to consider other diagnoses as well, including leptospirosis, Q fever, and Japanese encephalitis virus. Our research

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Figure 1. Location of Cowley Beach Training area within the state of Queensland, Australia.

explores the challenges of confirming a scrub typhus outbreak and provides statistical analysis of doxycycline chemoprophylaxis and permethrin treatment of clothing in preventing scrub typhus infection.

Methods

During the 2022 scrub typhus outbreak, the Australian Defence Force Malaria and Infectious Disease Institute staff communicated with treating military-based clinicians, Public Health Units, and diagnostic laboratories regarding the number of symptomatic persons, diagnostic tests being performed, and those test results. Existing military- and civilian-derived medical records and pathology results for affected soldiers were sourced and reviewed, with further testing requested if required.

Scrub typhus diagnosis is confirmed via positive nucleic acid testing of blood or eschar biopsy, seroconversion, or a 4-fold rise in total antibody titers in serologic testing (*2*). Confirmed and probable cases of scrub typhus in this outbreak were determined by using the Western Australia case definition (*16*) (Appendix, https://wwwnc.cdc.gov/EID/article/30/13/24-0056-App1.pdf). A clinical case definition was also established to capture soldiers with a compatible illness and epidemiologic links to the outbreak but with no disease cause identified through laboratory testing. We determined exposed, noncase soldiers, defined as infantry soldiers who conducted the same activities as case patients in the same environment but did not become ill, by using unit nominal rolls of personnel involved in the exercises.

Our research aimed to compare disease-risk data for a subset of personnel with scrub typhus (Brisbane-based case-patients) and a control group of exposed, noncase soldiers. Researchers interviewed scrub typhus case patients and asked exposed, noncase soldiers to complete an anonymized questionnaire. Questions included details regarding training area movement and activities, level of compliance with individual protection measures, and barriers to prophylaxis compliance. We entered data into an electronic database (Microsoft Excel 2016; Microsoft, https://www.microsoft.com) on a restricted military network and used Stata version 14.0 statistical software (StataCorp LLC, https://www.stata.com) for analysis. We used a χ^2 test of independence to investigate use of uniform permethrin dipping and doxycycline prophylaxis for case-patients versus noncase personnel. Because of a very low response from Townsville-based, noncase personnel, we did not include the 12 Townsville-based case-patients in our statistical comparison of data.

Results

We identified 24 cases of scrub typhus among military members from the 2 infantry units after training exercises conducted at CBTA during June 7–24, 2022. We determined a total of 337 soldiers from both units to be exposed personnel. Scrub typhus attack rate was 18.8% (12/64; 5 confirmed cases, 6 probable cases, 1 clinical case) for the Brisbane-based cohort conducting jungle warfare training and 4.4% (12/273; 8 confirmed cases, 3 probable cases, 1 clinical case) for the Townsville-based cohort conducting amphibious landings.

The incubation period range for the 24 cases, determined from the last possible date of exposure at CBTA to the onset of illness, was 8–20 days (median 12 days) (Figure 2). None of the soldiers had received a prior diagnosis of scrub typhus. Seven soldiers (29%, 7/24) visited a hospital, 5 (21%, 5/24) of whom were admitted to a hospital for an average length of stay of 5.6 days (range 2–8 days). Symptoms included seizures caused by meningoencephalitis (requiring intensive care unit admission), multisystem inflammatory response with evidence of hemodynamic instability, pulmonary congestion (requiring high-flow oxygen supplementation), acute kidney injury and hepatosplenomegaly, and uncomplicated cases of electrolyte disturbances and

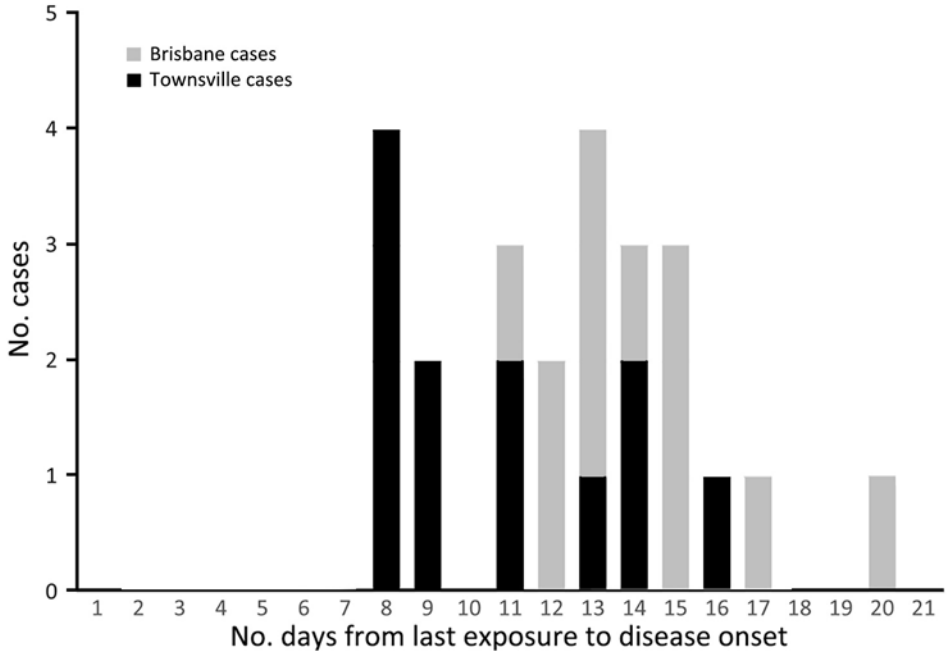

Figure 2. Epidemiologic curve of the 2022 scrub typhus outbreak occurring at Cowley Beach Training Area, Queensland, Australia. Training participants were from either Brisbane or Townsville in Queensland.

jaundice. The mean age for the soldiers was 27 years, and all were physically fit, with few or no comorbidities. Of the hospitalized soldiers, we categorized 4 as having a confirmed case of scrub typhus and 3 as having a probable case of the illness (maximum *O. tsutsugamushi* titer demonstrated on initial blood testing and therefore no capacity to demonstrate seroconversion or a rise in titer). We compiled data relating to symptom onset, blood sampling, and *O. tsutsugamushi* serologic results for all 24 case-patients (Appendix Table 2). We noted headache and fever to be the most common symptoms reported (Table), a finding consistent with previous literature (*2*). All soldiers responded well to treatment with doxycycline, and no soldiers died as a result of this outbreak.

At the time of illness onset, some soldiers were on leave and dislocated from their home unit location, delaying initial assessment. In addition, several soldiers were dispersed once they clinically improved, creating challenges in obtaining the blood samples required for serologic investigation. We conducted scrub typhus nucleic acid testing by using primary blood samples from 17 (71%) of the 24 soldiers. We collected 14 samples beyond the recommended 5-day window from illness onset; however, 3 (18%, 3/17) of the 14 were positive; of note, those samples were from hospital testing.

Many soldiers had positive IgM serologic responses to other pathogens. We observed 36 positive or equivocal IgM results for 11 diseases (Figure 3) for 17 of the 22 case-patients who underwent serologic testing. Results for the case patients who were retested revealed no increase in titer or immunological progression to an IgG response to other pathogens. Because a scrub typhus outbreak was eventually confirmed, we assumed that all of those results were related to IgM cross-reactivity between pathogens. Clinical improvement of all cases after commencement of treatment with doxycycline supported a diagnosis of scrub typhus, although that treatment is also appropriate for some differential diagnoses, such as leptospirosis and Q fever (*18*).

All personnel who attended the exercises at CBTA received prophylactic doxycycline. We gathered self-reported adherence data for 64% (41/64) of the Brisbane-based soldiers who participated in jungle training and for 56% (29/52) of the exposed, noncase soldiers (Figure 4). Most soldiers reported the intention to comply with the regimen. Circumstances contributing to omitted doses included irregular or absent food intake, damage to tablets (i.e., wet or crushed) in packs, secondary health complications (e.g., gastric reflux, vomiting), and loss of

Table. Common symptomatology among 24 case-patients investigated during a scrub typhus outbreak at Cowley Beach Training Area, Australia, 2022

Symptom or sign	No. (%) case-patients
Headache	20 (96)
Fever	21 (92)
Abnormal liver function*	18 (86)
Rash	16 (67)
Myalgia or arthralgia	18 (75)
Fatigue	14 (58)
Eschar or bites	13 (54)
Nausea or vomiting	12 (50)

*Three case patients did not have liver function checked.

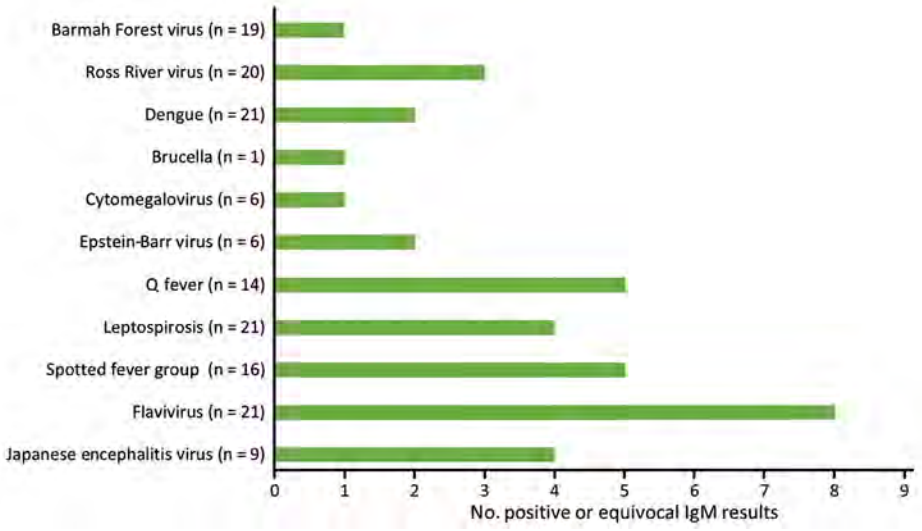

Figure 3. Cross-reactive positive or equivocal IgM serologic results in cohort of 24 scrub typhus cases investigated during a 2022 outbreak at Cowley Beach Training Area, Queensland, Australia. n values indicate number of case-patients tested for each pathogen/disease.

routine. Seven (58%) of 12 Brisbane-based case patients and 26 (90%) of 29 exposed, noncase soldiers reported complete or partial compliance (≥1 tablet taken while at CBTA) with the doxycycline regimen. Analysis showed that any doxycycline intake was protective and that soldiers who did not take any doxycycline were 6 times more likely to be infected with scrub typhus than those who took any doxycycline (OR 6.2, 95% CI 1.2–33.3; p = 0.03).

Case-patients and Brisbane-based exposed, noncase soldiers generally adhered to supplied personal protective measures. Questions regarding the appropriate wearing of uniforms and use of DEET insect repellent garnered consistent (>90%) "well adhered to" responses from both case-patients and noncase personnel. Of note, all scrub typhus case-patients reported sleeping or lying on the ground, compared with 72% of the Brisbane-based exposed, noncase soldiers. We collected information regarding uniform dipping for 36 Brisbane-based soldiers (9 case-patients; 27 exposed, noncase soldiers), revealing an overall compliance of 83.3% (30/36), including 5 of the 9 case-patients having dipped their uniforms. Data suggested that uniform dipping was protective, and soldiers who did not wear dipped uniforms were 10 times more likely to be infected with scrub typhus than those who wore dipped uniforms (OR 10, 95% CI 1.42–71.43; p = 0.021). Soldiers reported varying tolerability and attitudes toward doxycycline prophylaxis compliance and barriers to individual protection measure adherance.

Discussion

This most recent outbreak of scrub typhus at an Australian military training area underscores the continuing propensity of this illness to hinder military operations throughout the Indo-Pacific region. Despite established prophylactic regimens among military personnel at CBTA, the 2 infantry units involved in this outbreak had scrub typhus attack rates of 18.8% (Brisbane-based cohort) and 4.4% (Townsville-based cohort). The higher attack rate in the Brisbane-based

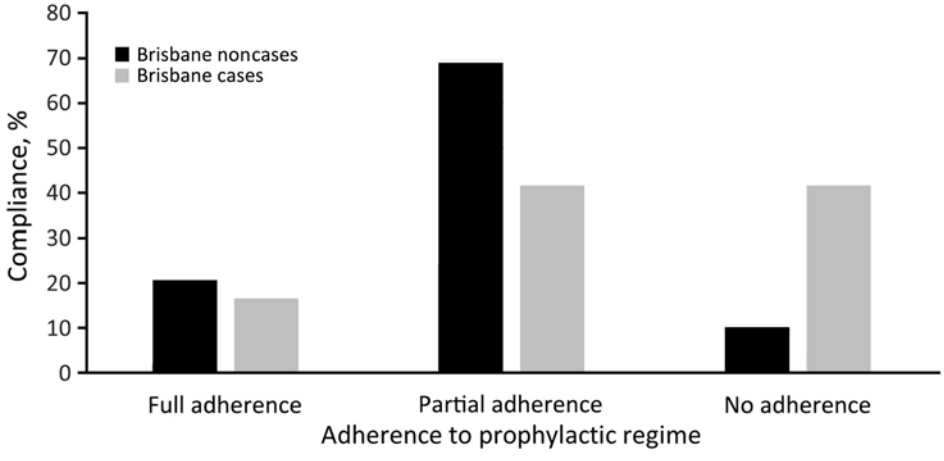

Figure 4. Reported adherence to prophylactic doxycycline among 12 case patients from a Brisbane, Australia–based military unit compared with 29 Brisbane-based exposed, noncase soliders obtained as part of an investigation of scrub typhus at Cowley Beach Training Area, Queensland, Australia, 2022.

cohort might reflect more intimate exposure to vegetation during jungle warfare training, and hence to mites, when compared with activities associated with amphibious landings.

Several factors contributed to a delay in the identification of the outbreak. Disease manifestations in all cases commenced ≥1 week after exercise completion, when a large proportion of personnel were on leave, many having traveled interstate. The scattered nature of personnel and unavailability of blood samples and medical and pathology records contributed to the delay in paired serology for some patients. In addition, some soldiers were unwilling or unable to undergo further laboratory investigation after clinical improvement.

Because testing of many ill soldiers revealed IgM seropositivity to several pathogens, serologic results of which arrived prior to those for scrub typhus, we were delayed in establishing diagnostic confirmation of the outbreak. Most clinicians requested rickettsial serology at the time of initial patient evaluation. However, the rickettsial screening offered by some pathology providers did not include *O. tsutsugamushi* serology, further delaying confirmatory results. Laboratory standard of intermittent, batched serologic testing for *O. tsutsugamushi* indirect fluorescent antibodies added to the delay. It therefore took an average of 23 days after sample collection to receive scrub typhus serologic test results for all patients.

We acknowledge several limitations of our investigation, including a relatively small case population; statistical analyses should be therefore interpreted with caution. It is possible that some nonconfirmed cases experienced a disease other than scrub typhus (e.g., leptospirosis), posing a misclassification risk to our analyses. However, when we removed the single clinical case from our analysis, the findings remained consistent and statistically significant. In addition, although we collected data regarding prophylactic compliance, we could not confirm that information, which might have influenced results.

In conclusion, this outbreak investigation confirmed a statistically significant association between scrub typhus disease occurrence and noncompliance with the prescribed doxycycline regimen, supporting the use of doxycycline chemoprophylaxis against scrub typhus disease (*18*,*19*). We also noted a strong association between nonuse of permethrin-treated uniforms and scrub typhus disease occurance, consistent with existing knowledge of this preventative measure in reducing exposure to disease vectors (*20*).

This outbreak highlights the need for increased awareness of this pathogen threat in both military and civilian health settings in Australia and among Pacific and Asian partners. The illness severity, even among young healthy soldiers, and occasional lethality of scrub typhus, together with reports of the illness throughout northern Australia, Southeast Asia, the Western Pacific, and China's South China Sea regions, make scrub typhus a dangerous, lingering issue for military health planners throughout the Indo-Pacific region.

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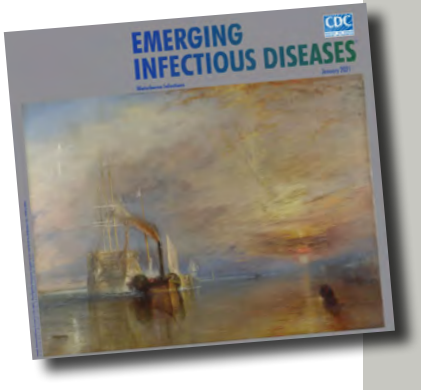
etymologia revisited

Petri Dish

[pe'tre 'dish]

The Petri dish is named after the German inventor and bacteriologist Julius Richard Petri (1852–1921). In 1887, as an assistant to fellow German physician and pioneering microbiologist Robert Koch (1843–1910), Petri published a paper titled "A minor modification of the plating technique of Koch." This seemingly modest improvement (a slightly larger glass lid), Petri explained, reduced contamination from airborne germs in comparison with Koch's bell jar.



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Molecular Surveillance of Multidrug-Resistant Bacteria among Refugees from Afghanistan in 2 US Military Hospitals during Operation Allies Refuge, 2021

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In 2021, two US military hospitals, Landstuhl Regional Medical Center in Landstuhl, Germany, and Walter Reed National Military Medical Center (WRNMMC) in Bethesda, Maryland, USA, observed a high prevalence of multidrug-resistant bacteria among refugees evacuated from Afghanistan during Operation Allies Refuge. Multidrug-resistant isolates collected from 80 patients carried an array of antimicrobial resistance genes, including carbapenemases (bla_{NDM-1}, bla_{NDM-5}, *and* bla_{OXA-23}) and 16S methyltransferases (*rmtC* and *rmtF*). Considering the rising transmission of antimicrobial resistance and unprecedented population displacement globally, these data are a reminder of the need for robust infection control measures and surveillance.

Antimicrobials are among the most widely used drugs worldwide and are essential for treating infections. However, antimicrobial drug effectiveness depends on the susceptibility of the targeted pathogens. The emergence of widespread antimicrobial resistance (AMR) among pathogens is limiting the use of these essential drugs and is a major threat to global health. An estimated 5 million deaths are associated with drug-resistant infections annually; without intervention, that number could increase to 10 million by 2050 (*1*).

The wars in Iraq and Afghanistan have highlighted the prevalence of AMR across the Middle East and South Asia. Isolation of multidrug-resistant (MDR) bacteria was common in combat-related infections, such as wound infections. Many of those infections were caused by ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) and had varying levels of resistance and potential for nosocomial spread (*2*–*6*).

In 2009, the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN) at the Walter Reed Army Institute of Research (Bethesda, Maryland, USA) was established to combat AMR emergence within the US military healthcare system. The MRSN serves as the primary surveillance organization for MDR bacteria across the Department of Defense (DoD). Since 2009, the MRSN has collected >120,000 bacterial isolates from patients treated at military hospitals across the world. Together, the MRSN and the DoD Global Emerging Infections Surveillance Branch coordinate a worldwide network of AMR surveillance across both military treatment facilities and partner nation settings.

In May 2021, the Taliban in Afghanistan began a military offensive that led to the fall of the Islamic Republic of Afghanistan 3 months later. More than 500,000 citizens of Afghanistan were displaced in 2021 and joined >5 million other refugees who have been displaced throughout the world during the past 2 decades. In July 2021, the US State Department established Operation Allies Refuge (OAR) to support a special

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immigrant visa program for eligible Afghanistan nationals who assisted the US government during the war, along with their families. On August 26, 2021, a suicide bombing at Hamid Karzai International Airport in Kabul, Afghanistan, prompted emergent medical evacuation of injured Afghanistan civilians to Landstuhl Regional Medical Center (LRMC; Landstuhl, Germany) and Walter Reed National Military Medical Center (WRNMMC; Bethesda, Maryland, USA). MRSN performed genomic evaluation on MDR bacteria isolated from evacuated patients at LRMC and WRNMMC. We assessed genomic relatedness from AMR isolates among OAR patients and historical strains.

Methods

According to institutional infection control policy, perirectal and nasal surveillance swab samples were collected on all patients from Afghanistan at admission to LRMC and WRNMMC. Swab samples were used to screen for methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase (ESBL)–producing bacteria, carbapenemase-producing *Enterobacterales* (CPE), and vancomycin-resistant *Enterococcus* (VRE). We defined an MDR organism (MDRO) as MRSA, VRE, CPE, or isolates recovered from clinical or surveillance cultures that were resistant to ≥1 agent in 3 different antimicrobial classes. We considered recovered isolates that met those criteria surveillance culture to be positive. Patients with negative surveillance cultures were immediately released from isolation; patients with positive surveillance or clinical cultures remained in isolation and on contact precautions to prevent nosocomial transmission. Isolates from all MDROs were sent to the MRSN for whole-genome sequencing. During September–October 2021, WRNMMC and LRMC submitted 171 MDR bacterial isolates from 80 inpatients and outpatients from Afghanistan. We used Illumina Miseq and MiSeq Reagent Kit version 3 (Illumina, https://www.illumina.com) for sequencing (600 cycles, 2 × 300 bp).

Results

Among 80 patients with MDR isolates, 42 were male and 38 were female, and their median age was 26 years (range 1 week to 83 years). The top 5 clinical diagnoses were blast trauma (28%; n = 22), gunshot wound (15%; n = 12), urinary tract infection (11%; n = 9), abscess (10%; n = 8), and pregnancy (6%; n = 5). Among the isolates analyzed, *E. coli* was the most prevalent (58%; n = 99) bacterial species, followed by *K. pneumoniae* (13%; n = 22), *S. aureus* (12%; n = 20), and *A. baumannii* complex (5%; n = 9) (Figure 1).

After removal of serial isolates from the same patient (same sequence-type determined by in silico

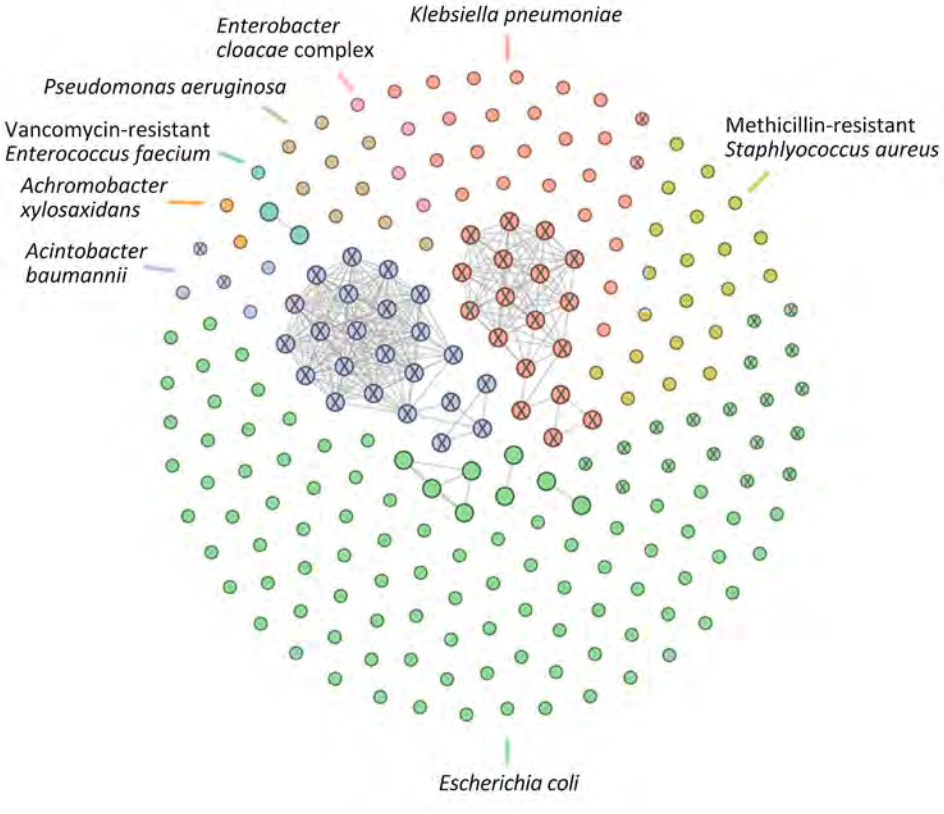

Figure 1. Cluster network of all isolates from molecular surveillance of multidrug-resistant bacteria among refugees from Afghanistan in 2 US military hospitals during Operation Allies Refuge, 2021. Isolates are grouped and colored by bacterial species. X indicates carbapenemase-producing isolates. Circles without connecting lines represent genetically unrelated isolates. Connected circles represent isolates that are ≤10 core alleles genetically distinct from Operation Allies Refuge isolates or historical isolates from Landstuhl Regional Medical Center, Landstuhl, Germany and Bagram Air Force Base, Parwan Province, Afghanistan. Clusters grouping serial isolates from single patients are not shown.

multilocus sequence typing), we used the deduplicated dataset of 132 isolates from 80 patients to investigate the prevalence of species and antimicrobial resistance genes. Of the 132 deduplicated isolates, *E. coli* was the most prevalent (64%; n = 85), followed by *K. pneumoniae* (12%; n = 16), *S. aureus* (11%; n = 14), and *A. baumannii* (5%; n = 6) (Table 1). Of the 80 distinct patients, 59 were identified with solitary MDRO-positive surveillance cultures of *E. coli* (76%; n = 45), *Klebsiella* (7%; n = 4), and *Enterobacter* spp. (2%; n = 1). Nine patients were colonized with multiple species in addition to ≥1 strain of *E. coli*: 5 (9%) with *E. coli* and *K. pneumoniae*, 2 (3%) with *E. coli* and VRE, and 2 (3%) with *E. coli* and 3 additional species. Overall, we found 92% (n = 54) of colonized patients carried an ESBL-positive *E. coli*. At LRMC, 85% (n = 30; data not shown) of patients who received surveillance cultures were colonized with ≥1 MDRO.

Among deduplicated *E. coli* isolates, 84% (n = 71) were ESBL-positive, mostly due to $bla_{\text{CTX-M-15}}$; 16% (n = 14) were carbapenemase-positive, including 9 isolates carrying $bla_{\text{NDM-5}}$ metallo-β-lactamase. We noted high diversity among the 85 *E. coli* isolates and identified 45 distinct sequence types (STs). Lineages ST10 (8 patients) and ST69 (6 patients) were most common. We identified only 2 patients with extraintestinal pathogenic *E. coli* lineage ST131. A total of 15 patients carried 2 (n = 11) or 3 (n = 4) distinct *E. coli* lineages. A single isolate of ST38 was shown to coproduce New Delhi metallo-β-lactamase (NDM) 5 and oxacillinase (OXA) 181 carbapenemases (Table 2).

We identified 16 distinct lineages among the 15 patients carrying *K. pneumoniae*. Overall, 81% (n = 13) of *K. pneumoniae* isolates carried $bla_{\text{CTX-M-15}}$, and 19% (n = 3) were carbapenemase-positive due to $bla_{\text{NDM-1}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{OXA-232}}$.

Among other identified MDRO isolates, we identified various antimicrobial resistance genes. Of the 6 patients with *A. baumannii*–positive cultures, 4 carried the epidemic clone ST2 that had $bla_{\text{OXA-23}}$ carbapenemase. Two of the *E. cloacae* isolates carried $bla_{\text{CTX-M-15}}$. All 14 *S. aureus* isolates carried the *mecA* gene, and all 3 *E. faecium* isolates carried the *vanA* gene. None of the 4 *P. aeruginosa* isolates carried high-risk AMR genes.

To identify clusters of high genetic relatedness, we compared genomes of all isolates from this study to historical isolates unrelated to OAR that were collected from LRMC and Bagram Air Force Base (AFB). Bagram AFB was located in the northeastern province of Parwan in Afghanistan, 40 km north of Kabul, and was evacuated by US military personnel in July 2021. Bagram AFB housed the Craig Joint Theater Hospital that provided healthcare to US and coalition forces. We identified a total of 6 genetic clusters: 3 *E. coli*, and 1 cluster each of *K. pneumoniae*, *A. baumannii*, and *E. faecium* (Figure 2). We detected 3 clusters of epidemic *E. coli*, ST44, ST69, and ST648 (Figure 2, panel A). The ST44 cluster contained isolates from 2 OAR patients whose admissions overlapped at WRNMMC; those isolates differed by 14–15 single-nucleotide polymorphisms (SNPs). The ST69 and ST648 clusters comprised 1 OAR patient from 2021 and 1 Bagram AFB patient whose isolates were collected in 2018; isolates in those clusters differed by 30 SNPs.

In the *K. pneumoniae* cluster, 3 serial ST15 isolates that carried $bla_{\text{OXA-232}}$, $bla_{\text{CTX-M-15}}$, and *rmtF* (16S rRNA methyltransferase) were isolated from a single OAR patient at WRNMMC. Those isolates were highly related (17–45 SNPs difference) to isolates previously collected from 5 patients at Bagram AFB in 2018 (Figure 2, panel B). That OAR patient was also colonized with VRE and *E. coli* ST38 and ST648. In the *A. baumannii* cluster, 3 OAR patients from LRMC carried ST2 isolates that were highly related to a protracted nosocomial outbreak at Bagram AFB during 2019–2020 (Figure 2, panel C), and differed only by 11–38 SNPs. Last, 3 patients in the *E. faecium* cluster each had an isolate belonging to the ST80 epidemic clone (Figure 2, panel D). Two of those *E. faecium* isolates only differed by 7 SNPs from 2 patients in isolation at the same time at LRMC. Those patients were wounded in Afghanistan 2–3 weeks before evacuation to LRMC and were admitted 1 day apart from each other.

Among the *S. aureus* isolates, we identified ST1482 in 43% (n = 6) of patients; the other patients

Table 1. Sources of 132 deduplicated isolates collected during molecular surveillance of multidrug-resistant bacteria among refugees from Afghanistan in 2 US military hospitals during Operation Allies Refuge, 2021

Species	No. by patient source					Total
	Respiratory	Urine	Wound	Blood	Rectal swab	
Achromobacter xylosoxidans	0	0	1	0	0	1
Acinetobacter baumannii	2	0	4	0	0	6
Enterobacter cloacae complex	0	0	1	0	2	3
Enterococcus faecium	0	0	0	0	3	3
Escherichia coli	0	10	3	1	71	85
Klebsiella pneumoniae	0	3	3	0	10	16
Pseudomonas aeruginosa	0	1	1	0	2	4
Staphylococcus aureus	3	0	11	0	0	14
Total	5	14	24	1	88	132

Table 2. Distribution of high priority antimicrobial resistance genes among isolates collected for molecular surveillance of multidrug-resistant bacteria among refugees from Afghanistan in 2 US military hospitals during Operation Allies Refuge, 2021

Species	bla_{NDM-1}	bla_{NDM-5}	bla_{OXA-23}	$bla_{OXA-943}$	$bla_{OXA-181}$	$bla_{OXA-232}$	*vanA*	*mecA*	*rmtC*	*rmtF2*	*rmtF1*	*rmtB1*
Escherichia coli	0	10	0	0	8	0	0	0	0	0	0	2
Acinetobacter baumannii	0	0	6	2	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	1	0	0	0	1	3	0	0	1	1	2	0
Enterococcus faecium	0	0	0	0	0	0	3	0	0	0	0	0
Staphylococcus aureus	0	0	0	0	0	0	0	14	0	0	0	0
Total	1	10	6	2	9	3	3	14	1	1	1	2

carried clones ST30 (n = 4), ST22 (n = 3), and ST772 (n = 1). Although many patients shared the same ST, we did not identify any clusters of isolates sharing high levels of genetic relatedness.

Discussion

In this study, MDRO surveillance was conducted at 2 US military hospitals that provided medical care to refugees from Afghanistan during OAR. We observed high rates of MDRO colonization with *E. coli* and found that many isolates carried ESBL, carbapenemase, and 16S methyltransferase genes. Furthermore, we observed many AMR *K. pneumoniae*, *A. baumannii*, *E. faecium*, and *S. aureus* clinical isolates. We found that >75% of ESBL-producing *E. coli* isolates produced CTX-M-15. Among the 14 carbapenemase-producing *E. coli* isolates, we isolated *E. coli* ST648 coproducing NDM-5 and RmtB in samples from 5 patients. ST648 has been characterized by a combination of multidrug resistance, high-level virulence, and biofilm formation, similar to the global high-risk ST131 clonal lineage (*7*). One patient was colonized with *K. pneumoniae* ST11 that coproduced NDM-1 and RmtC, a lineage that is commonly known for high virulence and resistance to all β-lactams, including carbapenems, and resistance to aminoglycosides (*8*).

One patient from WRNMMC had 3 serial *K. pneumoniae* ST15 isolates from an abdominal wound and surveillance cultures. Surveillance cultures from that patient were also positive for VRE, *P. aeruginosa*, and 2 ESBL-producing strains of *E. coli*, ST38 and ST648. In addition to $bla_{OXA-232}$, $bla_{CTX-M-15}$, and *rmtF*, the *K. pneumoniae* ST15 isolates carried markers of hypervirulence and hypermucoviscosity (data not shown). The siderophores, yersiniabactin and aerobactin, *rmpA2* (regulator of mucoid phenotype), and *peg344* (a drug and metabolite

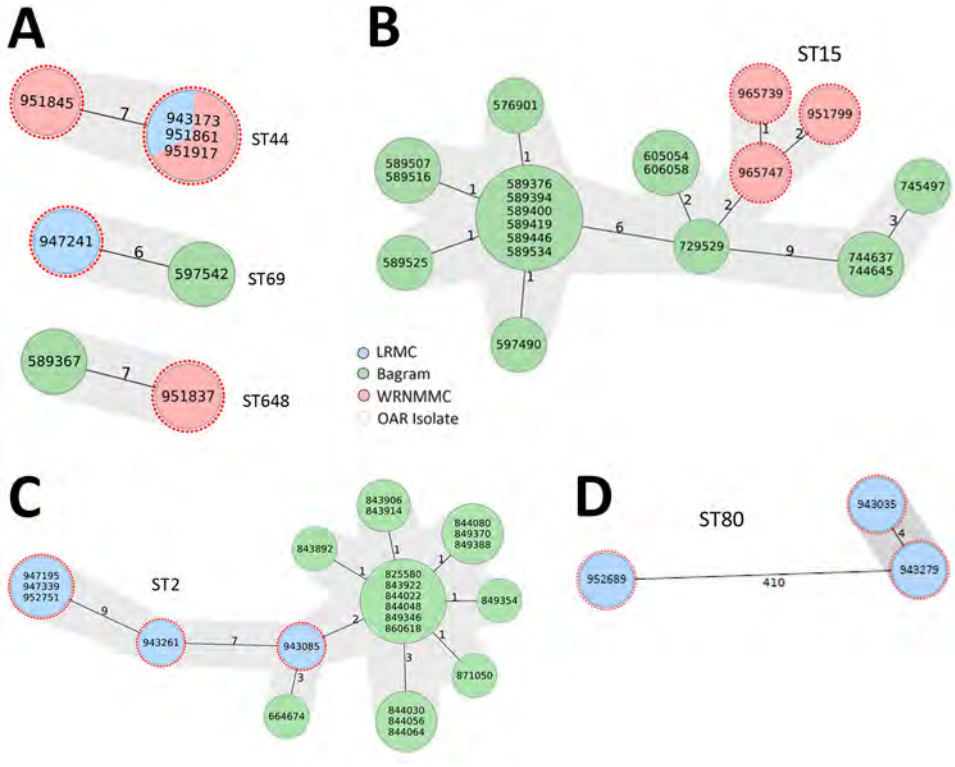

Figure 2. Phylogenetic analysis of highly related isolates from molecular surveillance of multidrug-resistant bacteria among refugees from Afghanistan in 2 US military hospitals during OAR, 2021. A) *Escherichia coli* ST44, ST69, and ST648 isolates. B) *Klebsiella pneumoniae* ST15 isolates; OAR isolates were closely related to isolates collected in 2018. C) *Acinetobacter baumannii* ST2 isolates; OAR isolates were closely related to isolates collected during 2019–2020. D) *Enterococcus faecium* ST80 isolates. Numbers inside circles indicate isolate identification numbers. Numbers along lines connecting circles indicate the number of allelic differences between isolates. Gray shading indicates clustered isolates. Bagram, Bagram Air Force Base, Bagram, Afghanistan; LRMC, Landstuhl Regional Medical Center, Landstuhl, Germany; OAR, Operation Allies Refuge; ST, sequence type; WRNMMC, Walter Reed National Military Medical Center, Bethesda, Maryland, USA.

transporter) were detected in those isolates, and all of those have been associated with invasive disease in immunocompetent patients (*9*). OXA-232–producing *K. pneumoniae* ST15 predominantly is found in China, where multiple nosocomial outbreaks have been reported, but the lineage also has been described globally (*10*).

Nosocomial outbreaks causing severe illness and death have been reported in medical treatment facilities in Iraq and Afghanistan and are a great concern for the US military healthcare system (*11*). Although phylogenetic analysis in this study revealed multiple clusters of highly related isolates, patients in those clusters were likely colonized or infected before entering US military hospitals. Prevalence of high-risk AMR genes is high in Afghanistan, and patients could have acquired environmental MDRO; however, those possibilities would be difficult to determine because microbiologic sampling was conducted after evacuation to LRMC. Alternatively, patients could have acquired MDR bacteria during hospitalization in Afghanistan before evacuation. However, we do not know the extent of medical treatment patients received in Kabul.

The *A. baumannii* ST2 isolates collected during this study were genetically related (11–38 SNPs) to nosocomial ventilator-associated pneumonia and bloodstream infections collected at Bagram AFB during 2019–2020 (C. Anderson et al., unpub. data). Two clusters were identified in that outbreak, comprising 10 patients from Afghanistan with *A. baumannii* ST2. The 2 clusters containing ST2 were separated by 80 allelic differences, which suggests 2 distinct strains of ST2 were involved in nosocomial transmission. Isolates within those clusters were found to differ by only 3–13 SNPs in 1 cluster and 1–4 SNPs in the other. Patients in that outbreak were transferred to Bagram AFB after initial treatment in hospitals in Afghanistan, but an outbreak investigation at Bagram AFB did identify lapses in infection control protocols that could have led to nosocomial transmission within those clusters after independent introductions of the strains from hospitals in Afghanistan. Despite the high prevalence of MDROs observed during OAR and the genetic similarities between some of the isolates, we found no evidence to suggest nosocomial spread occurred at LRMC or WRNMMC. That point highlights how crucial rapid screening, patient cohorting, and preemptive isolation upon hospital admission are to preventing nosocomial outbreaks within the military healthcare system.

We observed high rates of MDRO colonization in this study. Risk factors for MDRO acquisition include international travel, travel-related diarrhea, antibiotic use, and prolonged hospitalization (*12*). Among military personnel, Afghanistan-based personnel have been found to have a 5.5-fold higher prevalence of MDR *E. coli* colonization than US-based personnel (*13*). At LRMC, >80% of patients from Afghanistan had a positive MDRO surveillance culture, much higher than the 30% rate among US patients during the same timeframe (data not shown). Those rates are consistent with studies that describe high MDRO colonization rates or MDRO outbreaks among refugees from regions with high AMR prevalence. A 2016 study in Germany observed an MDRO prevalence in hospital-admitted refugees of up to 60.8%, and a preponderance of ESBL-producing *Enterobacterales* (*14*). Of note, >50% of the refugees in this study were from Syria or Afghanistan, and only 16.7% of nonrefugee patients were found to be positive for MRDOs. Similar observations in Germany, such as increased reports of NDM-1–producing *K. pneumoniae* in refugees and injured soldiers evacuated from Ukraine, have also been reported (*15*). Of note, prior hospitalization in Ukraine is now considered a major risk factor for MDRO colonization, because infection control measures have become inadequate due to limited resources and personnel since the 2022 invasion by Russia.

Although this study provides a unique snapshot of the AMR burden in Afghanistan, our results are limited by the small sample size and sampling bias because data were only available for refugees who were evacuated from Kabul and received care at LRMC or WRNMMC. This study also lacked detailed medical history before medical evacuation, such as the extent of hospitalization in Kabul. Further, microbiologic sampling was conducted after patients were evacuated from Afghanistan. Therefore, whether MDRO acquisition occurred during hospitalization or within the community environment because of high AMR prevalence in this region cannot be determined.

In summary, this study describes a high prevalence of MDRO among refugees from Afghanistan evacuated to US military hospitals during OAR. Molecular surveillance identified multiple high-risk clonal lineages that were characterized by extensive AMR and hypervirulence profiles and were genetically related to historical isolates collected during the war in Afghanistan. Unlike other reports that focused primarily on war wounds among military personnel injured in Afghanistan, this study uniquely provides detailed genomic AMR data, from both clinical and surveillance isolates, on refugees from Afghanistan who received care within the US military healthcare system. When viewed in the context of rising global AMR transmission and unprecedented population displacement, these data are a reminder of how crucial robust infection control measures and surveillance are to protecting public health.

Sequences have been deposited into GenBank (BioProject no. PRJNA1065584).

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Using SARS-CoV-2 Sequencing Data to Identify Reinfection Cases in the Global Emerging Infections Surveillance Program, United States

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The Centers for Disease Control and Prevention defines SARS-CoV-2 reinfection as a positive COVID-19 test result >90 days after the collection date for the initial positive test or if sequencing confirms a different lineage is causing the reinfection. Reinfection dynamics have been examined by using PCR or antigen surveillance data. We identified patients in the US Military Health System who had >1 positive SARS-CoV-2 test during March 2020–July 2022 by using whole-genome sequencing data to identify reinfection cases, then compared those data with patient demographics, symptoms, and vaccination status. We identified 267 reinfections, of which 90% were caused by the SARS-CoV-2 Omicron variant. Reinfection symptom severity correlated with initial symptom severity and time since first infection. Furthermore, we found intrahost mutation rates varied greatly in 72 cases of continuing infections with the same variant. Continued investigations of reinfections caused by emerging SARS-CoV-2 variants of concern is needed to maintain US military readiness.

Most of the human population in the United States has been infected ≥1 time with SARS-CoV-2 (*1*); much of that exposure occurred during the emergence of the SARS-CoV-2 Omicron variant. As the Omicron variant emerged globally in November 2021 and was first reported in the United States in December 2021 (*2*), the frequency of reinfections also increased (*3*). The Centers for Disease Control and Prevention (CDC) defines a reinfection as a positive COVID-19 test result >90 days after the initial positive test date (*4*). Reinfections have been examined by using PCR or antigen testing (*3*,*5*), and those studies used the >90-day definition. In addition, some studies have used genomic sequencing to define reinfections (*6*–*9*); meta-analyses have been performed in some of those works (*9*–*11*). Others have defined reinfections by using the rate of single-nucleotide variant (SNV) accumulation and have compared those rates with expected rates of mutation (e.g., 1 SNV/2 weeks) (*7*,*8*,*12*). However, reinfection dynamics might be influenced by the infecting SARS-CoV-2 variant; as few as 7 days between Omicron variant reinfection have been reported (*6*).

The Omicron variant has shown a remarkable ability to evade both vaccine-derived immune responses and those from prior infections (*3*,*13*), and waning immunity can occur faster for the Omicron variant than other variants (*9*–*11*). Hybrid immunity from antigen exposure through previous infection plus vaccination might provide better protection against the Omicron variant than infection or vaccination alone, but to a lesser extent than for other variants (*9*–*11*). Since the Omicron variant emerged and a greater understanding of different SARS-CoV-2

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variants has evolved (*14*), it is crucial to continue investigating reinfection dynamics.

Because of the variability of host immune responses to SARS-CoV-2, a single reinfection phenotype or outcome likely does not exist (*15*). Vaccine-derived neutralizing antibodies decrease over time and do not completely prevent infection (*16*), and antibody titers wane after infection as well (*17*). In addition, time intervals between exposures to different SARS-CoV-2 antigens can influence the breadth of the immune response (*18*).

We used retrospective clinical testing and sequencing data from public health surveillance specimens to characterize the dynamics of reinfections in the Military Health System (MHS), leveraging the activities of the Department of Defense (DoD) Global Respiratory Pathogen Surveillance Program (GRP-SP). We analyzed continuing infections (the same virus clade at 2 collection timepoints) and reinfections (different clades at the first and second collections timepoints). We collected demographic and symptoms data from persons who had reinfections determined by using whole-genome sequencing. Furthermore, we identified continuing infections, for which longitudinal specimens were collected, and analyzed genetic variations in those putative continuing infections over time. We conducted this study under a not research determination according to the Air Force Research Laboratory Institutional Review Board (protocol no. FWR20220269N).

Methods

Data Collection

This study encompasses the beginning of the COVID-19 pandemic through the emergence of the Omicron BA.5 variant (March 2020–July 2022). The DoDGRPSP is a global program that characterizes respiratory infections in US military service members and military healthcare beneficiaries (*19*,*20*). We used 2 approaches to capture reinfection specimens. First, a primary function of the program is to collect and test specimens weekly from a random set of 6–10 patients manifesting influenza-like illness at each of >100 DoD treatment facilities globally. Influenza-like illness is defined as a fever (≥38°C) and cough or sore throat; or fever accompanied by ≥2 symptoms associated with influenza or COVID-19; or a physician-diagnosed influenza-like illness (*20*). Each influenza-like illness encounter includes a patient questionnaire that collects demographic (sex, age, and location), symptomatic (onset, temperature/fever, cough, sore throat, fatigue, aches, chills, headache, dyspnea, loss of taste/smell, nausea, vomiting, and diarrhea), and vaccination information. In cases where questionnaires were not available or incomplete, we used codes from the International Classification of Diseases, 10th Revision, for symptoms obtained from MHS Data Repository records. Second, an additional activity of the DoDGRPSP is routine sequencing of residual clinical specimens from throughout the MHS that are positive for SARS-CoV-2, influenza, or other respiratory pathogens. Because of the unlikelihood of identifying reinfections from random encounters characterized in the influenza-like illness program alone, we augmented our dataset by including convenience samples of SARS-CoV-2–positive specimens tested at the US Air Force School of Aerospace Medicine epidemiology laboratory. We combined genotypic data from both initiatives to identify as many reinfection cases as possible.

To quantify severity, we used questionnaire and MHS Data Repository data for hospitalization, ventilation, and specificity of care. We slightly modified symptom severity indexes according to the codes from the International Classification of Diseases, 10th Revision, from previously described definitions (*21*) and grouped them as follows: asymptomatic, no symptoms; mild, any number of symptoms without fever; moderate, any number of symptoms with fever (>100.4°F); severe, respiratory distress, such as chest pain or shortness of breath; and hospitalization. If a patient record only indicated symptomatic and no specific symptoms were listed, we defined symptom severity as mild.

Laboratory Testing

We identified all SARS-CoV-2–positive specimens by PCR in the epidemiology laboratory at the US Air Force School of Aerospace Medicine by using the TaqPath COVID-19 Multiplex assay (Thermo Fisher Scientific, https://www.thermofisher.com), CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (https://www.cdc.gov), or the cobas SARS-CoV-2 test (Roche Diagnostics, https://diagnostics.roche.com). For a subset of SARS-CoV-2–positive specimens, we subsequently ran quantitative PCR (qPCR) to determine RNA genomic equivalents (*22*). We used the SARS-CoV-2 Research Use Only qPCR Primer & Probe Kit (Integrated DNA Technologies, https://www.idtdna.com), which targets 2 regions (N1 and N2) of the SARS-CoV-2 nucleocapsid gene and has an additional control that detects the human ribonuclease P gene. We used a standard curve consisting of 4 virus RNA concentrations (10^2–10^5) (*22*).

We selected specimens for sequencing if they had a qPCR cycle threshold of ≤30. We sequenced samples by using a 1,200-bp amplicon tiling approach (*23*). In brief, we extracted specimens and amplified 1,200-bp fragments, prepared libraries by using the Illumina Nextera XT Library Prep Kit, and then sequenced the libraries on an Illumina sequencing platform (Illumina, https://www.illumina.com) (*24*). We processed sequencing data by using the Mad River analysis pipeline (https://github.com/usafsam/mad_river_wf). We genotyped consensus genomes at 10× depth by using Nextclade software (*25*) and used the genotypes to differentiate between reinfections and continuing infections. We submitted consensus genome sequences to GenBank (accession nos. PP258063–640).

Data Analysis

We used the infecting SARS-CoV-2 clade that was identified through sequencing to differentiate between COVID-19 reinfection and continuing infection cases; we used those case categories to examine the influence of time, demographics, and vaccination on reinfection dynamics. In addition, we sought to define symptom outcomes for confirmed reinfection cases. For statistical analysis, we performed 1-way analysis of variance to determine differences in overall symptom severity between groups. Furthermore, we performed odds ratio analyses to determine associations between symptom severity and variables, adjusting for confounders (first infection severity, age, time since previous infection, vaccination status, time since vaccination, and sex of patient).

We defined cases as patients who had >1 positive SARS-CoV-2 test during March 2020–July 2022. We categorized each case according to the following criteria: sequenced specimens from the first and second encounters had >80% of 10× genome coverage, and the clade was determined by using Nextclade; 1 or both specimens had lower genome coverage but enough coverage to determine the clade; or the clade from the first infection was unknown, but the clade from the second infection was determined and was not present during the first infection timepoint (Figure 1). Overall, if clades from the first and second collections differed, we considered this to be a reinfection. If clades were the same at the 2 collection timepoints, we considered that to be a continuing infection.

For demographic comparisons, we matched control datasets according to the first specimen collection date; control patients had only 1 positive SARS-CoV-2 test. We performed all statistical analysis by using R version 4.2.3 (The R Project for Statistical Computing, https://www.r-project.org). We performed alignment and genomic analyses by using MEGA version 11.0.10 (https://www.megasoftware.net) and Geneious version 2023.21 (https://www.geneious.com).

Results

We identified 1,029 patients who had >1 positive SARS-CoV-2 test during March 2020–July 2022. After sequencing the positive specimens from those patients, we included 379 cases in our analyses. A total of 112 cases had the same virus genotype (continuing infection), whereas 267 were classified as reinfections (Figure 1). In addition, 338/379 cases were identified through residual clinical sample sequencing, whereas 41/379 were identified by a specimens collected through influenza-like illness surveillance and included questionnaires.

The number of days between the first and second specimen collection timepoints was determined for both continuing infection and the reinfection cases (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0231-App1.pdf). For continuing infections, the mean number of days between collections was 9 (range 1–43) days (Appendix Figure 1), except for 2 patients who were infected with the same clade >90 days apart (Appendix Table 1). For reinfections, the number of days between the first and second infections varied; 3 reinfection cases occurred <90 days apart and were caused by the Omicron clade 21K variant (Appendix Table 2, Figure 1). We determined the timeline of collection dates for first infection and reinfection in relation to the emergence of variants of concern and important vaccine dates (Appendix Figure 2).

Reinfections

We calculated the frequency of each reinfection clade according to the number of days between specimen collection dates (Figure 2). Before the Omicron variant emerged, only 9% (2/23) of reinfections occurred within 180 days of the first infection (1 each of clade 21J [Delta] and clade 20B [B.1.1]). After Omicron emerged, 21% (50/243) of reinfections occurred within 180 days. Although that difference was not statistically significant, it is consistent with the finding of an increased rate of reinfections associated with the Omicron variant, including reinfections with multiple Omicron clades (Appendix Table 3) (*26*).

We also examined an independent control dataset that was randomly matched with each first specimen collection date for reinfections, but for which patients only had 1 SARS-CoV-2–positive test. Age was the only significantly different demographic factor but was only marginally lower in the reinfection group (29.8 control vs. 27.7 reinfection; p = 0.048).

In cases where symptom severity was known for both the first and second infection, we observed that patients who experienced more severe symptoms during their first infection were more likely to have greater symptom severity upon reinfection (Figure 3). No trends were observed in continuing infections (Appendix Figure 3). Furthermore, we observed that, in severe first infection cases, symptom severity increased when reinfection occurred within 6 months, compared with reinfections that occurred 12–15 ($p = 0.0438$) or >15 ($p = 0.0366$) months after the first infection (Tukey post hoc analysis controlling for vaccination status at the time of reinfection). No hospitalized case-patients were identified in this study. Female patients had greater odds of having more severe symptoms upon reinfection than did male patients (Appendix Figure 4). Age, vaccination status, time since vaccination, and time since infection did not affect the odds of increased symptom severity upon reinfection (Appendix Table 4, Figure 4). Not enough data existed to perform analyses of clade-specific effects.

RNA Quantification

For continuing infections, we observed a decrease in the amount of virus RNA (N1 quantitation) in specimens between first and second collections; average time was 8.7 (range 1–43) days between collections (Figure 4, panel A). However, we observed no difference in the amount of virus RNA between the 2 specimen collections in reinfection cases (Figure 4, panel B). We found that vaccinated persons had significantly less virus RNA present at the time of the first infection

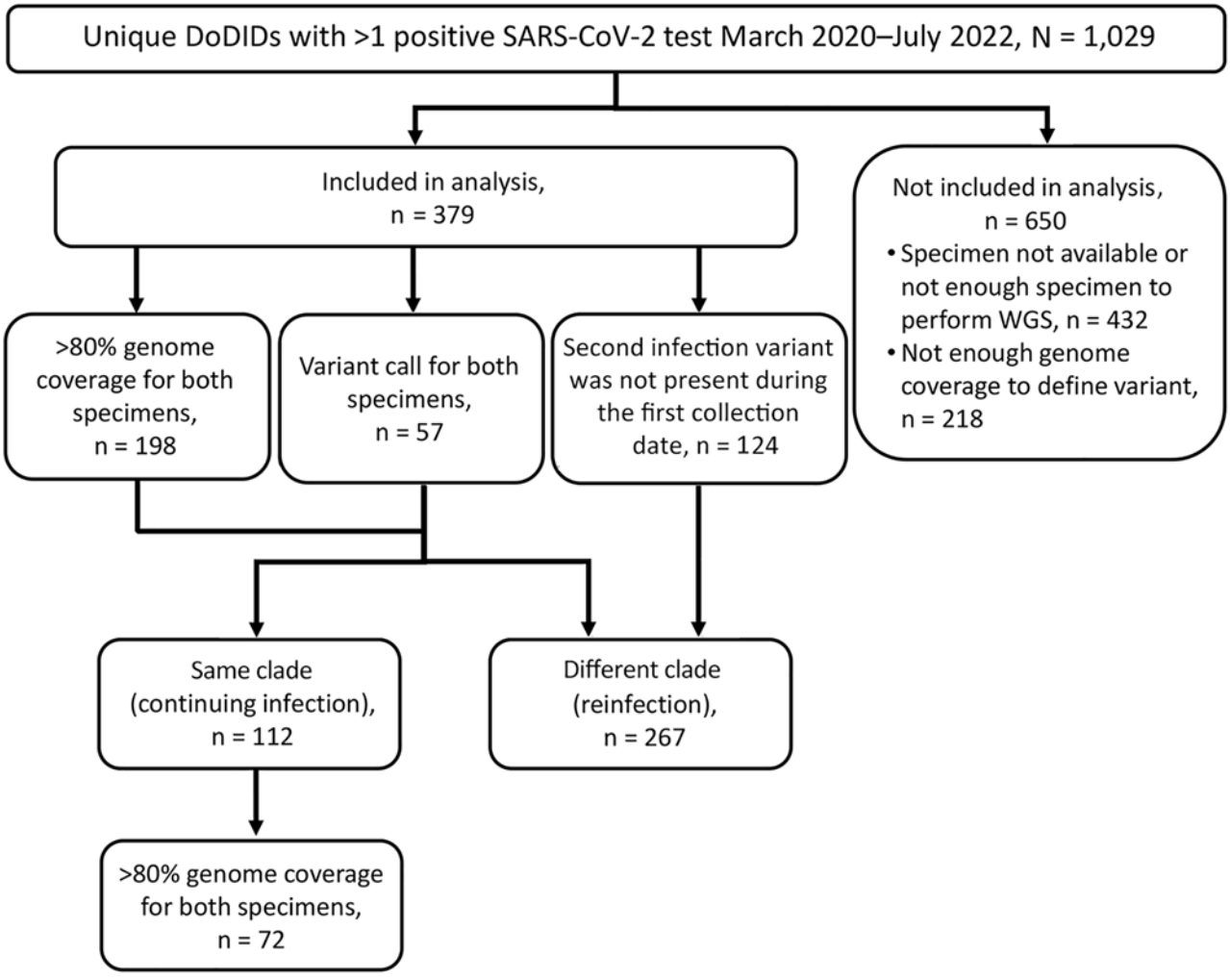

Figure 1. Case selection in study using SARS-CoV-2 sequencing data to identify reinfection cases in Department of Defense Global Respiratory Pathogen Surveillance Program, United States. Flowchart shows case selection criteria used to identify a SARS-CoV-2 reinfection according to whole-genome sequencing. Patient had either a reinfection if the Nextstrain (https://nextstrain.org) clade was different between the first and second specimen collection timepoints or had a continuing infection if the same clade was identified at both timepoints. DoDID, Department of Defense identification number; WGS, whole-genome sequencing.

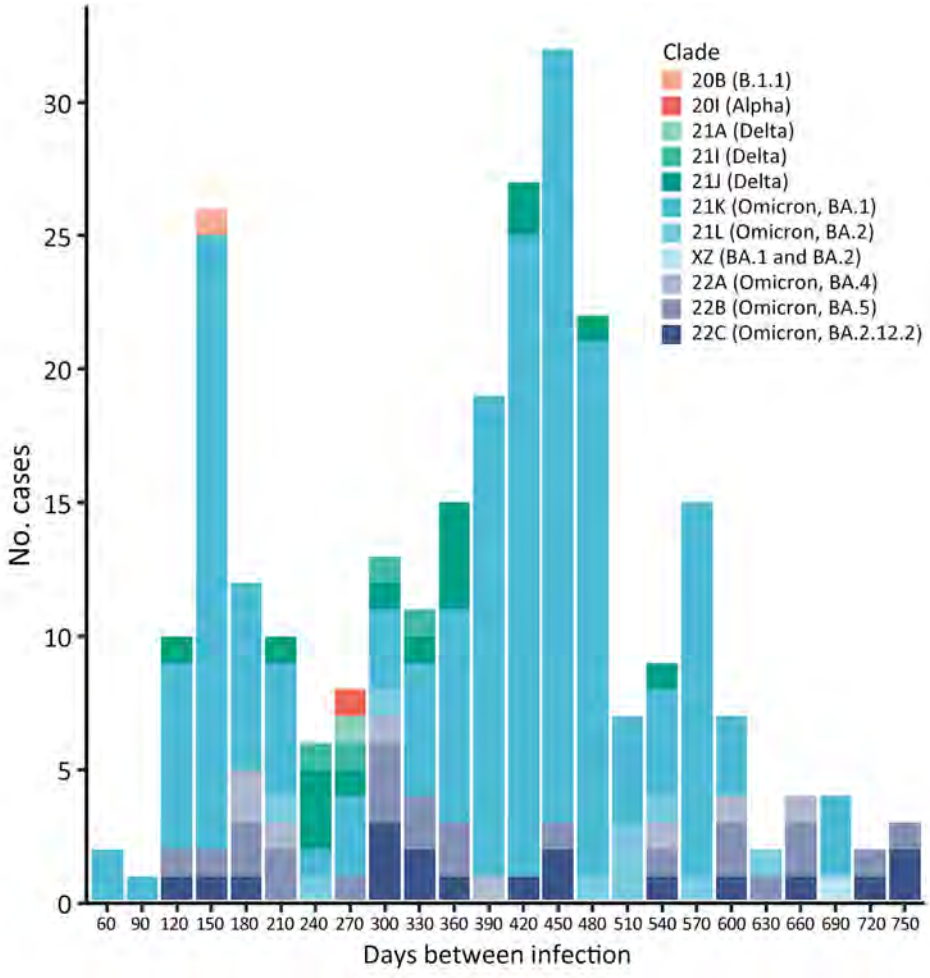

Figure 2. Number of SARS-CoV-2 reinfection cases in study using sequencing data in Department of Defense Global Respiratory Pathogen Surveillance Program, United States. Frequency of different SARS-CoV-2 variants relative to the number of days between the first and second specimen collection dates. One reinfection case was caused by a pre–variant of concern lineage, 1 case was a reinfection with an Alpha variant, and several cases were reinfections with a Delta variant. However, most reinfections were caused by Omicron variants. In addition, reinfections that occurred <90 days from the first infection were caused by Omicron 21K. One reinfection was caused by the XZ variant, a recombination of Omicron 21K and 21L.

than unvaccinated persons (Figure 4, panel C). Upon reinfection, the amount of virus RNA was significantly higher in the vaccinated group than in the unvaccinated group (Figure 4, panel C); however, most of our study population had received the COVID-19 vaccine (86% vaccinated; 1 patient received a booster dose) by that time.

Genetic Analysis of Continuing Infections

We compared sequencing data from continuing infection cases that had both the first and second collection timepoints (n = 72) to determine if nucleotide substitutions accumulated in the virus during the course of infection. The average number of days between collection dates in those cases was 7.7 (range 1–27). Using Tamura-Nei p-distance in MEGA software to quantify nucleotide changes, we found a significant relationship between the number of substitutions and time between specimen collections during continuing infections (Figure 5, panel A), which was not observed in reinfection cases (Figure 5, panel B). In addition, we saw no significant relationships between the number of nucleotide substitutions and patient sex, age, or symptom severity.

Discussion

We leveraged SARS-CoV-2 sequencing data from the DoDGRPSP, a global DoD public health surveillance network monitoring influenza-like illness, to identify reinfection cases in the MHS. The use of this type of increasingly available public health sequencing data bolsters epidemiologic investigations pertaining to clinical manifestations of disease in patients. Although many previous studies have relied on PCR surveillance data and a 90-day threshold to define a reinfection, we show that sequencing data can differentiate between first and second SARS-CoV-2 infections by identifying variant genotypes and can also support that 90-day threshold. In addition, symptom severity during the first infection tended to predict clinical manifestations upon reinfection.

The number of SARS-CoV-2 cases in the United States increased considerably during the emergence of the Omicron variant. Many of those infections were

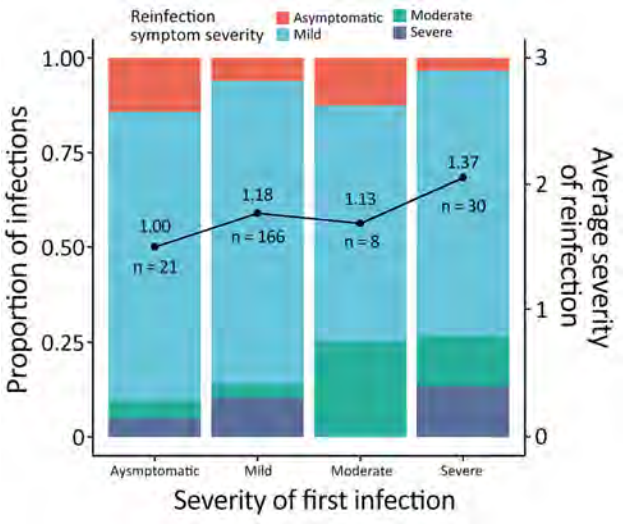

Figure 3. Reinfection symptom severity in study using SARS-CoV-2 sequencing data to identify reinfection cases in Department of Defense Global Respiratory Pathogen Surveillance Program, United States. Proportions of reinfections with different symptom severity at the second specimen collection timepoint are compared with the first specimen collection date. Symptom severity was assigned numeric values: 0, asymptomatic; 1, mild; 2, moderate; and 3, severe. Numbers along data line indicate the average infection symptom severity (top number) and number of reinfections (bottom number). Reinfection symptom severity correlated with symptom severity during the first infection. Relationships were determined by linear regression; adjusted p value = 0.0131, adjusted for sex and age.

found in persons who had already been infected with other variants and represented a substantial shift in SARS-CoV-2 epidemiology, where reinfections became commonplace (*26*). Of the 267 reinfections identified in our dataset, most occurred >90 days after the first infection; only 3 occurred under that threshold, and 2 of those 3 reinfections occurred in children (Appendix Table 2). Most reinfections in this study were caused by the 21K Omicron variant (Pangolin BA.1 lineage), which might have led to shortened time intervals between infections (*27*). As was seen for the Omicron variant, we observed an increased number of reinfections during the predominant Delta variant wave, which has been previously reported and was likely because of immune evasion over time after both vaccination and infection-acquired immunity (*3*). The amount of time needed for Omicron reinfection in this study was less than that seen for other variant waves, consistent with the shortened timeframe associated with the Omicron variant (*6*; M. Stegger et al., unpub. data, https://doi.org/10.1101/2022.02.19.22271112).

Previous studies have shown that increased disease severity is expected when reinfections occur in patients <90 days from the first positive test date, particularly when the first infection was also critical or severe (*28*). Our findings support this result and suggest that, in reinfections defined by using sequencing data, symptom severity during the first infection correlated with the symptom severity during reinfection. Our findings also showed an influence of time between the first and second infections; it was more likely for patients to have increased symptom severity upon reinfection if the first infection was severe, particularly if reinfection occurred within 6 months. Our data did not have enough variability to determine differences according to the reinfecting virus clade; further investigation will be required because little is known about how different variants might contribute to SARS-CoV-2 reinfection rates.

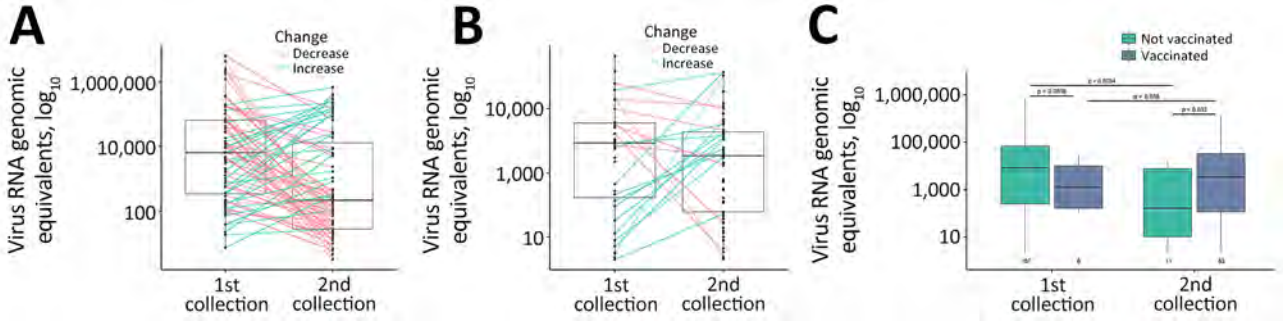

Figure 4. Virus load in patient specimens in study using SARS-CoV-2 sequencing data to identify reinfection cases in Department of Defense Global Respiratory Pathogen Surveillance Program, United States. Virus load was determined in specimens collected during the first and second timepoints by using quantitative PCR of the N1 region of the SARS-CoV-2 nucleocapsid gene for patients who had continuing infections (A) and reinfections (B) or who were vaccinated versus unvaccinated (C). Middle horizontal lines within each box plot are the median virus RNA genomic equivalents, outer horizontal lines indicate the interquartile range, and whiskers (vertical lines) indicate minimum and maximum data points. A) Significant decrease in virus load was observed between the first and second collection timepoints for patients who had continuing infections (p = 0.039 by Student *t*-test); average number of days between collection dates was 8.7 (range 1–43) days. B) No significant difference in virus RNA load was observed between the first and second collection points for patients who had reinfections (p = 0.290 by Student *t*-test). C) First collection group shows all first infections for patients who had either continuing or reinfections. Second collection group shows only reinfections. Numbers under box plots indicate the number of cases within each group.

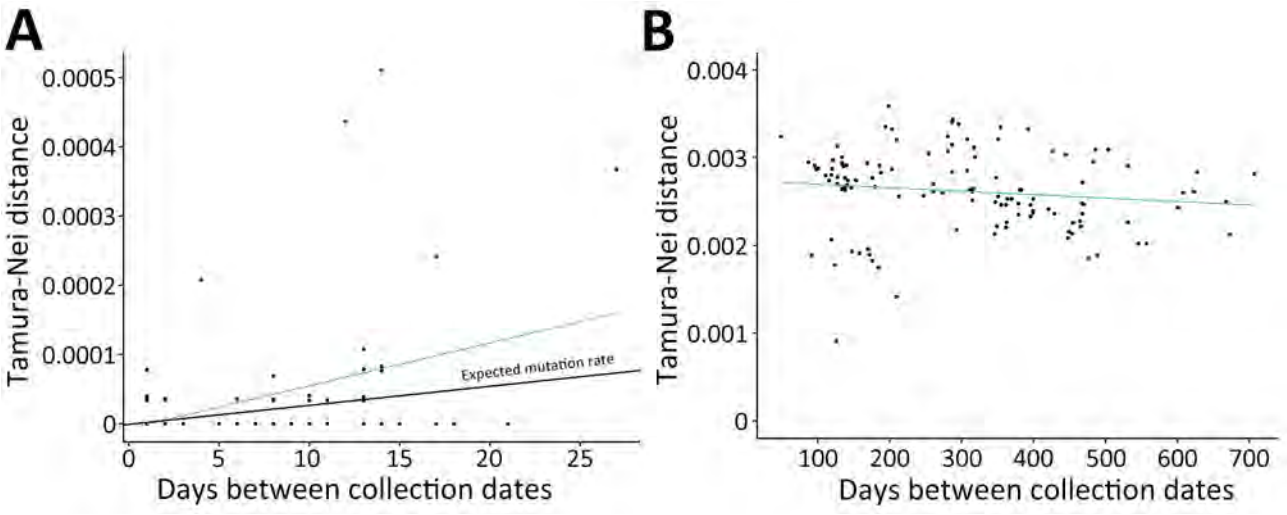

Figure 5. SARS-CoV-2 nucleotide changes in study using sequencing data to identify reinfection cases in Department of Defense Global Respiratory Pathogen Surveillance Program, United States. Tamura-Nei p-distances were determined relative to the number of days between specimen collection dates for continuing infections (A) and reinfections (B). A) Number of nucleotide substitutions correlated with the amount of time between specimen collections in patients who had continuing infections (p = 0.0021). Expected SARS-CoV-2 mutation rate was 1 single nucleotide variant per 2 weeks. B) No relationship was observed between number of nucleotide substitutions and time in reinfection cases (p = 0.137).

We did not observe a relationship between vaccination status and symptom severity in reinfection cases. During the first infection, the amount of virus RNA in vaccinated persons was significantly lower than that in unvaccinated persons. However, vaccinated persons had higher amounts of virus RNA detected after reinfection than unvaccinated persons. That finding might suggest that qPCR is a poor method to determine infectious virus burden. Alternatively, the observed increase in virus RNA in vaccinated persons in this study might have been caused by immune imprinting from the initial monovalent vaccine received by the study population (*29*,*30*). Studies have shown that hybrid immunity can influence immune response upon virus reexposure (*31*).

Before the Omicron variant emerged, 1 study used a substitution rate of >1 SNV/2 weeks as a threshold to define reinfection by using SARS-CoV-2 sequencing data, observing 18 reinfections 116–342 days apart (*7*). Also, in that study, continuing infections showing substitution rates <1 SNV/2 weeks were observed >90 days apart (*7*). After the Omicron variant emerged, that same substitution rate measure was used to document reinfections involving the same Omicron clade, including some reinfections that had only 27 days between specimen collection dates (*8*). Using clade definitions to define reinfections in this study, we found many continuing infections in which the mutation rate for the SARS-CoV-2 virus was greater than expected. Accordingly, if we had used the previously reported substitution rate threshold of >1 SNV/2 weeks (*7*), 23 of our continuing infections would have been identified as reinfections, some having only a 1-day difference between collection dates. In this study, we excluded 7 cases that were inferred to not be continuing infections but were more likely co-infections by very closely related clades. Our findings highlight several considerations when using SARS-CoV-2 sequencing data to define reinfection status. Although the average mutation rate for SARS-CoV-2 viruses is 1 SNV/2 weeks, considerable interhost variation is likely because the virus interacts with more complex immune responses in populations continually exposed to emerging clades (*32*) and because patients might be immunocompromised (*33*). It will be crucial to continue investigating how emerging clades cause reinfections, which might shift our current understanding and definition of reinfection.

The first limitation of our study is that we leveraged a public health surveillance system that collects data on MHS beneficiaries who manifest influenza-like illness at clinics, as well as opportunistic sampling of SARS-CoV-2–positive specimens. Thus, this study is not a clinical observation study following persons over time, which would be a more powerful study design to assess reinfection and continuing infection dynamics. The data were collected without knowledge of prior infection history, except for those data that were captured in the medical and testing records available for public health surveillance. Using molecular testing data combined with our inability to gather symptom onset information

for every case limits our ability to control for when samples were collected. Second, analysis of the military population is not generalizable because of health, age, and gender distribution limitations. Although any active-duty military, military dependent (child or spouse), or retired military member could be included in the analysis, most (66%) patients were male, and the average age was 29.7 years. The surveilled populations consisted of generally healthy persons, which limits our analysis of any underlying illnesses. Furthermore, active-duty members were required to receive a COVID-19 vaccine during this study period. Therefore, the percentage of vaccinated persons in this study (86% vaccinated by their second collection date) was significantly higher than the percentage of vaccinated persons nationwide (62%–63% in January 2022; $p<0.0001$ by χ^2 test) (*34*). Vaccination reduces symptom severity (*35*), which might skew the data toward persons who have less severe symptoms.

In conclusion, we used sequencing data to differentiate SARS-CoV-2 variant genotypes and analyze infection dynamics of emerging clades in a military population. Symptom severity during the first infection tended to predict clinical severity after reinfection. Continued investigations of reinfections caused by emerging SARS-CoV-2 variants of concern by using advanced molecular methods, such as whole-genome sequencing, is needed to maintain DoD's military readiness, and the additional clinical information gathered will benefit the general population.

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Common Patterns and Unique Threats in Antimicrobial Resistance as Revealed by Global Gonococcal Surveillance, 2014–2022

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The rapid emergence of antimicrobial-resistant strains of *Neisseria gonorrhoeae* threatens treatment options and control efforts. The Uniformed Services University Gonococcal Reference Laboratory and Repository of the Global Emerging Infections Surveillance Program receives isolates from several geographically distinct regions worldwide. We analyzed 962 isolates collected during 2014–2022 for genomic and phenotypic antimicrobial resistance. Resistance to antimicrobial drugs previously used for gonococcal infections was high, but of most concern were increases of resistance to currently used antibiotic drugs, such as extended-spectrum cephalosporins and the alternative antibiotic treatment gentamicin. The percentage of isolates with reduced susceptibility to ceftriaxone was 3.6%, to cefixime was 2.5%, and to gentamicin was 15.0%. Although isolates were collected from populations of limited diversity, 706 (73.4%) of isolates demonstrated novel multiantigen sequence types, and 225 (23.4%) had novel multilocus sequence types. Continued surveillance of *N. gonorrhoeae* is essential to monitoring the prevalence and spread of resistant organisms worldwide.

Neisseria gonorrhoeae infections cause substantial illness globally, and control is challenged by increasing antimicrobial resistance. The World Health Organization (WHO) reported 82.4 million new *N. gonorrhoeae* infections worldwide among persons 15–49 years of age (*1*). In the United States, an estimated 1.5 million new cases of gonorrhea are reported each year (*2*).

Gonococcal urogenital tract infections can cause severe complications, especially in women, who are often asymptomatic and go undiagnosed. Untreated cervical infections can cause upper genital tract

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disease, such as pelvic inflammatory disease, chronic pelvic pain, and ectopic pregnancy, and also increases the risk for tubal infertility. Urethral infections in men can ascend to cause epididymitis or orchitis; however, unlike cervical infections, urethral infections are usually symptomatic. The resulting discharge and dysuria increase the likelihood that male patients will seek testing and treatment.

Effective infection control is challenged by underdiagnosis of asymptomatic infections, lack of point-of-care diagnostics, and increasing persistent antimicrobial resistance. *N. gonorrhoeae* has developed resistance to all antibiotic drugs that have been used for routine treatment because of its ability to readily acquire genes through horizontal gene transfer or spontaneous mutations. The prevalence of antimicrobial resistance (AMR) within *N. gonorrhoeae* strains has steadily increased across the antibiotic era, necessitating frequent changes in treatment recommendations. The initial emergence of high-level penicillin and tetracycline resistance was followed by the introduction of fluoroquinolones for gonorrhea treatment in the mid-1980s, which were subsequently removed from treatment in 2007 (*3*). Dual therapy using extended-spectrum cephalosporins (ESCs) and azithromycin then became the primary recommended therapy for a decade. Azithromycin was removed in 2021 because of increasing resistance, leaving only ESCs for first-line treatment of gonorrhea. Globally, ceftriaxone is the sole remaining primary therapy for first-line treatment of gonorrhea in most guidelines (*4*–*6*). However, isolates with reduced susceptibility to ceftriaxone have proliferated worldwide, and multidrug-resistant, ceftriaxone-resistant strains have been reported in several countries (*7*–*10*), threatening simple outpatient therapy.

Because of the threat of untreatable gonorrhea, *N. gonorrhoeae* is classified by the Centers for Disease Control and Prevention as an urgent threat (*11*) and by WHO as a high-priority pathogen (*12*) for which new treatments are critically needed. Global rates of *N. gonorrhoeae* infections have been reported since 1992 through the WHO Enhanced Gonococcal Antimicrobial Surveillance Programme (EGASP). Data for 2017–2018 from 73 countries demonstrated resistance to ESCs of 0%–22%, azithromycin resistance of 0%–60%, and ciprofloxacin resistance of 0%–100% (*13*). Although several countries report AMR data to the EGASP, *N. gonorrhoeae* surveillance data from many global regions, such as Central America, Eastern Europe, Southeast Asia, sub-Saharan Africa, and the Eastern Mediterranean, remain scarce.

The Sexually Transmitted Infection (STI) National Strategic Plan for the United States (2021–2025) recognizes the need to improve STI prevention at the local, state, and federal levels. The plan also recommends that specific groups, such as the military and fraternal organizations, include services that address men's sexual health and their role in transmitting STIs (*14*). Military service members are at high risk for STI because of social demographics including age; however, factors such as increased alcohol consumption, diversification of sexual networks, and infrequent condom use also exacerbate risk in military populations (*15*). In addition, sexual assault, which carries an inherent risk for STI, has been reported in 1.0% of men and 4.9% of women in military service (*16*).

In alignment with the National Action Plan for Combating Antibiotic-Resistant Bacteria (*14*), and to monitor this urgent, ever-changing AMR threat, the Uniformed Services University (USU), in collaboration with the Armed Forces Health Surveillance Division's Global Emerging Infections Surveillance (GEIS) Branch, established the USU Gonococcal Reference Laboratory and Repository (GC Repository) within the USU Department of Microbiology and Immunology (Bethesda, Maryland, USA). This report analyzes trends in the susceptibility of *N. gonorrhoeae* isolates from different geographic regions to 8 different antibiotic drugs during 2014–2022 as part of the GEIS STI surveillance program. We also report the distribution of key alleles on the basis of genomic analysis to help define the prevalence of specific AMR determinants in different geographic regions. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25.

Methods

The GEIS STI initiative was established in 2010 to improve the health of the US armed forces and support force health protection decision-making. The GC Repository was established in 2014 to serve as a central entity for confirmatory testing and both phenotypic and genotypic characterization (*17*–*19*). As part of the surveillance program, a proficiency testing program was also established for quality assurance of partner laboratory methods for *N. gonorrhoeae* AMR testing.

Sampling Methods

We collected samples from persons enrolled in clinical care or public health surveillance activities during 2014–2022, which included military populations,

civilians, and high-risk populations from 5 geographic regions. We gram-stained from urethral, vaginal, cervical, pharyngeal, or rectal swab samples, plated them on selective media such as modified Thayer-Martin agar, and incubated for 24 hours at 37°C in 5% CO_2 or in a candle jar. We froze isolates of presumptive *N. gonorrhoeae* in 25% glycerol and tryptic soy broth and shipped to the GC Repository. We assessed AMR using Etest (bioMérieux, https://www.biomerieux.com) (Appendix, https://wwwnc.cdc.gov/EID/article/30/14/24-0296-App1.pdf) and performed agar dilution to confirm MICs for isolates with reduced susceptibility to azithromycin, ceftriaxone, cefixime, and gentamicin.

Reference Laboratory Testing

As of December 2023, the GC Repository received a total of 1,244 presumptive isolates from 6 countries: Thailand (n = 557), the Philippines (n = 35), Ghana (n = 73), Peru (n = 237), Kenya (n = 211), and Georgia (n = 95). We confirmed isolates by culture on modified Thayer-Martin agar, Gram staining, oxidase test positivity, superoxol test positivity, and API NH biochemical test (bioMérieux). We used detection of the *porA* pseudogene to resolve inconclusive API NH test results (Appendix). We determined MICs for all 962 isolates (Appendix).

Whole-Genome Sequencing and Bioinformatic Analysis

We sent *N. gonorrhoeae* isolates to the Walter Reed Army Institute of Research's Multidrug-Resistant Organism Repository and Surveillance Network (Silver Spring, Maryland, USA) for whole-genome sequencing (Appendix) and genotypic characterization. Multilocus sequence typing (MLST) was performed in silico using the *N. gonorrhoeae* scheme curated by Maiden (*20*). We performed additional in silico molecular typing using *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) and *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) with ngmaster version 1.0.0 (*21*) (Appendix).

Results

Of the 1,244 frozen suspensions of presumptive *N. gonorrhoeae* from 5 geographic regions received by the GC Repository, 962 (77.3%) were confirmed as *N. gonorrhoeae* isolates. Among isolates for which the type of sample was recorded, most came from urethral swab samples taken from men. Limited, inconsistent demographic data were available to the partner laboratories involved in public health surveillance.

Antimicrobial Susceptibility Testing

We compiled phenotypic AMR data for all 962 isolates (Table 1). Benzylpenicillin resistance was most commonly observed (917/962 [95.3%]), followed by tetracycline (902/962 [93.7%]) and ciprofloxacin (882/962 [91.7%]). Resistance to those antibiotic drugs varied among sites; ≈50% resistance to each of those antibiotic drugs was observed in Georgia, whereas other sites exhibited up to 90% resistance. Elevated MICs (IR≥1; R≥2) to azithromycin was found in 10 isolates, 8 of which had azithromycin MICs of 1 and 1.5 μg/mL (2 from Georgia, 2 from Peru, and 4 from Thailand); 2 isolates had MICs >256 μg/mL (Kenya). Among isolates from Kenya, 5 exhibited reduced susceptibility to the ESCs: 1 for cefixime, 2 for ceftriaxone, and 2 for both cefixime and ceftriaxone. Similarly, 11 Georgia isolates exhibited reduced susceptibility to ESCs. We observed that 84% (809/962) of the isolates were susceptible to gentamicin (S≤4; IR = 8–16; R≥32). Of the remaining isolates, 7 (0.7%) had MICs of 16 μg/mL (4 from Peru, 2 from Georgia, and 1 from Ghana). All 962 isolates were susceptible to spectinomycin. Multidrug resistance was common among all international collection sites. The frequency of resistance to any 3 antibiotic drugs ranged from 11% (Ghana) to 92% (Peru).

Table 1. Summary of phenotypic antimicrobial resistance in study of common patterns and unique threats in antimicrobial resistance as demonstrated by global gonococcal surveillance*

Region	Isolates with reduced susceptibility or resistance, no. (%)						
	Tetracycline	Benzylpenicillin	Ciprofloxacin	Azithromycin	Cefixime	Ceftriaxone	Gentamicin
Thailand, n = 516	500 (96.9)	502 (97.3)	500 (97)	4 (0.77)	2 (0.4)	16 (3.1)	31 (6.0)
Ghana, n = 19	19 (100)	19 (100)	17 (89.5)	0	1 (5.3)	0	7 (36.8)
Peru, n = 208	195 (93.7)	205 (98.5)	186 (89.4)	2 (0.96)	7 (3.4)	3 (1.4)	63 (30.3)
Nairobi, Kenya, n = 27	27 (100)	26 (96.3)	23 (85.1)	0	0	0	1 (3.7)
Kisumu, Kenya, n = 110	108 (98.2)	105 (95.5)	106 (96.4)	2 (1.8)	3 (2.72)	4 (3.63)	26 (23.6)
Uganda, n = 10	9 (90)	10 (100)	9 (90)	0	0	0	2 (20)
Georgia, n = 72	44 (61.1)	50 (69.4)	41 (56.9)	2 (2.8)	11 (15.2)	11 (15.2)	16 (22.2)
Total, N = 962	902 (93.7)	917 (95.3)	882 (91.7)	10 (1.02)	24 (2.5)	34 (3.6)	146 (15.2)

*MICs interpreted according to Clinical and Laboratory Standards Institute criteria when available (*22*). CLSI resistance breakpoints used for penicillin (I>0.06; R≥2.0 μg/mL), tetracycline (I>0.25; R≥2.0 μg/mL), and ciprofloxacin (I>0.06; R≥1.0 μg/mL) (*22*). Gonococcal Isolate Surveillance Project breakpoints used for azithromycin (I≥1; R≥2.0 μg/mL), cefixime (I>0.06; R≥0.25 μg/mL), and ceftriaxone (I>0.06; R≥0.125 μg/mL) (*23*,*24*), because CLSI has not established criteria for resistance to those antimicrobial drugs. Gentamicin breakpoints (I≥8–16 μg/mL; R I≥32.0 μg/mL) were determined according to research published by the Centers for Disease Control and Prevention (*25*).

Table 2. Presence of antimicrobial-resistant genetic determinants in study of common patterns and unique threats in antimicrobial resistance as demonstrated by global gonococcal surveillance*

	No. (%) isolates								
	Tetracycline resistance		Benzylpenicillin resistance		Ciprofloxacin resistance		Azithromycin resistance	Cefixime and ceftriaxone resistance	
Region	V57	*tetM*	β-lactams	*ponA*$_{L421P}$	*gyrA*	*parC*	*mtrD*	*Mtr*	*penA*
Thailand, n = 516	506 (98)	449 (87)	*bla*$_{TEM-1}$, 327 (63.4); *bla*$_{TEM-135}$, 77 (15)	80 (15.5)	S91, D95, 318 (61.6)	D86, 217 (42); S87, 218 (42.2); E91, 21 (4); S88, 8 (1.5)	*MtrD* S821A K823E, 7 (1.35)	Internal stop codon, 27 (5.3)	I312M V316T G545S, 2 (0.38)
Ghana, n = 19	19 (100)	15 (79)	*bla*$_{TEM-1}$, 13 (68.4)	16 (84.2)	S91, D95, 17 (89.5)	D86, 3 (17.6); S87, 12 (70.6); E91, 1 (5.9)	0	G45D, 7 (36.8)	I312M V316T G545S, 1 (5.26)
Peru, n = 208	208 (100)	87 (41.8)	*bla*$_{TEM-1}$, 111 (53.3); *bla*$_{TEM-135}$, 13 (6.25); *bla*$_{TEM-22}$, 1 (0.48)	120 (57.6)	S91; D95, 188 (90.3)	D86, 52 (28); S87, 46 (24.7)	*MtrD* mosaic 2, *MtrR* mosaic 2, 2 (0.96)	G45D, 21 (10)	I312M V316T G545S, 17 (8.17)
Nairobi, Kenya, n = 27	27 (100)	23 (85.2)	*bla*$_{TEM-1}$, 22, 81.5)	12 (44.4)	S91, D95, 21 (77.7)	D86, 1 (4.76)	0	0	0
Kisumu, Kenya, n = 110	110 (100)	99 (90)	*bla*$_{TEM-1}$, 32 (29); *bla*$_{TEM-135}$, 3 (2.72); *bla*$_{TEM-239}$, 5 (4.54)	66 (60)	S91, D95, 106 (96.3)	D86, 10 (9); S87, 9 (8.2); E91, 65 (59)	23s rDNA A2045G, 2 (1.8)	A39, 84 (76.5); G45, 2 (1.8); D79, 11 (10); M197, 1 (0.9)	A501, F504, A516 N512, 4 (3.6)
Uganda, n = 10	10 (100)	10 (100)	*bla*$_{TEM-1}$, 8 (80); *bla*$_{TEM-135}$, 1 (10); *bla*$_{TEM-239}$, 1 (10)	5 (55)	S91, D95, 10 (100)	D86, 4 (40); S87, 2 (20); E91, 4 (40)	0	A39, 8 (80); D79, 2 (20)	F504, 10 (100); A516, 10 (100)
Georgia, n = 72	47 (65.3)	14 (19.4)	*bla*$_{TEM-1}$, 11 (15.3)	40 (55.5)	S91, D95, 41 (57)	D86, 12 (19.5); S87, 31 (75.6); E91, 12 (29.3)	*MtrD* mosaic 2, *MtrR* mosaic 2, 2 (2.7); *MtrD* S821A K823E, 5 (6.9)	A39, 28 (38.9); G45, 6 (8.3); D79, 14 (19.4); M197, 2 (2.7)	I312M V316T G545S, 9 (12.5%)
Total, N = 962	928 (96.4)	697 (72.5)	*bla*$_{TEM-1}$, 524 (54.5); *bla*$_{TEM-135}$, 94 (9.77); *bla*$_{TEM-239}$, 6 (0.62); *bla*$_{TEM-22}$, 1 (0.10)	339 (35.2)	S91, D95, 701 (72.9)	D86, 299 (31); S87, 318 (33); E91, 103 (10.7)		A39, 641 (66.6); G45D, 36 (3.74)	

*Percentages calculated on total number of *Neisseria gonorrhoeae* isolates confirmed for the country.

Molecular Determinants of AMR and Genomic Characterization

All 676 isolates with high-level tetracycline resistance (Tet^R) (MIC >8 μg/mL) (676/962 [97.7%]) isolates) harbored the *tetM* gene and the *rpsJ* V57M mutation, whereas isolates with MICs of 0.5–3 μg/mL did not carry the *tetM* gene but had the *rpsJ* V57M mutation (Table 2). Among the 917 benzylpenicillin-resistant isolates carrying β-lactamase–producing plasmids, 4 different β-lactamase resistance genes were detected; *bla*$_{TEM-1}$ was detected in 57.1% of isolates and *bla*$_{TEM-135}$ was detected in 10.3% of isolates. One isolate from Peru harbored the *bla*$_{TEM-22}$ plasmid. The *bla*$_{TEM-239}$ plasmid was present in 6 isolates from East Africa (1 from Uganda and 5 from Kenya).

In contrast, the number of isolates harboring chromosomally mediated determinants of AMR varied widely. Mutations in the *mtrR* gene (G45D) were

present in 3.7% of isolates, and mutations in the *mtr* promoter region (−35Adel) were present in 10% percent of isolates, whereas the A39THTH mutation was more prevalent (66.6% of isolates). Overall, we identified MtrR disruptions in 12% of isolates. The *ponA*L421P mutation was found in 35.2% of isolates, whereas *porB* mutations A121N, G120K, and A121D were less common and found in 2.2% of isolates (A121N), 8.6% of isolates (G120K), and 3.8% of isolates (A121D). All isolates with reduced susceptibility or resistance to ciprofloxacin (MICs 1 to >32 μg/mL) harbored S91F and D95G/A/N mutations in *gyrA*. Mutations in *parC* (D86, S87, or E91K) were found in 74.8% of isolates. We found the *fusA* A563V mutation, which confers reduced susceptibility to gentamicin, in 1 isolate from Peru (*26*,*27*).

Isolates with reduced susceptibility to azithromycin harbored myriad chromosomal resistance determinants. Mosaic *mtrD* and *mtrR* alleles were found in 4 isolates (2 from Georgia and 2 from Peru). One of those Georgia isolates also carried the *penA* mosaic allele XXXIV. One isolate from Peru carried the *mtrR* mosaic allele but lacked the *mtrD* mosaic allele. We found that 7 isolates from Thailand harbored *mtrD* S821A K823E mutations associated with azithromycin resistance (*28*), but only 4 of the 7 isolates had reduced susceptibility to azithromycin (MICs ≥1 μg/mL). The 23s rDNA A2045G mutation was present in 2 isolates from Kenya (MIC >256 μg/mL). Examination of ESC resistance determinants showed that 32 of the 962 isolates carried mosaic *penA* alleles. We detected 4 mosaic *penA* alleles: XXXIV (24 isolates [2.5%]), 166 (2 isolates [0.2%]), and 217 (5 isolates [0.5%]); 1 isolate (0.1%) had a novel allele. Of those 32 isolates, 29 had reduced susceptibility to cefixime and 11 had reduced susceptibility to both cefixime and ceftriaxone. We found that 5 isolates with reduced susceptibility to both ceftriaxone and cefixime did not carry a mosaic *penA* allele.

We monitored the type of *porB1* allele present, which encodes the major outer membrane porin (PorB). *N. gonorrhoeae* strains express 1 of 2 *porB1* alleles. The *porB1A* allele is associated with strains that cause disseminated infection, whereas strains with *porB1B* more frequently cause localized infections (*29*). Although *porB1B* strains are usually more common, the frequencies of *porB1A* and *porB1B* alleles were similar among the 962 isolates, except for Thailand, Georgia, and Peru isolates. Thailand isolates cultured before 2016 (n = 88) carried *porB1A* more frequently (81/88 [92.0%]) than *porB1B* (7/88 [8.0%]). After 2016, *porB1B* strains were isolated more often in Thailand. Among isolates from Georgia, only 5 (6.9%) isolates expressed *porB1A*, and in Peru, 62 (47.3%) of 131 *porB1A*-expressing isolates were collected during 2014–2017, compared with 14 isolates (18.2%) of 77 *porB1A* strains collected during 2018–2022.

Molecular typing identified 98 NG-MAST, 198 MLST, and 199 NG-STAR sequence types (STs) among the 962 isolates (Figure 1). We found that 706 isolates belonged to a novel NG-MAST ST. The most common defined NG-MAST STs were ST6211 (n = 36), ST8058 (n = 21), ST2318 (n = 14), ST5573 (n = 12), and ST681 (n = 10). We identified novel MLST STs in 225 of 962 isolates. The most common MLST STs were ST1587 (n = 133), 1588 (n = 80), 7363 (n = 55), 8756 (n = 55), 8143 (n = 44), and 7827 (n = 40). Those isolates were all ciprofloxacin resistant. Using NG-STAR, we identified 173 novel types. The most common defined NG-STAR STs were ST719 (n = 69), ST271 (n = 24),

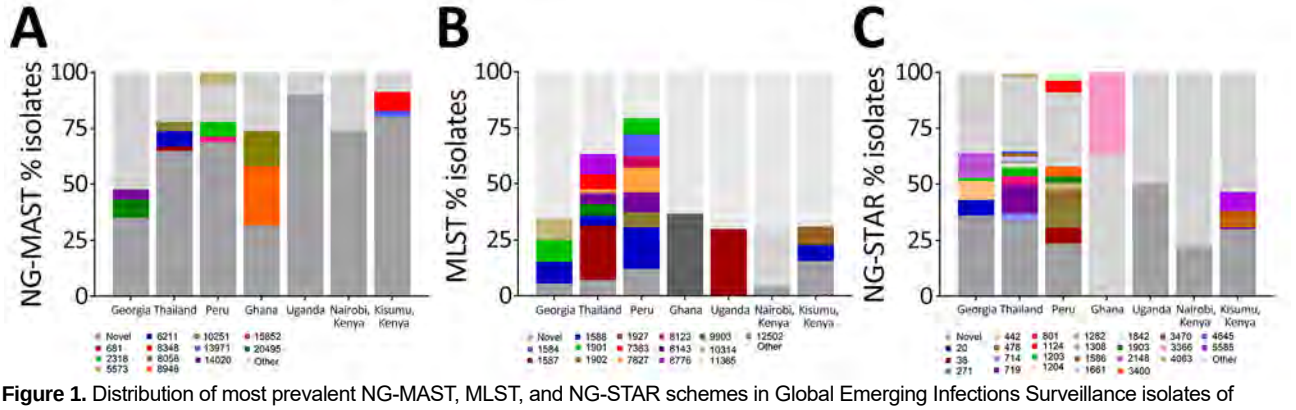

Figure 1. Distribution of most prevalent NG-MAST, MLST, and NG-STAR schemes in Global Emerging Infections Surveillance isolates of *Neisseria gonorrhoeae* received at Uniformed Services University, Bethesda, Maryland, USA, from sites outside the United States (n = 962) in study of common patterns and unique threats in antimicrobial resistance as demonstrated by global gonococcal surveillance. A) Percentage of isolates assigned to the most common NG-MAST types in each region. B) Percentage of isolates assigned to the most common MLST types in each region. C) Percentage of isolates assigned to the most common NG-STAR types in each region. MLST, multilocus sequence typing; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing; NG-STAR, *N. gonorrhoeae* sequence typing for antimicrobial resistance.

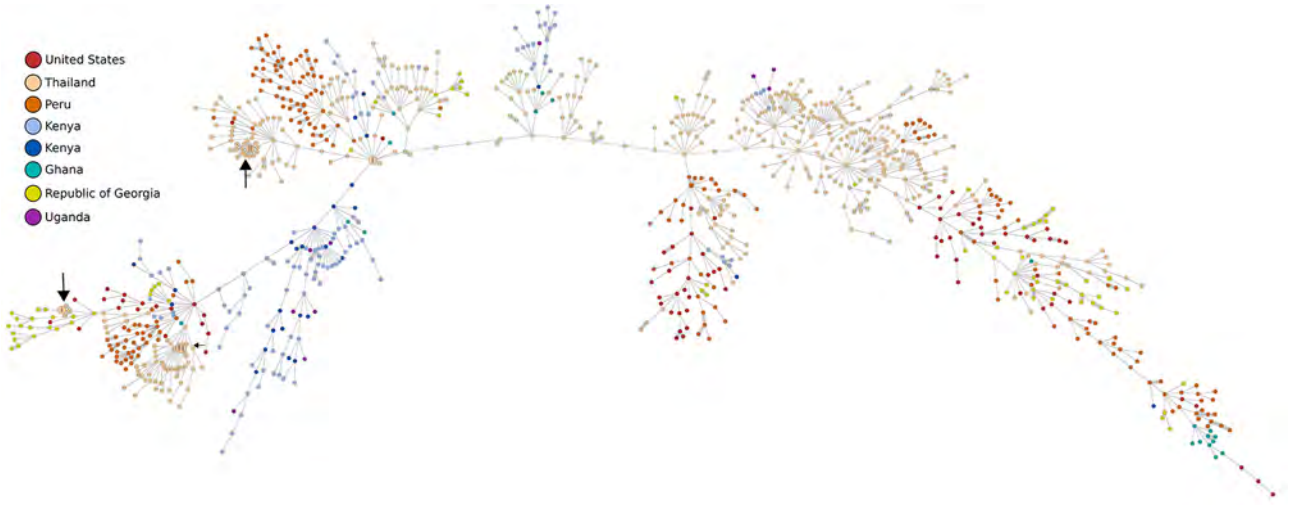

Figure 2. Minimum-spanning tree showing genome-based genetic relatedness of all *N. gonorrhoeae* isolates received at Uniformed Services University (n = 1,044), Bethesda, Maryland, USA, in study of common patterns and unique threats in antimicrobial resistance as demonstrated by global gonococcal surveillance. Tree was generated using core genome multilocus sequence typing. Each circle represents ≥1 isolates; isolates with 1–10 allelic differences are emphasized by gray shading around the lines and are considered highly genetically related with suspicion of nosocomial origin. Isolates are colored corresponding to their country of origin. Possible clonal isolates are shown with black arrows.

ST801 (n = 23), and ST1203 (n = 22). The distribution of NG-MAST, MLST, and NG-STAR STs also revealed that certain STs are specific to various regions (Figure 1). We generated a minimum-spanning tree on the basis of core genome MLST of all isolates, categorized by geographic location, to examine genomic diversity and possible clonal spread (Figure 2). Isolates from Thailand clustered into 4 major groups, and 3 appear to be clonal isolates (black arrows). Georgia isolates also clustered, but some were closely related to isolates from Thailand (≈300 core genome allele differences). Isolates from Peru grouped into 5 clusters.

Discussion

Increasingly resistant *N. gonorrhoeae* infections present a major public health burden for civilian communities, military force health protection, and US military readiness. Surveillance programs incorporating specimen culture are critical for linking genotypic and phenotypic AMR data to enable AMR prediction. The WHO EGASP program provides data from 68 countries in 6 regions as of 2018 (*30*). The GEIS network fills some key gaps in surveillance, including Eastern Europe and East Africa, where *N. gonorrhoeae* AMR data remain scarce.

This study reports phenotypic and genotypic analyses of geographically and temporally diverse NG isolates collected through the GEIS STI surveillance program. Isolates from international sites displayed high frequencies of resistance to benzylpenicillin, tetracycline, and ciprofloxacin, ranging from 50% to 100%. Those data are similar to data from previously published literature. Investigators from Peru identified ≈95% resistance to ciprofloxacin (*31*–*33*), whereas reduced susceptibility or resistance to penicillin was observed in 99.4% of isolates and to tetracycline in 94.5% of isolates (*31*). In Peru, 76% of isolates were reported to have reduced susceptibility to gentamicin (*31*). In comparison, however, our study identified reduced susceptibility to gentamicin in ≈15% of isolates. Regional differences in AMR patterns can be driven by community-based factors, including limited access to care and lack of available diagnostics, leading to empiric treatment. Similarly, lack of access to recommended antibiotic drugs and readily available access to other over-the-counter antibiotic drugs in the absence of valid healthcare encounters can also drive selection for *N. gonorrhoeae* AMR. For example, ciprofloxacin is still used empirically to treat STIs in Peru and other countries in Latin America. In Uganda, cefixime therapy is recommended but not easily available (*34*).

In Georgia, the recommended treatment for *N. gonorrhoeae* remains 1 g ceftriaxone plus 2 g azithromycin. Isolates from Georgia displayed lower frequencies of resistance to penicillin, tetracycline, and ciprofloxacin (≈50%) than did isolates from Africa and Asia (>90%). However, isolates from Georgia were more likely to exhibit reduced susceptibility to ESCs (≈15%) than were isolates from Asia and Africa (≈3.6%). *N. gonorrhoeae* can develop resistance to antibiotic drugs within a few decades of introduction

(*35*). Earlier uptake of ESCs in Georgia might account for the decrease in susceptibility seen, compared with our isolates collected from the global south. Antimicrobial susceptibility among Georgia isolates might also be affected by population changes caused by neighboring political unrest. Several studies of STIs in migrants, refugees, and internally displaced persons observe that these populations might be at higher risk for sexual assault and STI (*36*). However, the potential association between migration and *N. gonorrhoeae* AMR requires further study (*37*).

Many multidrug-resistant *N. gonorrhoeae* isolates originate in Asia (*1*). However, isolates from Thailand tested at the GC Repository exhibited low overall frequencies of resistance to primary therapies such as cefixime (0.4%), ceftriaxone (3%), and azithromycin (0.77%). The findings are surprising given the regional history of resistant *N. gonorrhoeae*; however, other recent surveillance studies in Thailand have observed similar results (*38*). The GC Repository recently received 18 isolates collected from high-risk patients in Pattaya, Thailand, that exhibited higher frequencies of resistance to macrolides and ESCs.

Recently, the US Centers for Disease Control and Prevention published guidelines on preventive treatment for bacterial STIs using doxycycline postexposure prophylaxis (doxyPEP) (*39*). Multiple prospective studies observed a reduction in incident bacterial STIs among men who have sex with men who were taking doxyPEP (*40*–*42*). Those studies have largely focused on syphilis, but the effect on *N. gonorrhoeae* infection has been noted. For example, in South Africa, doxyPEP reduced *N. gonorrhoeae* infections in men by 50%, but no difference was observed in cisgender women in Kenya taking doxyPEP compared with women in the standard care group (*43*). Many isolates tested at the GC Repository had the *tetM* gene, which is harbored in the easily spread pCONJ plasmid and can be transferred with *pbla* (*44*,*45*), which might counter the potential effectiveness of doxyPEP for gonorrhea prevention. Although doxycycline therapy is not commonly used for contemporary treatment of *N. gonorrhoeae*, continued surveillance is essential to understand the potential effects of doxyPEP on transmission and AMR.

Limitations of this study include low sample size and a study population that might be neither population-representative nor representative of the United States or partner nation militaries. As previously mentioned, most isolates originated from urethral samples taken from men, largely because of both local clinical standards of care at collection sites and ease of sample collection and culture. Genital specimens from female patients, in contrast, are more difficult to culture, possibly because of the robust female urogenital microbiome. Extragenital isolates, which were infrequent in this study, are particularly relevant because of their proximity to commensal *Neisseria*, which may provide opportunities for horizontal gene transfer and acquisition of genetic determinants of AMR. In addition, the GC Repository has limited access to demographic and clinical data, such as sex or military status.

As of January 2024, two new antibiotic drugs for the treatment of gonorrhea infections, zoliflodacin and gepotidacin (*46*,*47*), have undergone Phase III clinical trials with promising results. Even with impending availability, however, the ease of AMR development in *N. gonorrhoeae* still portends a grim outlook for long-term treatment effectiveness. Without a vaccine, enhanced surveillance of *N. gonorrhoeae* AMR that combines culture, epidemiologic information, and molecular data must continue to identify genetic determinants of AMR and inform appropriate treatment recommendations.

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Molecular Characterization of Noroviruses Causing Acute Gastroenteritis Outbreaks among US Military Recruits, 2013–2023

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Norovirus is the leading cause of acute gastroenteritis (AGE) worldwide. Norovirus outbreaks at military facilities can cause loss of training and working days and increased healthcare utilization, affecting force readiness. During 2013–2023, we enrolled 2,304 US military recruits from 4 basic training facilities to investigate AGE outbreaks among this population. Among enrollees, we detected norovirus in 433 (18.8%) AGE cases, and norovirus caused 49 AGE outbreaks during our longitudinal study. On average, each norovirus case-patient missed 1.2 training days due to illness, and 6.2% required infusion care. Whole-genome sequencing of selected samples from each outbreak produced full-length genomes (6,989–7,787 bp) for 39 samples. Norovirus GII.4 Sydney was the most (12/39, 30.8%) identified genotype over the study period. Phylogenetic and comparative genomic analyses revealed that several outbreak strains were responsible for causing ≥1 outbreak, even across different training sites. Our findings can inform infection control practices at military installations and overall norovirus vaccine development.

In the United States, norovirus causes 20 million acute gastroenteritis (AGE) cases, 110,000 hospital visits, and 900 deaths annually (*1*,*2*). Because norovirus is highly infectious and frequently causes outbreaks, the virus is of particular concern for populations in crowded environments, including military installations (*3*). Norovirus outbreaks can cause lost training and working days, increased healthcare utilization, and disruptions to military missions and training exercises (*4*,*5*).

Norovirus is a nonenveloped, single-stranded, positive-sense RNA virus in the family *Caliciviridae* (*6*). Currently, 10 norovirus genogroups (GI–GX) and 49 genotypes are recognized (*7*). Genogroups I and II (GI and GII) are the major causes of norovirus infection in humans (*8*).

The Global Emerging Infections Surveillance Branch and Naval Health Research Center's (NHRC) Operational Infectious Diseases (OID) Directorate have conducted AGE surveillance among US Department of Defense (DoD) recruit populations since 2011. We conducted a retrospective analysis to investigate the genomic composition of norovirus strains identified as the etiologic agents of AGE outbreaks at US military recruit training facilities during 2013–2023.

Methods

Study Design

Recruits meeting the case definition were enrolled in the AGE surveillance study during April 22, 2013–February 13, 2023. Participants signed consent forms at enrollment and their participation was voluntary. Upon enrollment, participants in AGE surveillance completed a case report form detailing demographics, clinical

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data, symptoms, and illness impact on training. The AGE case definition was ≥3 episodes of vomiting, diarrhea, or both within 24 hours; or ≥2 episodes of vomiting, diarrhea, or both within 24 hours along with ≥2 of the following symptoms: abdominal cramps, abdominal pain, fever, nausea, or blood or mucus in stool.

We conducted a retrospective study of AGE surveillance across 4 DoD recruit basic training facilities: Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri. The AGE study protocol was approved for human research by the NHRC institutional review board (protocol no. NHRC.2011.0012) and includes norovirus genomic analysis.

Norovirus Case and Outbreak Definitions

We defined a norovirus case as a laboratory-confirmed sample of norovirus GI or GII by real-time reverse transcription PCR (rRT-PCR). We defined a sporadic case as a single norovirus case occurring >5 days after another case at the same location. We defined a cluster was 2 Norovirus cases occurring within 5 days of each other but >5 days after any other case at the same location. We defined an outbreak as ≥3 norovirus cases where each case occurred within 5 days of the previous case or after a case at the same location.

Sample Collection and Storage

At the time of study enrollment, participants provided a stool sample or 2 rectal swab samples for clinical testing. Rectal swabs were stored in universal transport medium. Raw stool and rectal swabs were stored and shipped to NHRC-OID at 4°C.

Nucleic Acid Extraction

We extracted total nucleic acid from stool or rectal swab suspensions by using the QIAamp Viral RNA Mini kit (QIAGEN, https://www.qiagen.com) according to the manufacturer's protocol except for addition of 1 μL of MS2 bacteriophage to each sample before extraction as a control. We stored extracted nucleic acid from all samples at −80°C for future use.

rRT-PCR and Whole-Genome Sequencing

We tested each sample by multiplexed rRT-PCR by using the Ag-Path One-Step RT-PCR Kit (Thermo Fisher Scientific, https://www.thermofisher.com) and primers specific for norovirus GI, GII, and MS2, following previously described conditions and procedures (*9*). We selected a single norovirus sample from each outbreak across the sites for whole-genome sequencing (WGS).

To ensure sufficient nucleic acid was available for sequencing, we prioritized selected norovirus samples with rRT-PCR cycle threshold values ≤26 and sufficient volume. We thawed samples stored at −80°C and processed for total nucleic acid extraction, as described above for WGS. We generated full-length norovirus GI and GII amplicons by following previously described conditions and procedures (*9*), with separate reactions for GI and GII primers (*10*). We used Nextera XT DNA Library Preparation Kit (Illumina, https://www.illumina.com) to prepare libraries, following the manufacturer's instructions, then sequenced on the MiSeq (Illumina) system using the MiSeq Reagent version 2 Sequencing Kit (Illumina) for 151 paired-end reads.

Norovirus WGS Data Analysis

We used the MetaDetector pipeline (*11*) to process raw reads. To remove adaptor and primer sequences and trim sequences based on quality scores, we used bbduk version 38.96 embedded in BBMerge software, then assembled the resulting data by using metaSPAdes and SPAdes version 3.15.3 (*12*–*14*). Then, we mapped reads back to contigs by using BBmap version 38.96 (*15*) and classified reads and contigs by using DIAMOND BLAST (*16*) against the National Center for Biotechnology Information (NCBI) nonredundant protein database (*15*,*17*). We used MEGAN (*18*) to analyze results from MetaDetector and note blastx (https://blast.ncbi.nlm.nih.gov) results for the largest assembled contig for each sample (*19*). We trimmed whole-genome nucleotide sequences to RNA-dependent RNA polymerase (RdRp) sequences and determined P-types by using blastn. To identify open reading frames (ORFs), we used the CLC Genomics Workbench (QIAGEN) to analyze nucleotide sequences representing the full-length genomes of 39 norovirus strains.

Phylogenetic Tree Construction

We used the MUSCLE algorithm in MEGAX version 11.0 (*20*) to align consensus norovirus WGS and ORF2 sequences from the study strains, along with reference WGS nucleotide and protein sequences of norovirus GI and GII obtained from GenBank and CaliciNet (https://www.cdc.gov/norovirus/php/reporting/calicinet.html). We used MEGAX to construct phylograms of WGS and ORF2 sequences and calculated sequence identities on the basis of distance matrices prepared by using the p-distance algorithm in MEGAX (*20*).

Results

Across the 4 training centers, AGE surveillance enrolled 2,304 military recruits in the study (Table 1). Of those, 433 (18.8%) were positive for norovirus GI, GII, or both (Table 1). We identified norovirus GII in

290 (67.0%) cases and GI in 142 (32.8%) cases. Of the 433 confirmed norovirus cases, recruits missed an average of 1.2 training days per case; 27 (6.2%) case-patients received infusion care, and 4 (<1.0%) were hospitalized (Table 1). No deaths were reported.

During the study period, 47 sporadic norovirus cases, 23 clusters, and 49 outbreaks occurred across all 4 sites (Table 2). We detected norovirus GI in 14 (28.6%) and GII in 35 (71.4%) of the outbreaks. In total, 340 norovirus cases were associated with outbreaks, and outbreaks had an average of 6.9 (SD 4.8) confirmed cases.

WGS Data Analysis

We selected a single Norovirus-positive sample from each outbreak for retrospective genomic analyses. Of the 49 selected samples, we were able to assemble 39 WGS sequences with lengths of 6,989–7,787 bp (Appendix 1 Table, https://wwwnc.cdc.gov/EID/article/30/14/24-0307-App1.pdf). Ten samples failed to produce quality norovirus genomes, and we removed those from further analyses. Norovirus GII.4 was the most identified genotype, causing 12 outbreaks across 3 sites (Appendix 1 Table). Overall, 12/39 (30.8%) sequences were identified as GII.4 Sydney. Norovirus GI.3 was the most identified GI genotype, causing 4 outbreaks across 2 sites (Appendix 1 Table).

Phylogenetic and Sequence Analyses

We used WGS data to perform phylogenetic and similarity matrix analysis of the 39 norovirus outbreak study strains and ORF2 sequences (Figures 1–3; Appendix 2, https://wwwnc.cdc.gov/EID/article/30/14/24-0307-App2.xlsx). We further segregated the norovirus strains in both WGS GI and GII groups according to the genotypes and P-types. Within the GI group, we identified 2 paraphyletic clusters, and study and reference strains occupied the same cluster (Figure 1). Norovirus GI cluster 1 consisted of 7 study strains grouped according to genotypes with published GenBank strains. We observed the highest (99.9%) similarity between South Carolina strains ESP70516.V/SC/2017/GI.7[P7]/OB24 and ESP70546.V/SC/2017/GI.7[P7]/OB26 (Appendix 2 Table 1). Norovirus GI cluster 2 consisted of 6 study strains and previously reported strains retrieved from the GenBank database. Within cluster 2, three of the California study strains, ESP20296.V/CA/2013/GI.6[P11]/OB1, ESP20581.V/CA/2016/GI.6[P11]/OB6, and ESP20577.UTM/CA/2016/GI.6[P11]/OB5, were closely related phylogenetically and shared a high similarity (range 98.3%–100%) but were distantly related (72.1%–80.4%) to GenBank strains included in the analysis.

Norovirus GII strains segregated into 2 major clusters (Figure 1). Cluster 1 consisted of 5 study strains grouped according to genotypes and 3 GII reference strains. Within cluster 1, the study strains were distantly related (Appendix 2 Table 2). Norovirus GII cluster 2 included 21 study strains grouped according to genotypes along with several reference strains. Several study strains from South Carolina and Missouri clustered phylogenetically and shared high similarity with each other. We also noted high similarity (99%) between Illinois study strains ESP10614.V/IL/2017/GII.4 Sydney[P16]/OB33 and ESP10649.UTM/IL/2017/GII.4 Sydney[P16]/OB34.

The norovirus GI ORF2 sequences were phylogenetically segregated into 2 major clusters and were

Table 1. Characteristics of AGE enrollments, NoV cases, and training impacts in a study of molecular characterization of noroviruses causing acute gastroenteritis outbreaks among US military recruits at 4 basic training facilities, 2013–2023*

Characteristics	California	South Carolina	Illinois	Missouri	Total
No. AGE enrollments	567	435	734	568	2,304
Norovirus genotypes detected					
GI/GII†	132 (23.3)	131 (30.1)	78 (10.6)	92 (16.2)	433 (18.8)
GI‡	54 (40.9)	37 (28.2)	26 (33.3)	25 (27.2)	142 (32.8)
GII‡	78 (59.1)	93 (71.0)	52 (66.7)	67 (72.8)	290 (67.0)
GI and GII‡	0	1 (0.8)	0	0	1 (0.2)
No norovirus detected†	433 (76.4)	290 (66.7)	650 (88.6)	471 (82.9)	1,844 (80.0)
Failed PCR†§	2 (0.4)	14 (2.3)	6 (0.8)	5 (0.9)	27 (1.2)
Norovirus cases					
Complete case report form‡	128 (97.0)	124 (94.7)	74 (94.9)	92 (100)	418 (96.5)
Missed 1–2 training days¶	111 (86.7)	106 (85.5)	62 (83.8)	66 (71.7)	345 (82.5)
Received infusion care¶	5 (3.9)	13 (10.5)	9 (12.2)	0	27 (6.5)
Hospitalized¶	1 (0.8)	0	2 (2.7)	1 (1.1)	4 (1.0)

*Values are no. (%). Percentages may not add to 100% due to rounding and MS2 control. The 4 facilities were Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri. AGE, acute gastroenteritis.
†Percentages are of AGE cases.
‡Percentages are of norovirus GI/GII cases.
§PCR reactions negative for all norovirus GI/GII targets.
¶Percentage of norovirus GI/GII cases with complete case report form.

Table 2. Outbreak descriptions in a study of molecular characterization of noroviruses causing acute gastroenteritis outbreaks among US military recruits at 4 basic training facilities, 2013–2023*

Outbreak description†	California	South Carolina	Illinois	Missouri	Total
Sporadic	17	5	13	12	47
Cluster	5	6	4	8	23
Outbreak	13	17	6	13	49
Norovirus GI outbreak, no. (%)	5 (38.5)	5 (29.4)	1 (16.7)	3 (23.1)	14 (28.6)
Norovirus GII outbreak, no. (%)	8 (61.5)	12 (70.6)	5 (83.3)	10 (76.9)	35 (71.4)
No. confirmed NoV cases from all outbreaks	105	114	57	64	340
Mean cases per outbreak (SD)	8.1 (5.8)	6.7 (4.1)	9.5 (7.0)	4.9 (2.5)	6.9 (4.8)
Combined length of outbreaks, d	90	98	56	90	334
Mean length per outbreak, d (SD)	6.9 (5.0)	5.8 (±4.9)	9.3 (8.3)	6.9 (5.5)	6.8 (5.5)

*The 4 facilities were Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri.
†Sporadic = 1 norovirus case occurring >5 days after another norovirus case at the same location; cluster = 2 norovirus cases occurring within 5 days of each other but >5 days after any other norovirus case at the same location; and outbreak ≥3 cases occurring >5 days after another norovirus case at the same location.

separated according to genotypes (Figure 2). GI ORF2 cluster 1 consisted of 6 study strains, and cluster 2 included 7 study strains grouped together with GenBank strains (Figure 2; Appendix 2 Table 3). Study strains ESP20296.V/CA/2013/GI.6[P11]/OB1, ESP20577.UTM/CA/2016/GI.6[P11]/OB5, and ESP20581.V/CA/2016/GI.6[P11]/OB6 in cluster 1 were identical to each other. In cluster 2, study strains ESP20837.V/CA/2019/GI.3[P3]/OB11 and ESP70598.V/SC/2019/GI.3[P3]/OB28, as well as ESP70516.V/SC/2017/GI.7[P7]/OB24 and ESP70546.V/SC/2017/GI.7[P7]/OB26 shared an absolute amino acid similarity with each other.

Phylogenetically, norovirus ORF2 GII study strains and cognate gene sequences of reference strains used in this analysis mapped into 2 major clusters. GII strains were grouped according to their genotypes and P-types. GII ORF2 strains were segregated into cluster 1 and cluster 2. Cluster 1 consisted of 14 study strains spread across different genotype groups. Of note, several study strains demonstrated high amino acid similarity across geographic sites; strains from Missouri and South Carolina showed similarity, as did strains from Illinois and South Carolina. All GII.4 study strains shared a high (93.4%–100%) similarity with GII.4 reference strains (Appendix 2 Table 4).

Discussion

This 10-year retrospective study characterized norovirus-related AGE outbreaks across DoD recruit training facilities. Norovirus GII.4 Sydney was the most (12/39, 30.8%) identified genotype observed in this study, which is consistent with outbreak data from CaliciNet covering 2013–2016 (Appendix 1 Table) (*21*). Further, a novel [P16] polymerase type emerged in November 2015 that was associated with GII.4, causing 60% of outbreaks during 2015–2016 (*21*). We identified GII.4 Sydney[P16] in this study in December 2016, and it was responsible for 6/12 (50%) outbreaks through January 2018 (Appendix 1 Table).

Phylogenetic analysis of the ORF2 sequences showed that the GI and GII study strains were segregated into small clusters according to genotypes. Those same norovirus strain segregation patterns have been reported in previous studies (*22*,*23*). Similarity distance analysis of the WGS and ORF2 sequences showed that study strains ESP70516.V/SC/2017/GI.7[P7]/OB24 and ESP70546.V/SC/2017/GI.7[P7]/OB26 from South Carolina were genetically similar and shared an absolute percentage identity, indicating that the same strain was responsible for causing those 2 outbreaks. Similarly, study strain ESP20837.V/CA/2019/GI.3[P3]/OB11 from California shared 99.2% similarity with strain ESP70598.V/SC/2019/GI.3[P3]/OB28 from South Carolina, suggesting that the same strain was responsible for both outbreaks in 2019. Those findings could indicate cross-country transmission between the 2 Marine Corps training centers.

Molecular epidemiologic studies suggest that norovirus GII.4 caused multiple AGE pandemics in persons of all ages (*24*,*25*). In the current study, we identified norovirus GII.4 genotypes as the cause of multiple outbreaks in South Carolina, Missouri, and Illinois. Phylogenetically, the GII.4 Sydney[P16] from those 3 sites and the GII.4 Sydney[P31] from South Carolina and Missouri clustered together (Figure 3), and GII.4 Sydney strains were detected across the globe in previous years (*24*,*26*–*28*). Those data suggest that the origins of the GII.4 Sydney[P16] and GII.4 Sydney[P31] strains detected in this study might have been imported internationally or were circulating locally before causing outbreaks at the military facilities in our study. Similarly, 5 GII.2[P2] strains from South Carolina, Missouri, and Illinois clustered together with reference strains from abroad (Figure 3) (*29*,*30*).

One limitation of this study was the small number of samples. On average, 5 samples were collected per outbreak, but using more samples for sequencing would

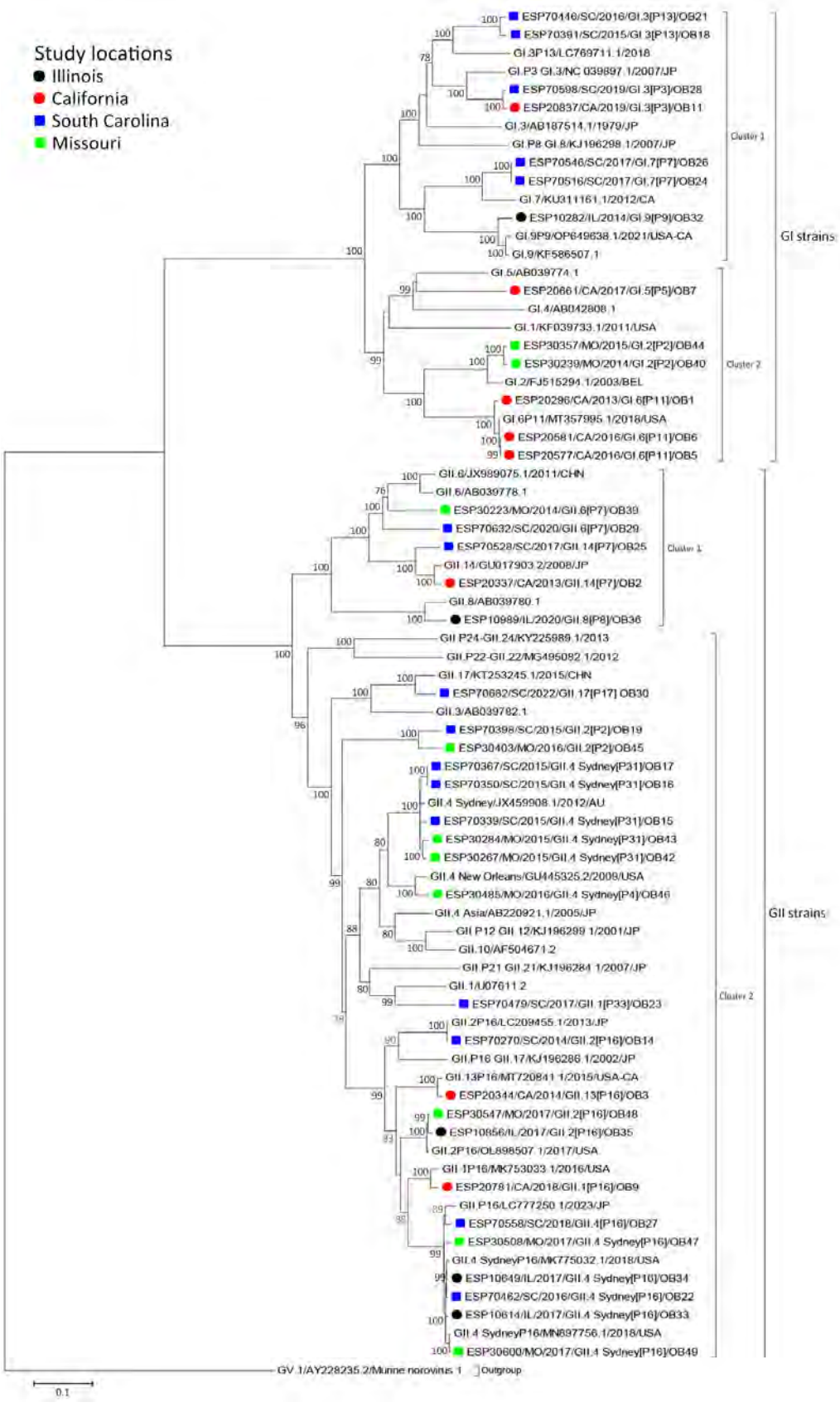

Figure 1. Maximum-likelihood phylogram in a study of molecular characterization of noroviruses causing acute gastroenteritis outbreaks among US military recruits at 4 basic training facilities, 2013–2023. Phylogram reveals genetic relatedness of whole-genome sequences (±7,500 bp) for 39 of the 49 selected outbreak virus study strains characterized by whole-genome analysis. Outbreak study strains are labeled from left to right as follows: sample identification/location sample was collected/year sample was collected/genogroup and P-type/outbreak number. The 4 facilities were Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri. Bootstrap values ≥70% are indicated at branch nodes where applicable. Scale bar indicates nucleotide substitutions per site.

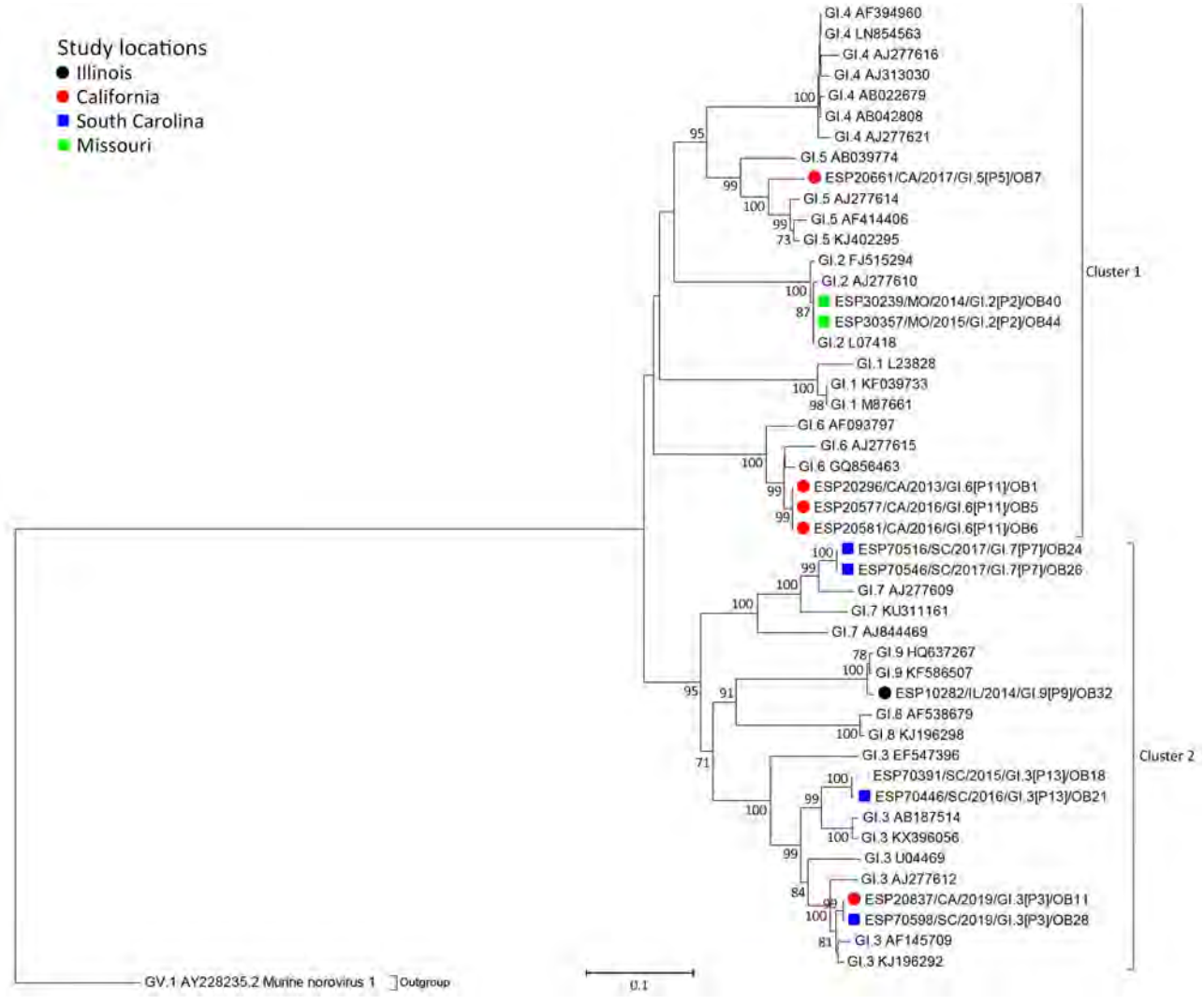

Figure 2. Maximum-likelihood phylogram of GI ORF2 (VP1) in a study of molecular characterization of noroviruses causing acute gastroenteritis outbreaks among US military recruits at 4 basic training facilities, 2013–2023. Phylogram reveals genetic relatedness of GI ORF2 (VP1) deduced amino acid sequences (±550 bp) for 13 of the 39 norovirus outbreak strains. Outbreak study strains are labeled from left to right as follows: sample identification/location sample was collected/year sample was collected/genogroup and P-type/outbreak number. The 4 facilities, identified by color, were Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri. Bootstrap values ≥70% are indicated at branch nodes where applicable. Scale bar indicates nucleotide substitutions per site. ORF, open reading frame; VP, virus capsid protein.

have provided more detailed information regarding the genomic epidemiology of each outbreak. In addition, we used cycle threshold values ≤26 as the selection criteria, thereby reducing the number of samples eligible for sequencing. Another limitation was the lack of detailed questionnaire data and limited questionnaire administration. Including more detailed data points in the questionnaire, such as decreased work and training performance metrics, would have clarified the detrimental impacts of norovirus in this population. Similarly, administering questionnaires to trainees experiencing symptoms but declining study enrollment would have enhanced our knowledge of the extent and effects of outbreaks.

Conclusions

Despite its limitations, this study enhances our knowledge of the genetic code of norovirus strains among US military recruit populations. Norovirus can quickly cause large outbreaks among trainee and deployed military populations, potentially causing decreased mission readiness for entire units at a time for several days. As illustrated in this study, the average norovirus case-patient missed multiple training days due to infection,

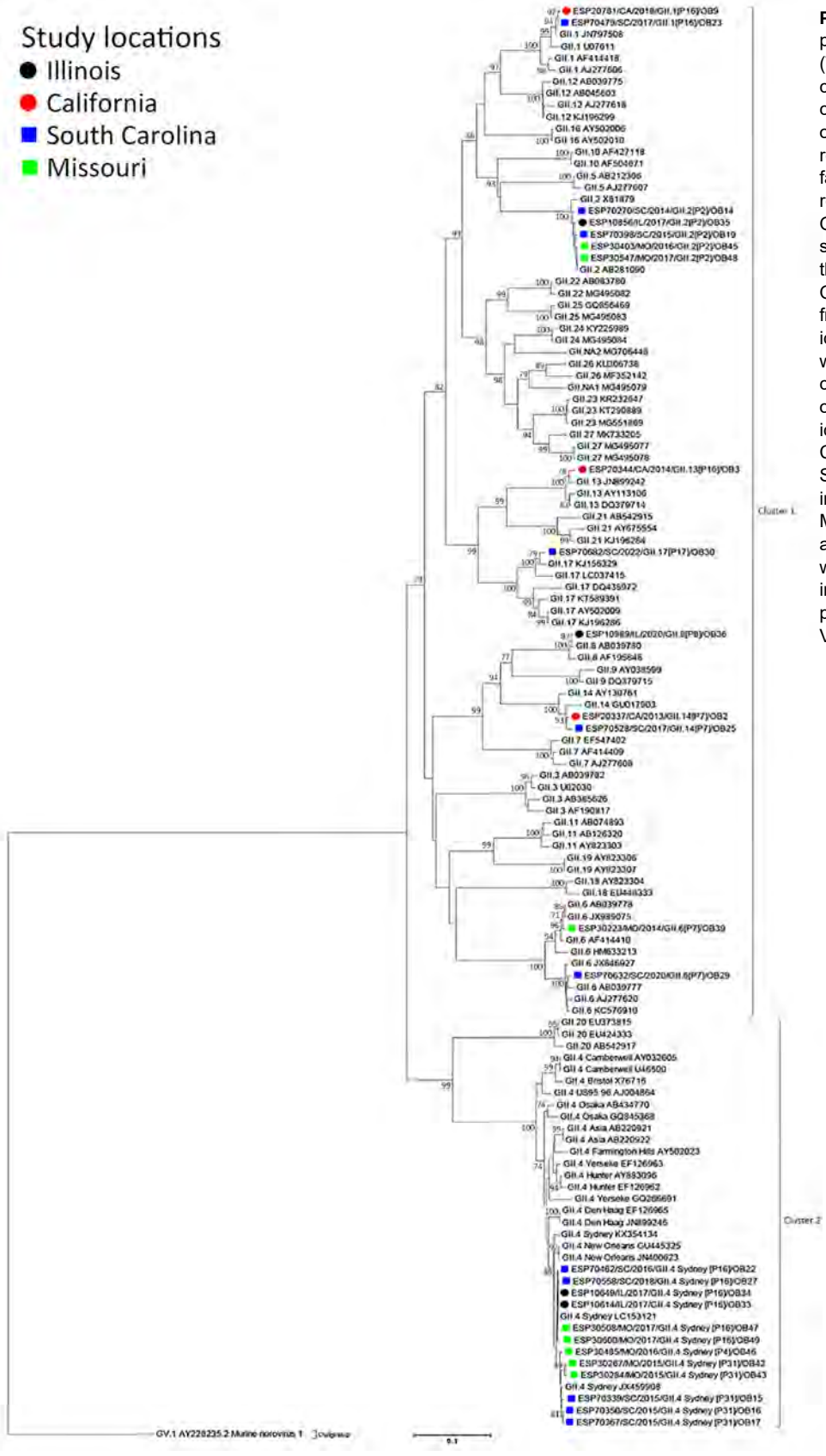

Figure 3. Maximum-likelihood phylogram of GII ORF2 (VP1) in a study of molecular characterization of noroviruses causing acute gastroenteritis outbreaks among US military recruits at 4 basic training facilities, 2013–2023. Phylogram reveals genetic relatedness GII ORF2 (VP1) deduced amino acid sequences (±580 bp) for 26 of the 39 norovirus outbreak strains. Outbreak study strains are labeled from left to right as follows: sample identification/location sample was collected/year sample was collected/genogroup and P-type/ outbreak number. The 4 facilities, identified by color, were Marine Corps centers in California and South Carolina, a Navy center in Illinois, and an Army center in Missouri. Bootstrap values ≥70% are indicated at branch nodes where applicable. Scale bar indicates nucleotide substitutions per site. ORF, open reading frame; VP, virus capsid protein.

and 6.2% required infusion care. However, those numbers do not capture the full spectrum of norovirus impacts, including decreased workdays or training performance, which likely are greater than reported. Maintaining surveillance systems and sample repositories with associated WGS information can be critical to developing effective preventive measures against norovirus, such as vaccines (*31*–*33*).

In conclusion, understanding Norovirus epidemiology could help inform military public health practices and support military health readiness. Having near-real-time genomic information can assist infection control and preventive medicine teams in pinpointing the outbreak etiologies and transmission dynamics to mitigate active outbreaks and prevent future norovirus outbreaks. Our findings will enable us to characterize and monitor the spread of norovirus strains, anticipate future patterns, pinpoint outbreak sources, and advance vaccine technology to enhance public health response efficacy.

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Serosurveillance for *Plasmodium falciparum* Malaria in Peruvian Army Peacekeeping Personnel, Central African Republic, 2021–2022

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Plasmodium falciparum infection threatens military populations deployed to highly malaria-endemic regions, such as Peruvian Army peacekeepers deployed to Central African Republic. During deployment, malaria cases were identified by microscopy and rapid diagnostic tests. After deployment, we performed malaria diagnosis by malachite green loop-mediated isothermal amplification and photo-induced electron transfer PCR assays. We used ELISA to test for *P. falciparum* C-terminal 19-kDa region merozoite surface protein 1–specific IgG from 97 peacekeepers. Malaria prevalence during deployment was 33.33% and we detected 4 cases after deployment: *P. falciparum* (n = 2), *P. ovale* (n = 1), and *Plasmodium* spp. (n = 1). IgG surveillance showed a seroprevalence of 31.96% in peacekeepers, who had a high *P. falciparum* exposure during deployment. Our findings reinforce the necessity of active surveillance in military populations to reduce the risk for introduction of new *Plasmodium* species and strains into the Americas from malaria-endemic areas.

Malaria is a vectorborne disease that affects persons living or traveling within tropical and subtropical regions around the world. According to the World Health Organization, an estimated 249 million malaria cases occurred in 2022 globally; 93.6% of cases and 95.4% of deaths occurred in Africa (*1*). Military personnel are at particularly high risk for malaria during deployments. Malaria caused by *Plasmodium falciparum* can lead to severe symptoms such as fever, chills, headache, and even death, if not identified and treated promptly. Those symptoms can seriously affect the performance of military personnel during the execution of critical operations (*2*).

A continuous surveillance system using rapid diagnostic tests (RDTs), microscopy, and molecular and serologic diagnostic tools is necessary to determine the absolute risk during deployments to highly malaria-endemic areas. For example, in 2003, a malaria outbreak was reported in 44 US Marines deployed to Liberia who had laboratory-confirmed or suspected *P. falciparum* infections and required immediate medical evacuation (*2*). A review concluded that the outbreak was associated with inefficient preventive measures, such as partial adherence to mefloquine and the inadequate use of repellent and bed nets (*3*). In contrast, military personnel from the United Kingdom successfully deployed to the Democratic Republic of the Congo in the same year and had no reported malaria cases during 512 person-weeks (*4*). Their success was associated with the use of the ABCD (awareness, bite avoidance, chemoprophylaxis, and diagnosis) program to educate and enforce mission objectives (*4*). Both scenarios highlight the role of numerous factors, such as preventive measures, the complexity and objectives of the mission, duration of deployment, and respective risk for malaria transmission (*5*).

The Central African Republic (CAR) also reports high transmission of malaria; ≈2.0 million (36.4%) persons were reported to have suspected or confirmed *P. falciparum* infections during 2021. United Nations military peacekeeping operations in CAR consist of ≈200 armed forces personnel from Peru who promote and maintain the local security of civilians, support democratic efforts, and provide global humanitarian

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assistance. Peacekeepers from Peru are at risk for severe clinical manifestations of malaria because they are immunologically naive. Infected peacekeepers could introduce new *Plasmodium* species or new strains to the Americas upon their return to Peru from deployment. In this study, we evaluated the exposure to *P. falciparum* malaria infection in military peacekeepers from Peru deployed to CAR during 2021–2022.

Materials and Methods

Epidemiologic Information and Blood Collection

We collected basic demographic, epidemiologic, and clinical information for malaria case-patients identified in CAR (Figure 1). Whole-blood samples were collected again in Hospital Militar Central Luis Arias Schreiber (the Peruvian Army hospital) in Lima, Peru, 1 month after deployment to evaluate active malaria infection. Upon arrival, military personnel were quarantined for 30 days at an army base in Lima, a nonendemic area for malaria; movement outside the base was completely restricted according to guidelines issued by the Peruvian Army Health Unit. In addition, we randomly selected 97 military peacekeepers (because of limited available testing reagent materials) and collected plasma samples to evaluate exposure to *P. falciparum* (Figure 1).

Detection of Active Cases

Active *Plasmodium* infection was detected by Boil and Spin malachite green loop-mediated isothermal amplification (LAMP) assay (*6*). In brief, every 20-μL reaction contained 2× in-house LAMP buffer 0.2% Tween-20, 1.5 mol Betaine, 2 mmol of dNTP, 0.004% malachite green dye, 320 U/mL of Bst DNA Polymerase (New England Biolabs, https://www.neb.com), and 5 μL of Boil and Spin DNA template from whole blood. We performed an amplification reaction at 63°C for 60 minutes using a mini heat block (BioExpress, https://www.bioexpress.com). Positive samples showed a green color and were confirmed by 2 independent laboratory technician readers.

We performed malaria species determination using photo-induced electron transfer (PET) PCR (*7*). In brief, the PET genus reaction was performed in a 20 μL volume containing 5 μL of purified DNA from whole blood, 2X TaqMan Environmental Buffer 2.0 (ThermoFisher Scientific, https://www.thermofisher.com), and 250 nmol of genus-forward and FAM-reverse primer. The singleplex PET species-specific reactions contained the same mix but with a concentration of 125 nmol of the HEX-labeled species-specific primer (ThermoFisher Scientific). We used thermal cycling conditions for both genus- and species-specific

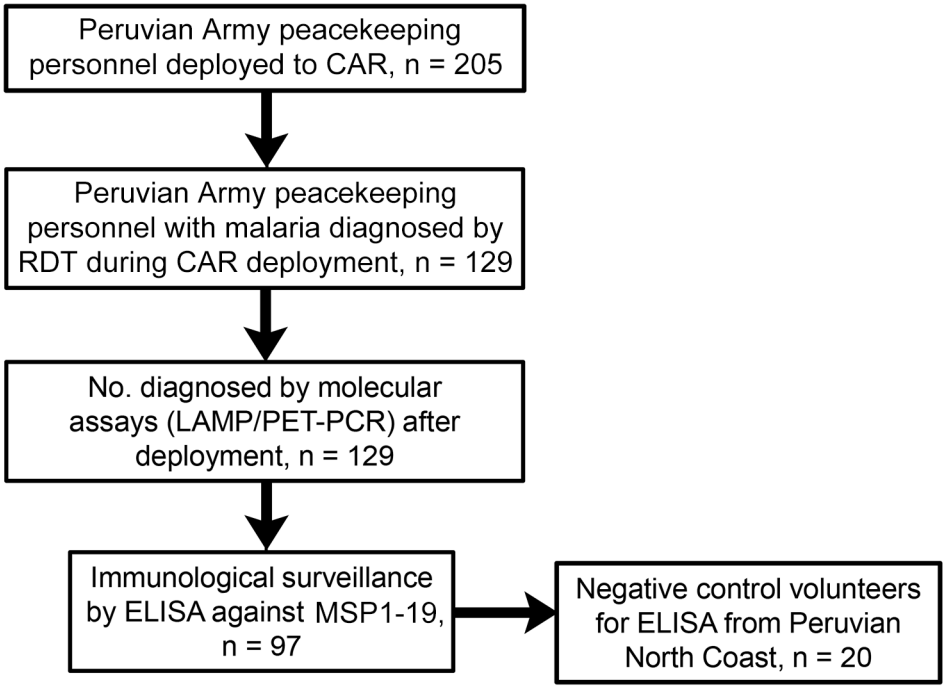

Figure 1. Flow diagram of participants included in study of serosurveillance for *Plasmodium falciparum* malaria in Peruvian Army peacekeeping personnel, Central African Republic, 2021–2022. CAR, Central African Republic; LAMP, loop-mediated isothermal amplification; MSP1-19, C-terminal 19-kDa region merozoite surface protein 1; PET-PCR, photo-induced electron transfer PCR; RDT, rapid diagnostic test.

Table 1. Demographic, clinical, and laboratory characteristics of 129 participants in study of serosurveillance for *Plasmodium falciparum* malaria in Peruvian Army peacekeeping personnel, Central African Republic, 2021–2022*

Characteristic	Value
Demographics	
Sex	
M	119 (92.2)
F	10 (7.7)
Mean age, y (SD)	42.4 (7.4)
Rank	
Enlisted	33 (25.6)
Warrant officers	74 (57.4)
Officers	22 (17.0)
Clinical	
Malaria diagnosed in CAR	43 (33.3)
No. malaria episodes, n = 43	
1	34 (79.1)
2	7 (16.3)
3	1 (2.3)
4	1 (2.3)
Malaria diagnosed in Peru	4 (3.1)
Hospitalization required	2 (50.0)
Laboratory, n = 97	
OD values against *P. falciparum* MSP1-19	
Mean (SD)	0.17 (0.31)
Median (IQR)	0.08 (0.07–0.12)
Seroprevalence to *P. falciparum* MSP1-19	
Positive	31 (31.9)
Negative	66 (68.1)

*Values are no. (%) except as indicated. CAR, Central African Republic; IQR, interquartile range; MSP1-19, C-terminal 19-kDa region merozoite surface protein 1; OD, optical density.

assays of initial denaturation at 95°C for 10 minutes, 45 cycles of denaturation at 95°C for 10 seconds, and annealing at 60°C for 40 seconds. We used a cycle threshold (Ct) value of <41 to separate positive and negative samples.

ELISA of Human Samples

We used plasma samples to screen for IgG seropositivity for *P. falciparum* C-terminal 19-kDa region merozoite surface protein 1 (MSP1-19) by indirect ELISA as a marker of *P. falciparum* malaria exposure. We included *P. falciparum*–negative control plasma samples obtained from 20 persons from Piura Department on the north coast of Peru, a region that has very low malaria incidence. We used those plasma samples to calculate a positivity cutoff value using the average optical density value plus 3 SD on the basis of methods published elsewhere (*8*).

Statistical Analysis

We performed statistical analysis using Stata version 16.1 statistical software (StataCorp LLC, https://www.stata.com) and Prism software version 9 (GraphPad Software, Inc., https://www.graphpad.com). We used descriptive analysis to demonstrate demographic and epidemiologic characteristics of Peruvian Army peacekeepers and used bivariate analysis to compare characteristics between seropositive and seronegative participants.

Ethics Considerations

Data and sample collections were performed for clinical diagnostic support requested by the Peruvian Army to Naval Medical Research Unit SOUTH. Data and sample analyses were covered by the NAMRU6.2018.0002 protocol (NHSR protocol) approved by Naval Medical Research Unit SOUTH Institutional Review Board. Findings were reported to Peruvian Army authorities for malaria treatment administration, as needed.

Results

Study Population

Out of 205 total peacekeepers deployed to CAR during July 2021–June 2022, only 129 (62.9%) were tested for malaria by microscopy or RDT because they reported malaria-like symptoms. Most (92.2%) participants were men, and a high percentage (57.4%) were warrant officers; mean age was 42.4 (SD 7.4) years. The prevalence of malaria during deployment was 33.3% (43/129) by RDT or microscopy, and the number of malaria episodes experienced ranged from 1 to 4. From that group, 79.1% (34/43) had only 1 episode, 16.3% (7/43) had a second episode, and 1 (2.3%) participant reported having third and fourth episodes of malaria-like symptoms (Table 1). All participants received artemether/lumefantrine and responded adequately to malaria treatment.

In after-deployment samples, LAMP assays detected 4 positive cases out of 129 samples (3.1% positivity), and subsequent molecular method PET-PCR enabled us to further genotype those cases showing 2 *P. falciparum*, 1 *P. ovale*, and 1 *Plasmodium* spp. Of the 4 positive case-patients, 2 (50.0%) reported a malaria episode during their deployment in CAR, and they received the same malaria treatment (artemether/lumefantrine) during deployment as the other positive case-patients.

Regarding malaria exposure, ELISA for MSP1-19–specific IgG demonstrated that 31/97 (31.9%) Peruvian peacekeepers were positive for exposure to *P. falciparum* malaria (Table 1). Four persons had higher IgG titers than the average positive study population, suggesting a recent malaria infection (Figure 2). Finally, seropositivity was statistically significant between participants in whom malaria was diagnosed in CAR and those in whom it was not (p<0.001) and among persons who were enlisted, warrant officers, and officers (p = 0.026) (Table 2).

Discussion

Military populations are continuously exposed to *P. falciparum* during deployments in malaria-endemic regions, resulting in outbreaks, mainly in populations with poor preventive measures. However, disease identification on the basis of symptoms alone could lead to an underestimation of actual transmission and subsequently higher risk for malaria during deployments. In our study, we found that almost one third (31.9%) of peacekeepers deployed to CAR were immunologically exposed to *P. falciparum*. We also found a significant difference in seropositivity in military personnel in whom malaria was diagnosed in Africa during deployment. The seronegative participants who were malaria-positive in Africa (13.6%; 9/66) could be explained by antibody kinetics, which require weeks after infection to reach significant levels for detection, especially in naive malaria populations. On the other hand, almost half (41.9%; 13/31) of seropositive participants did not receive a malaria diagnosis; this finding could be caused by exposure to *Plasmodium* parasites that could activate an immune response without a clinical malaria episode. Another possibility could be related to the use of microscopy and RDTs, because these diagnostic methods depend on the skills of laboratory personnel, sample quality, and low parasitemia associated with submicroscopic infections. We do not have information on parasitemia levels to assess the diagnostic capacity of the microscopists and RDTs used during deployment. Diagnosis of active malaria (symptomatic or asymptomatic) should be prioritized using molecular and microscopic tools but could be complemented by serologic analysis in populations with long-term periods of exposure. That finding correlates well with other reports and shows the relevance of serologic surveillance to evaluate exposure to *Plasmodium* parasites in mobile populations like deployed military personnel (*9*). This information highlights the need to improve both preventive measures in military personnel and timing of malaria diagnosis (*2*).

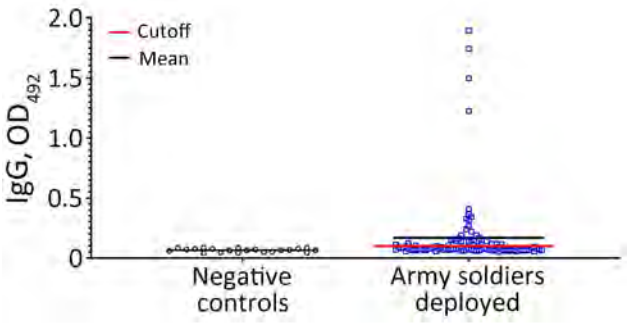

Figure 2. Seroprevalence against *Plasmodium falciparum* malaria in Peruvian Army peacekeepers deployed to Central African Republic, July 2021–June 2022. Dot plot of OD_{492} of *P. falciparum* C-terminal 19-kDa region merozoite surface protein 1 by ELISA assay, with negative control group (black circles, n = 20) and Peruvian Army peacekeepers (blue squares, n = 97), 31 (31.9%) of whom were seropositive. Red line represents the cutoff determined by the average value plus 3 standard deviations of negative OD_{492} control values; black line represents the mean of OD_{492} values per group. OD_{492}, optical density at 492 nm.

Information is limited about the incidence of malaria in military populations deployed to highly malaria-endemic areas in Africa, especially in social-military conflicted regions. Because of reports of malaria in Somalia and Afghanistan, several military health surveillance systems were implemented (*10*,*11*). The US Defense Medical Surveillance System uses a tracking system to determine the area in which malaria was acquired (*12*). Data from that tracking system are valuable for identifying risk factors related to malaria in military populations, but can be biased by the sample collection process or population type. Identification of military personnel with active malaria infections enables calculation of disease incidence and leads to a better understanding of malaria transmission in this population. However, negative results for malaria on the basis of symptom assessment alone do not necessarily mean that military personnel were not infected during deployment because persons could have been infected without clinical symptoms.

Table 2. Characteristics of 97 participants in a study of serosurveillance for *Plasmodium falciparum* malaria in Peruvian Army peacekeeping personnel, Central African Republic, 2021–2022*

Characteristic	Total, n = 97	Seronegative, n = 66	Seropositive, n = 31	p value
Sex				
M	90 (92.8)	61 (92.4)	29 (93.6)	1.000†
F	7 (7.2)	5 (7.6)	2 (6.4)	
Mean age, y (SD)	43.2 (7.7)	42.7 (7.5)	44.3 (8.0)	0.347
Rank				
Enlisted	24 (24.7)	21 (31.8)	3 (9.7)	0.026
Warrant officers	60 (61.9)	39 (59.1)	21 (67.7)	
Officers	13 (13.4)	6 (9.1)	7 (22.6)	
Malaria diagnosis in CAR				
Y	27 (27.8)	9 (13.6)	18 (58.1)	<0.001
N	70 (72.2)	57 (86.4)	13 (41.9)	

*Values are no. (%) except where indicated.
†By 2-tailed Fisher exact test.

Serologic surveillance in highly malaria-endemic areas offers a tool to determine previous malaria exposure, thereby helping in surveillance efforts to diagnose malaria parasites during symptomatic infection (*13*). Different antigens can be used for diagnosis, including the liver stage antigen-1 and MSP1-19 (*10*,*11*). *P. falciparum* MSP1 antigens, including MSP1-19, are highly immunogenic during blood-stage *Plasmodium* and result in sustained IgG titers up to several months after infection (*14*). Serologic testing can be applied to military populations to determine malaria seroprevalence at the end of deployment, offering complementary information to other diagnostic tools, especially in naive military or civilian populations deployed to highly malaria-endemic regions.

Another relevant finding is the 2 positive non–*P. falciparum* malaria cases diagnosed after deployment. That result differs from the 2023 World Malaria Report, in which CAR reported >2 million malaria cases, 100% of which were *P. falciparum* (*1*). Non–*P. falciparum* malaria has different biologic and clinical manifestations than *P. falciparum* malaria. Accurate *Plasmodium* species diagnosis is key to reducing complications, including relapse because of inadequate malaria treatment for hypnozoites in *P. vivax* or *P. ovale* cases. The 2 cases we detected were not the first reported *P. ovale* cases in military personnel returning to Peru from CAR, highlighting the need for better postdeployment evaluation of personnel to prevent introduction of new *Plasmodium* species (*15*). The possibility of contracting non–*P. falciparum* malaria from other countries in Africa is low because returning personnel were quarantined in Lima, a non–malaria-endemic area, for 30 days after deployment.

The first limitation of our study is that we did not have a predeployment sample to evaluate serologic performance against *P. falciparum* MSP1-19; only self-reports of no previous travel to malaria-endemic regions were available. Second, malaria diagnosis was performed only in symptomatic persons during deployment, and some subjects could have had asymptomatic malaria. Finally, data about compliance to preventive measures (malaria prophylaxis, use of mosquito repellent or bed nets) or other factors that could modify the risk for malaria were unavailable. Those limitations should be considered in future studies in deployed military personnel to highly malaria-endemic areas.

Conclusions

Our results showed that one third of Peruvian Army military peacekeepers deployed to CAR during 2021–2022 were exposed to *P. falciparum*. Although few sporadic malaria cases were reported in personnel returning from the African region (*15*), those findings reinforce the need for additional tools to measure malaria exposure and to implement preventive measures to reduce malaria risk, thereby decreasing infections in civilian and military populations deployed to highly malaria-endemic areas.

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J.A.V. and J.F.S. conceived, designed, and supervised the project study. J.A.V. performed experiments for evaluation of immunogenicity by ELISA. E.M. and R.S. performed LAMP assays for malaria diagnosis. J.A.V. and J.F.S. performed the data and statistical analyses. M.O. and A.M.D.-F. participated in data and sample collection. J.A.V., J.F.S., H.O.V., and D.L.P. wrote the first draft of the manuscript. J.V.A., J.F.S., H.O.V., E.M., R.S., and D.L.P. wrote the final version of the paper. All authors reviewed, edited, and approved the manuscript.

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Azithromycin Resistance Patterns in *Escherichia coli* and *Shigella* before and after COVID-19, Kenya

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Escherichia coli and *Shigella* spp. are leading bacterial causes of acute diarrhea in sub-Saharan Africa and pose risks to global communities, travelers, and the US military. Increasing antimicrobial resistance (AMR) in those and other enteric pathogens creates treatment challenges for clinicians. Inappropriate use of antimicrobial drugs, such as azithromycin for viral respiratory infections, increased during the COVID-19 pandemic. We evaluated AMR trends of 116 *E. coli* and 109 *Shigella* spp. isolates obtained from 1,672 pre–COVID-19 (2017–2019) and 1,118 post–COVID-19 (2022–2023) human fecal samples from Kenya. Azithromycin resistance increased significantly from before to after COVID-19, from 6.3% to 40.4% (p = 0.001). Phenotypic AMR profiles from a subset of isolates were compared with genotypic AMR information derived from whole genome sequencing. The most common AMR gene detected was the macrolide *mph(A)* gene. This study highlights the need for continued AMR surveillance.

Enteric infections are preventable and treatable but remain a leading cause of illness and death globally by causing >1.6 million fatalities overall and >525,000 deaths in children <5 years old in low- and middle-income countries, such as Kenya, each year (*1*). Although diarrheal illnesses are typically self-limiting, antimicrobial treatment for bacterial enteric infections is used to reduce the duration and severity of symptoms and to prevent other severe illnesses and long-term sequelae (*2*–*4*). However, the growing global public health threat of antimicrobial resistance (AMR) is a major challenge in treating illnesses such as bacterial enteric infections (*2*–*4*).

Bacterial enteric infections are relevant to the US military because they can cause outbreaks and limit service members' abilities to work effectively. Bacterial enteric infections are consistently the number 1 infectious disease threat according to the Military Infectious Disease Research Program's threat prioritization panel (*4*). Therefore, effectively treating bacterial enteric infections is critical. Conducting surveillance to understand the epidemiology of diarrheal illness and AMR patterns among bacterial causes supports military and global health objectives to combat AMR and diarrheal illness (*3*).

Improper use of antimicrobial drugs can reduce bacterial susceptibility and contribute to AMR (*5*,*6*). Kenya faces major challenges in regulating antimicrobial access and use that were substantially exacerbated during the COVID-19 pandemic (*7*–*14*). The ease of access to antimicrobial drugs in many countries is a contributing factor to AMR (*15*). Furthermore, few published studies have described AMR patterns in enteric bacteria in Kenya. We investigated the potential effects of the COVID-19 pandemic on azithromycin and fluoroquinolone resistance in *Escherichia coli* and *Shigella* spp. isolates from enteric infections collected across various sites in Kenya before (2017–2019) and after (2022–2023) the COVID-19 pandemic.

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Methods

Case Definition

E. coli and *Shigella* spp. isolates were recovered from participants in Kenya who had symptomatic diarrheal illness. Participants were recruited from county hospital surveillance sites including Busia, Kericho, Kisii, Kisumu, Kombewa, Uasin Gishu, and Lamu counties (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0374-App1.pdf).

Enrollment Strategy

Study staff enrolled participants during 2 time periods: March 2017–December 2019 (pre–COVID-19) and January 2022–May 2023 (post–COVID-19). Persons who sought outpatient care for acute diarrheal illness and were willing to provide a fecal specimen were enrolled regardless of sex, age, or military status. A standard questionnaire was used to collect participant information. All participants consented to their inclusion in this study.

Process for Isolation and Selection

We plated fecal specimens on hektoen enteric, MacConkey, and MacConkey sorbitol agars (BD Diagnostic Systems, https://www.bd.com) and incubated the cultures overnight aerobically at 37°C to recover lactose fermenting (*E. coli*) and non–lactose- and non–sorbitol-fermenting (*Shigella* spp.) colonies. We conducted bacterial identification and antimicrobial susceptibility testing (AST) of suspected *E. coli* and *Shigella* spp. isolates by using the MicroScan WalkAway (Beckman Coulter, https://www.beckmancoulter.com), the Phoenix automated microbiology system (BD Diagnostic Systems), and Etest strips (bioMérieux, https://www.biomerieux.com). We cultured *E. coli* isolates for confirmatory PCR testing and extracted the isolate DNA by boiling at 100°C. We performed multiplex PCR (Appendix Table 1) by using the Veriti thermocycler (Thermo Fisher Scientific, https://www.thermofisher.com), and a 2% agarose gel (Millipore Sigma, https://www.sigmaaldrich.com), and gel documentation by using iBright 1000 (Thermo Fisher Scientific).

Antimicrobial Susceptibility Testing

We performed AST for ciprofloxacin and levofloxacin on all *E. coli* and *Shigella* spp. isolates by using the MicroScan WalkAway Gram negative NC66 panels (Beckman Coulter) for pre–COVID-19 isolates and the Phoenix Gram negative panels (BD Diagnostic Systems) for post–COVID-19 isolates. We interpreted MICs in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (*16*). We conducted additional AST with azithromycin Etest strips (bioMérieux) by using the manufacturer's instructions on Mueller Hinton agar plates (BD Diagnostic Systems) and incubating at 37°C for 16–20 hours. We used *E. coli* ATCC 25922 as the quality control strain for each day of testing. We selected isolates for sequencing on the basis of resistance to either azithromycin or fluoroquinolones of interest (ciprofloxacin and levofloxacin).

Genomic DNA Extraction

We extracted DNA from the *E. coli* and *Shigella* spp. isolates by using QIAmp Fast DNA Stool Mini Kit (QIAGEN, https://www.qiagen.com) according to the manufacturer's instructions. We quantified DNA concentrations by using Qubit 4 and the Qubit 1X dsDNA High Sensitivity assay kit (Thermo Fisher Scientific). We stored the DNA at –20°C before sequencing.

Genomic Sequencing

We prepared DNA libraries by using Nextera XT DNA Library Preparation Kit (Illumina, https://www.illumina.com) according to the manufacturer's instructions. We ran the library on a TapeStation 4200 (Agilent Technologies, https://www.agilent.com) to determine its average length and quality. We sequenced a 750-pM library spiked with 10% Phix on the NextSeq 2000 (Illumina) by using the P1 (300 cycles) paired end reagents (Illumina).

Bioinformatics Analysis

We assessed the quality of the raw reads by using FastQC (*17*). We trimmed the low-quality reads, string of Ns, and adaptor sequences by using fastp (*18*). We performed genome assembly by using Shovill (https://github.com/tseemann/shovill), and we assessed genomic features (e.g., genome size, number of contigs N50) by using QUAST (*19*). We screened for antimicrobial resistance and virulence genes by using abritAMR (*20*), whereas we screened the plasmid replicons by using ABRicate (https://github.com/tseemann/abricate) against the PlasmidFinder database (*21*). We determined sequence types by using MLST version 2.23.0 (https://github.com/tseemann/mlst), phylogroup by EzClermont (*22*), and fim types by FimTyper version 1.1 (*23*). We determined the *Shigella* spp. cluster type, serotype, and O and H antigens by using ShigEiFinder (*24*). Finally, we generated a maximum-likelihood single-nucleotide polymorphism–based core genome phylogenetic tree by using Parsnp (*25*) (*Shigella* spp. reference sequence

GCF_000022245.1 and *E. coli* reference sequence GCF_000005845.2) and annotated on iTOL (*26*).

Analytic Methods

We interpreted the results of the phenotypic analysis according to CLSI standards (*16*). We pooled data across sites after confirmation of outcome homogeneity (Appendix Figure 2). Acute diarrhea was defined in this study as 3–5 loose stools over a 24-hour period and severe acute diarrhea as >5 stools over a 24-hour period. When comparing resistance over time, we grouped partially resistant intermediate isolates with fully resistant isolates. We assessed the differences in antimicrobial resistance levels pre–COVID-19 and post–COVID-19 by using a 2-tailed Fisher exact test at significance level of 0.05 and analyzed by using R Statistical Software version 4.3.1 (The R Project for Statistical Computing,

Table 1. Characteristics of *Escherichia coli* and *Shigella* cases in Kenya before (2017–2019) and after (2022–2023) COVID-19*

Characteristic	*E. coli*, n = 116	*Shigella*, n = 109	p value†
Median age, y (interquartile range)	7 (3–25)	18 (4–28)	0.02
Age group			0.01
Children ≤18 y	75 (64.7)	52 (47.7)	
Adults ≥18 y	41 (35.3)	57 (52.3)	
Study period			0.17
Pre–COVID-19, 2017–2019	63 (54.3)	69 (63.3)	
Post–COVID-19, 2022–2023	53 (45.7)	40 (36.7)	
County site			0.96
Busia County Referral Hospital	18 (15.5)	15 (13.8)	
Kericho County Referral Hospital	40 (34.5)	38 (34.9)	
Kombewa County Hospital	10 (8.6)	7 (6.4)	
Kisii Teaching and Referral Hospital	30 (25.9)	31 (28.4)	
Uasin Gishu	17 (14.7)	18 (16.5)	
Lamu	1 (0.9)	0	
Diarrhea severity			0.75
No acute diarrhea	3 (2.6)	1 (0.9)	
Acute diarrhea‡	53 (46.5)	50 (45.9)	
Severe acute diarrhea§	58 (50.9)	58 (53.2)	
Water source§			
Municipal	54 (47.0)	56 (51.4)	0.51
Rain	22 (19.1)	30 (27.5)	0.14
Borehole	22 (19.1)	18 (16.5)	0.61
Spring	9 (7.8)	18 (16.5)	0.046
Well	7 (6.1)	11 (10.1)	0.27
Bottle	3 (2.6)	4 (3.7)	0.72
Tap	1 (0.9)	0	≥0.99
Stream	1 (0.9)	0	≥0.99
Other	0	1 (0.9)	0.49
Water treatment¶			
No treatment	81 (70.4)	74 (67.9)	0.68
Boil	20 (17.4)	22 (20.2)	0.59
Distillation	0	1 (0.9)	0.49
Chemical	1 (0.9)	1 (0.9)	≥0.99
Chlorine	0	1 (0.9)	0.49
Water guard	14 (12.2)	10 (9.2)	0.47
Ciprofloxacin susceptibility (*15*)			0.25
Susceptible	110 (97.3)	108 (100)	
Intermediate	0	0	
Resistant	3 (2.7)	0	
Levofloxacin susceptibility (*15*)			0.25
Susceptible	110 (97.3)	108 (100)	
Intermediate	0	0	
Resistant	3 (2.7)	0	
Azithromycin susceptibility (*15*)			<0.001
Susceptible	89 (76.7)	105 (96.3)	
Intermediate	1 (0.9)	0 (0)	
Resistant	26 (22.4)	4 (3.7)	

*Values are no. (%) except as indicated. One adult *E. coli* case was missing data and removed from denominators for the following variables: diarrhea severity, water source, and water treatment. Four cases (3 *E. coli* and 1 *Shigella*) had inconclusive ciprofloxacin and levofloxacin susceptibility results and were excluded from susceptibility profiles. Any missing data were excluded from analysis.
†We obtained p values by using the Wilcoxon rank sum test, Fisher exact test, or Pearson χ^2 test, as appropriate.
‡Defined as 3–5 loose stools over a 24-h period.
§Defined as ≥6 loose stools over a 24-h period.
¶Participants could select multiple water sources or treatments.

Table 2. Cases and antimicrobial susceptibility of *Escherichia coli* and *Shigella* before (2017–2019) and after (2022–2023) COVID-19, Kenya*

Characteristic	*E. coli*				*Shigella* spp.			
	Overall	2017–2019	2022–2023	p value†	Overall	2017–2019	2022–2023	p value†
All ages								
No. cases	116	63	53	NA	109	69	40	NA
No. tested	2,790	1,672	1,118	NA	2,790	1,672	1,118	NA
Cases/100 persons (95% CI)	4.2 (3.5–5.0)	3.8 (2.9–4.8)	4.7 (3.5–6.2)	0.21	3.9 (3.2–4.7)	4.1 (3.2–5.2)	3.6 (2.6–4.8)	0.47
% Resistant isolates‡								
Ciprofloxacin	2.7	1.6	3.9	0.59	0	0	0	>0.99
Levofloxacin	2.7	1.6	3.9	0.59	0	0	0	>0.99
Azithromycin	23.3	7.9	41.5	**<0.001**	3.7	0	10.0	**0.02**
Adults ≥18 y								
No. cases	41	19	22	NA	57	31	26	NA
No. tested	1,446	767	679	NA	1,446	767	679	NA
Cases/100 persons (95% CI)	2.8 (2.0–3.8)	2.5 (1.5–3.8)	3.2 (2.0–4.9)	0.38	3.9 (3.0–5.1)	4.0 (2.8–5.7)	3.8 (2.5–5.6)	0.84
% Resistant isolates‡								
Ciprofloxacin	5.1	5.3	5.0	≥0.99	0	0	0	>0.99
Levofloxacin	5.1	5.3	5.0	≥0.99	0	0	0	>0.99
Azithromycin	24.4	5.3	40.9	**0.01**	7.0	0	15.4	**0.04**
Children <18 y								
No. cases	75	44	31	NA	52	38	14	NA
No. tested	1,344	905	439	NA	1,344	905	439	NA
Cases/100 persons (95% CI)	5.6 (4.4–6.9)	4.9 (3.6–6.5)	7.1 (4.9–9.9)	0.10	3.9 (2.9–5.0)	4.2 (3.0–5.7)	3.2 (1.8–5.3)	0.37
% Resistant isolates‡								
Ciprofloxacin	1.4	0	3.2	0.42	0	0	0	>0.99
Levofloxacin	1.4	0	3.2	0.42	0	0	0	>0.99
Azithromycin	22.7	9.1	41.9	**0.002**	0	0	0	>0.99

*Four cases (3 *E. coli* and 1 *Shigella*) had inconclusive ciprofloxacin and levofloxacin susceptibility results and were excluded from susceptibility profiles. Boldface indicates significant values (p<0.05). NA, not applicable

†Pearson χ^2 test used to measure differences in prevalence across COVID-19 periods. Fisher exact test used to measure differences in resistance proportions between the pre–COVID-19 and post–COVID-19 periods.

‡One intermediate resistant isolate grouped with fully resistant.

https://ww.r-project.org). No adjustments were made for multiple observations.

Results

Sample Collection and Case Identification

During a 4.5-year period in Kenya, 2,790 fecal samples were collected and tested for *E. coli* and *Shigella* spp. Of those, 1,672 (59.9%) specimens were collected 3 years before the COVID-19 pandemic (March 2017–2019). Adult patients ≥18 years old provided 767 (45.9%) of the pre–COVID-19 samples and 905 (54.1%) were from children. The remaining 1,118 (40.1%) samples were collected during the 1.5-year period after the COVID-19 pandemic (January 2022–May 2023). Adult patients provided 679 (60.7%) samples and 439 (39.3%) samples came from children. We identified 116 *E. coli* isolates in total, 75 (64.7%) from children (44 [58.7%] pre– and 31 [41.3%] post–COVID-19) and 41 (46.3%) from adults (19 [46.3%] pre– and 22 [53.7%] post–COVID-19). We identified 109 *Shigella* spp. isolates in total, 57 (52.3%) from adults (31 [54.4%] pre– and 26 [45.6%] post–COVID-19) and 52 (47.7%) from children (38 [73.1%] pre– and 14 [26.9%] post–COVID-19) (Table 1).

Demographics and Prevalence of Cases

Across both study periods, *E. coli* case-patients were on average younger than *Shigella* spp. case-patients (median [interquartile range] 7 years [3–25] vs. 18 [4–28] years of age; p = 0.02). Nearly all *E. coli* (97.4%) and *Shigella* spp. (99.1%) case-patients reported either acute or severe acute diarrhea. Most *E. coli* (61.8%) and *Shigella* spp. case-patients were from Kericho and Kisii. Municipal water was the most frequently reported water source among both *E. coli* and *Shigella* spp. case-patients, followed by rain, boreholes, and spring water. Water treatment was uncommon; only 30.8% of all case-patients reported chemical or physical water treatment methods, which did not meaningfully vary by pathogen (Table 1). Overall, *E. coli* cases increased from 3.8 (95% CI 2.9–4.8) per 100 persons pre–COVID-19 to 4.7 (95% CI 3.5–6.2) per 100 persons post–COVID-19 (p = 0.21). Of note, recovery of *E. coli* isolates from children increased from 4.9 (95% CI 3.6–6.5) per 100 persons pre–COVID-19 to 7.1 (95% CI 4.9–9.9) per 100 persons in the post–COVID-19 period (p = 0.10). Among adults, there was a slight increase from 2.5 (95% CI 1.5–3.8) to 3.2 (95% CI 2.0–4.9) per 100 persons (p = 0.38). *Shigella* spp. prevalence remained steady

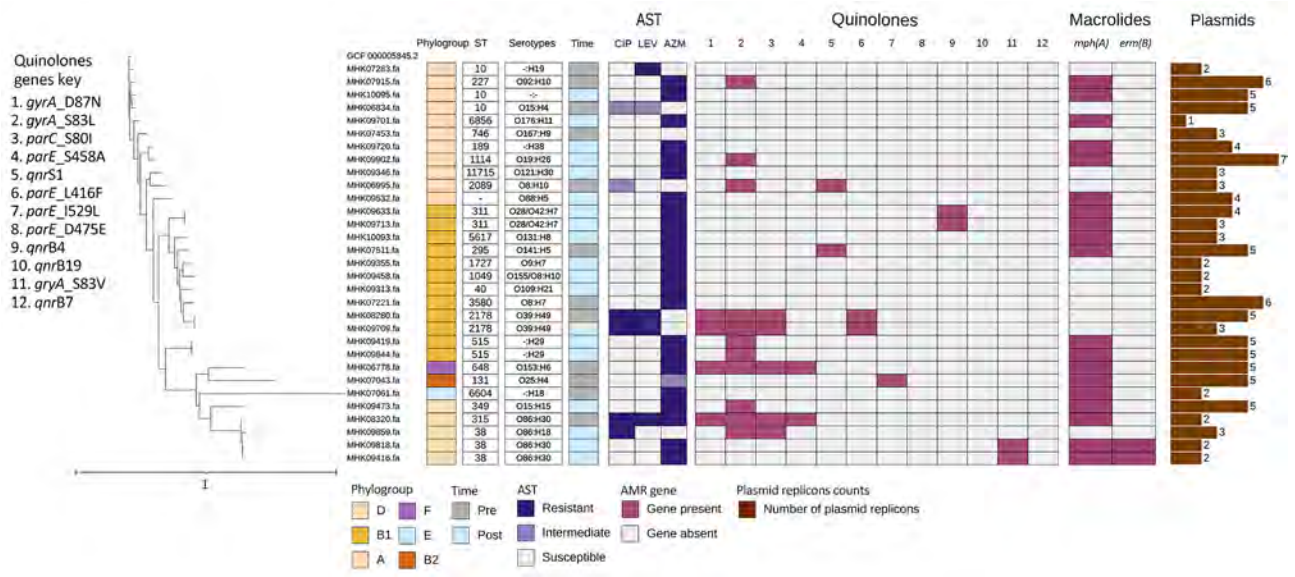

Figure 1. Phylogenetic tree and corresponding heatmap of 31 *Escherichia coli* isolates carrying antimicrobial resistance genes recovered from patients in Kenya with acute or severe diarrheal disease from pre–COVID-19 (2017–2019) and post–COVID-19 (2022–2023) periods. The phylogenetic tree was constructed by using a maximum-likelihood single-nucleotide polymorphism core genome alignment with a reference strain. Isolates are identified by reference genome identification numbers. Tree scale bar measures substitutions per site. AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing; AZM, azithromycin; CIP, ciprofloxacin; LEV, levofloxacin; Post, post–COVID-19; Pre, pre–COVID-19; ST, sequence type.

across COVID-19 periods and age groups, ranging from 3.2 to 4.2 per 100 persons (Table 2).

Antimicrobial Resistance Patterns before and after COVID-19

For the pre–COVID-19 period, <10% of *E. coli* isolates were resistant to ciprofloxacin (n = 1), levofloxacin (n = 1), or azithromycin (n = 4). For the post–COVID-19 period, *E. coli* resistance to ciprofloxacin, levofloxacin, or azithromycin increased to 45.1% (n = 23). This increase was predominantly because of an increase in azithromycin resistance, from 7.9% pre–COVID-19 to 41.5% post–COVID-19 (p<0.001) (Table 2). Ciprofloxacin and levofloxacin resistance increased from 1.6% to 3.9% (p = 0.59), at near identical magnitudes among both adults and children (Table 2), even when stratified by children <5 years and 5–17 years of age (Appendix Table 2). Of *Shigella* spp. isolates tested, 96.2% (n = 104) were susceptible to all 3 antimicrobial drugs. Only 4 isolates (3.7%) were resistant to only azithromycin. All 4 resistant isolates were from adults in the post–COVID-19 period (Table 2). All *Shigella* spp. isolates were susceptible to ciprofloxacin and levofloxacin (Table 2).

Similar resistance patterns were observed after excluding Busia and Lamu sites that only recruited participants in the post–COVID-19 period (Appendix Figure 2). Azithromycin resistance patterns did not significantly vary by reported water source or water treatment methods (Appendix Table 3).

Genomic Characteristics of *E. coli* and *Shigella* spp. Isolates

For *E. coli* isolates, phylogenetic groups, strain types, and plasmid replicons of the 31 *E. coli* isolates characterized by whole genome sequencing (WGS) are provided in detail (Figure 1). One post–COVID-19 isolate had a missing allele and could not be identified. Macrolide resistance gene *mph(A)* (n = 19) was detected in 6/12 pre–COVID-19 and 13/19 post–COVID-19 isolates, whereas *erm(B)* was detected in 2/19 post–COVID-19 isolates. Quinolone resistance genes (n = 31) detected were *gyrA_D87N* (n = 4), *gyrA_S83L* (n = 11), *gyrA_S83V* (n = 2), *parC_S80I* (n = 5), *parE_S458A* (n = 2), *parE_L416F* (n = 2), and *parE_I529L* (n = 1). Plasmid–mediated quinolone resistance genes *qnrS1* (n = 2) and *qnrB4* (n = 2) were detected; however, only 1 isolate was not susceptible to ciprofloxacin or levofloxacin. There was also co-occurrence of both macrolide and quinolone resistance genes: *mph(A)* with *gyrA* (n = 10), *mph(A)* and *erm(B)* with *gyrA* (n = 2), and *mph(A)* with *qnrB4* (n = 2) that were also resistant to azithromycin (Figure 1).

For *Shigella* spp., 6 isolates were characterized by WGS, belonging to 3 species: *S. flexneri* (n = 4), *S. boydii* (n = 1), and *S. dysenteriae* (n = 1). Sequence types, clusters, and serotypes are provided in detail (Figure 2). Macrolide resistance gene *mph(A)* was detected in 3/6 *Shigella* spp. isolates. All isolates phenotypically

resistant to azithromycin carried the *mph(A)* gene, except 1 that carried 2 multidrug efflux pump genes, *mdtM* and *acrF*. One isolate carried a quinolone resistance gene, *qnrS1*, but its phenotypic susceptibility to ciprofloxacin and levofloxacin was inconclusive, and it was therefore excluded from AST analysis.

Discussion

As with previous studies conducted in Kenya, acute bacterial enteric infections in this study were primarily caused by *E. coli* and *Shigella* spp. (*26*). Although *E. coli* prevalence was slightly higher in the post–COVID-19 period, the increase was not significant (p>0.05), suggesting a return to baseline circulation of enteric pathogens after the cessation of COVID-19 prevention measures. The largest increase in prevalence was observed among children <18 years of age, possibly because of the reentry of immune-naive children into public spaces and schools, as has been hypothesized for other infectious diseases.

Of note, this study revealed that *E. coli* isolates from adults and children after the COVID-19 pandemic were 5 times more likely to be resistant to azithromycin than those isolated before the pandemic. Potential explanations for those increases include Kenya's insufficient antimicrobial regulation and suboptimal clinical use of antimicrobial drugs, particularly during the COVID-19 pandemic (*7*–*10*,*13*,*27*). The likelihood of encountering antimicrobial drugs is increased, which might enable resistance development. It is possible the increases in azithromycin resistance observed in this study were part of the gradual increase in resistance patterns over time, but it is also possible resistance patterns were accelerated because of increased use of antimicrobial drugs such as azithromycin for viral respiratory infections during the COVID-19 pandemic (*12*).

Macrolide resistance genes, such as the *mph(A)* gene responsible for azithromycin resistance, are commonly found in *E. coli* (*28*). Of the isolates we sequenced, 19/31 contained macrolide resistance genes. Only 6 of those 19 *E. coli* isolates were from pre–COVID-19 samples, revealing an increase in azithromycin resistance genes after the pandemic. The *mph(A)* gene was found in different strain types, indicating the potential for transmission across *E. coli* species. Among 6 *Shigella* spp. isolates collected in the post–COVID-19 period, the 3 identified as azithromycin resistant carried the *mph(A)* gene. Literature suggests that spread of macrolide resistance genes among *Shigella* spp. is because of horizontal gene transfer rather than direct lineage (A. Asad, unpub. data, https://pubmed.ncbi.nlm.nih.gov/37461575).

Our findings are crucial for US military members who may be deployed to Kenya. *E. coli* and *Shigella* spp. infections can result in severe diarrhea and sequelae, which can reduce service members' ability to perform expected duties. Ciprofloxacin and azithromycin can shorten symptom duration and severity and accelerate recovery from bacterial enteric infections (*6*). However, reduced antimicrobial susceptibility might impede clinician efforts to treat infections effectively and return service members back to full operational capabilities. On a global public health level, bacterial enteric pathogens can cause large outbreaks, making antimicrobial drugs critical in mitigating their negative impacts.

The first limitation of this study is that participants were enrolled as a single encounter without follow-up, so it is possible some asymptomatic participants with a pathogen later became cases. Second, there was insufficient information about residence (rural or urban) or military status, which may affect the results

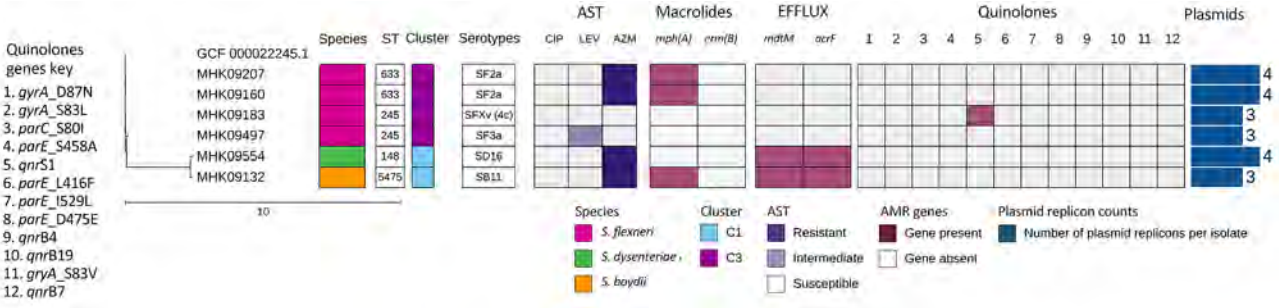

Figure 2. Phylogenetic tree and corresponding heatmap of 6 *Shigella* spp. isolates carrying antimicrobial resistance genes recovered from patients in Kenya with acute or severe diarrheal disease. The phylogenetic tree was constructed by using a maximum-likelihood single-nucleotide polymorphism core genome alignment with a reference strain. Isolates are identified by reference genome identification numbers. Tree scale bar measures substitutions per site. AMR, antimicrobial resistance; AST, antimicrobial susceptibility testing; AZM, azithromycin; CIP, ciprofloxacin; LEV, levofloxacin; ST, sequence type.

because of exposure differences to pathogens and antimicrobials. Third, no information was collected regarding treatment regimens and outcomes, which raised questions about the clinical effect of AMR phenotypes on patients. Fourth, the study did not include analyzable demographic data such as sex, which can influence healthcare-seeking behaviors and sensitivity to certain antimicrobial drugs. Fifth, different platforms were used for phenotypic fluoroquinolone resistance characterization before and after COVID-19 for MIC testing, which could have led to differences in AST results. However, results from both platforms were interpreted according to CLSI guidelines, limiting potential differences. Finally, data early in the COVID-19 pandemic (January 2020–December 2021) could have provided additional context to the increase in AMR, but this study was unable to capture samples during that time.

In conclusion, understanding of the AMR patterns of bacterial enteric infections, such as those observed in this study, is crucial for military and local clinicians when considering antimicrobial drugs for treating acute diarrhea. The US military must be adequately prepared to deploy into any area at any given time by understanding all potential threats, including pathogens. AMR can manifest anywhere because of globalized travel and gene transfer; therefore, continuous monitoring of phenotypic AMR and resistance gene markers against antimicrobial drugs for bacterial enteric pathogens is necessary, particularly in regions such as sub-Saharan Africa where AMR surveillance is underreported.

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The investigators have adhered to the policies for protection of human subjects as described in US Army Regulation 70–25 (approval no. WRAIR#1549/1549A). Ethics approval was obtained from the Kenya institutional review board (approval no. SERU#1549).

The nucleotide sequences from this study have been submitted to the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) (BioProject ID nos. PRJNA1104425 [*E. coli*], PRJNA1104399 [*Shigella*]).

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Characteristics of Madariaga and Venezuelan Equine Encephalitis Virus Infections, Panama

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Madariaga virus (MADV) and Venezuelan equine encephalitis virus (VEEV) are emerging arboviruses affecting rural and remote areas of Latin America. However, clinical and epidemiologic reports are limited, and outbreaks are occurring at an increasing frequency. We addressed the data gap by analyzing all available clinical and epidemiologic data of MADV and VEEV infections recorded since 1961 in Panama. A total of 168 human alphavirus encephalitis cases were detected in Panama during 1961–2023. We described the clinical signs and symptoms and epidemiologic characteristics of those cases, and also explored signs and symptoms as potential predictors of encephalitic alphavirus infection compared with those of other arbovirus infections occurring in the region. Our results highlight the challenges for the clinical diagnosis of alphavirus disease in endemic regions with overlapping circulation of multiple arboviruses.

Arthropodborne viruses (arboviruses) infect humans worldwide and cause significant illness and death. The emergence or resurgence of some arboviruses has been increasing and poses a major global health threat (*1*). US military personnel are frequently stationed in areas where arboviruses are endemic or may emerge, which could threaten military readiness.

Venezuelan equine encephalitis virus (VEEV) is widely distributed throughout the Americas; at least 14 subtypes and varieties have been described (*2*). VEEV subtypes IAB and IC can cause explosive, large-scale epizootics in horses and spillover epidemics in humans (*3*,*4*). VEEV enzootic subtypes (i.e., VEEV ID, IE) are associated with a regular incidence of human infections by spillover from enzootic cycles that involve rodents and sylvatic mosquitoes. Evidence suggests that equine-adaptive or mosquito-adaptive mutations in the VEEV enzootic subtype ID led to the emergence of epizootic and epidemic VEEV subtypes (*3*). VEEV enzootic and endemic subtype ID infection is highly prevalent in the eastern province of Darien, Panama, where human infections are sometimes

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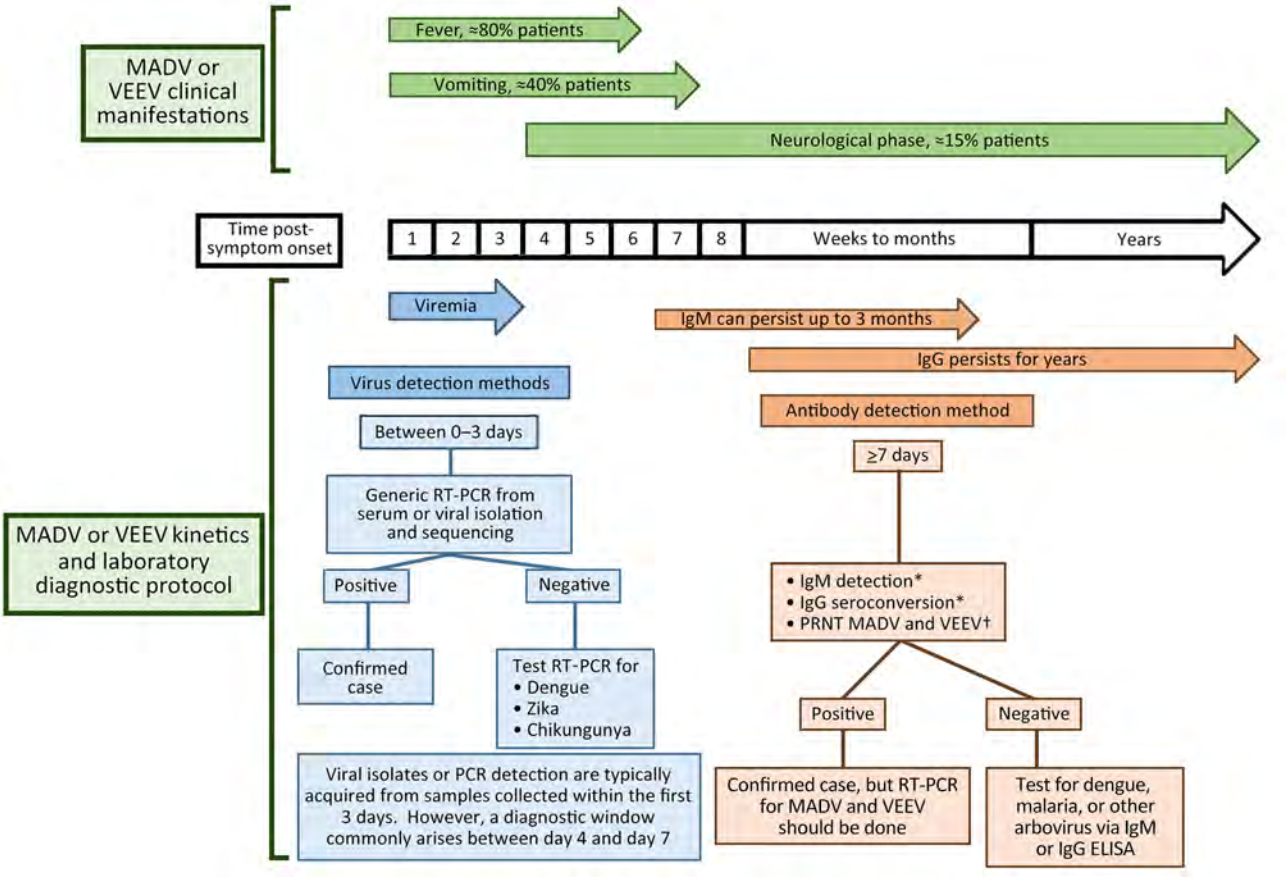

Figure 1. General laboratory algorithm used for diagnosis of MADV and VEEV virus infections, Panama. MADV and VEEV diagnosis made on the basis of days since symptom onset. *In paired samples showing 4-fold increase in antibody titers. †Confirmation after IgG or IgM testing. MADV, Madariaga virus; PRNT, plaque-reduction neutralization test; RT-PCR, reverse transcription PCR; VEEV, Venezuelan equine encephalitis virus.

fatal and seroprevalence in some villages is up to 75% of the population (*3*). Eastern equine encephalitis virus (EEEV) was reclassified as 2 different species in 2010: EEEV in North America and Madariaga virus (MADV) in Latin America (*5*). MADV was not associated with human outbreaks before 2010, when a human outbreak was reported in Darien (*6*). Both MADV and VEEV circulated simultaneously during that outbreak, and 99 acute cases and 19 hospitalizations for encephalitis were reported. Confirmed cases included 13 for MADV, 11 for VEEV, and 1 case of co-infection. A fatal MADV infection was confirmed in the same region in 2017. Modeling of 2012 and 2017 Darien Province serosurvey data suggested that alphavirus transmission is endemic in the region (*7*). Many alphavirus disease cases appear to clinically present as a self-limited febrile illness, but persistent neurologic signs and symptoms have been reported for up to 5 years after MADV and VEEV exposure (*3*).

VEEV and MADV infections are likely underdiagnosed because of limited diagnostic tools and the inability to clinically differentiate those infections from other arboviral diseases. Some estimates report that ≥10% of syndromically characterized dengue cases in Central and South America may be caused by VEEV (*8*). Further complicating the mischaracterization is the increasing trend in dengue incidence over the last several decades (*9*). Chikungunya virus (CHIKV) and Zika virus (ZIKV) had not previously circulated within the Western Hemisphere until the explosive emergence in 2013 (CHIKV) and 2014 (ZIKV). Both viruses became endemic in Latin America, where they now co-circulate in dengue virus (DENV)–endemic regions (*10*). The clinical presentation of those arboviral diseases can range from asymptomatic or undifferentiated mild febrile illness to severe disease (*10*).

The increasing geographic spread and disease incidence of arbovirus infections in the Americas is

Table. Sociodemographic characteristics of case-patients in study of characteristics of MADV and VEEV infections, Panama*

Characteristic	No. (%)
Sex	
F	163 (35.8)
M	292 (64.2)
Mean age, y (SD)	23.6 (19.7)
Age group, y	
0–5	98 (20.2)
6–20	159 (32.7)
≥21	229 (47.1)
Province	
Darien	319 (71.1)
Comarca Embera	32 (7.1)
Other provinces	98 (21.8)
VEEV	
Negative	400 (80.7)
Positive	96 (19.4)
MADV	
Negative	460 (92.7)
Positive	36 (7.3)

*Samples were submitted for encephalitic alphavirus testing during 1961–2023 (n = 496). Some variables may total <496 because of missing data. MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.

a major public health concern. Undifferentiated febrile illnesses remain a diagnostic and therapeutic challenge in arbovirus-prone regions because of the lack of available tools for identifying the pathogens responsible for those clinical syndromes. Shortly after disease onset, MADV and VEEV infections are often clinically indistinguishable from other arboviral syndromes, delaying prompt care for patients at risk for more serious outcomes; MADV and VEEV have been associated with severe or even fatal outcomes. Here, we describe the clinical signs, symptoms, and epidemiologic characteristics of all reported MADV and VEEV human infections occurring in Panama during 1961–2023. In addition, we explore potential symptoms as predictors of encephalitic alphavirus infection compared with those occurring from other arbovirus infections endemic to the region.

Materials and Methods

Alphavirus Surveillance

We heterogeneously sourced alphavirus surveillance data in Panama from samples submitted by health center clinicians upon suspecting MADV or VEEV; the national dengue surveillance system; and during outbreak response activities. Upon suspicion of MADV or VEEV (henceforth called encephalitic alphavirus infection) in Panama, health center clinicians submit blood samples to Instituto Conmemorativo de Gorgas de Estudios de la Salud (ICGES), which serves as the national reference center for infectious-disease diagnostics in Panama. Alphavirus infections are also often identified through the national dengue surveillance system or during encephalitis outbreak response activities. The national system, instituted in 1988, initially provided centralized testing of samples from suspected dengue cases submitted by clinicians during 1993–2009, but subsequently established diagnostic capacity in all local clinical units (*11*). Some alphavirus infections were identified when cases tested negative for DENV. In addition, several alphavirus outbreak investigations have been conducted since 2010 and consist of communitywide febrile surveillance and serosurveys.

Alphavirus Outbreak Case Definition

We defined a suspected alphavirus encephalitis case as one with fever and headache, and we defined a probable case as a suspected case with neurologic manifestations (e.g., somnolence, lethargy, or seizures). We defined a confirmed case as a suspected or probable case with laboratory confirmation through viral isolation, reverse transcription PCR (RT-PCR), IgM ELISA, or IgG ELISA or plaque reduction neutralization test (PRNT) seroconversion of paired clinical samples (Figure 1; Appendix, https://wwwnc.cdc.gov/EID/article/30/14/24-0182-App1.pdf).

Alphavirus Data Collection

We retrospectively searched and retrieved clinical and epidemiologic information of MADV and VEEV infections reported in clinical records and epidemiologic forms during 1961–2020, data available at ICGES, and extending data published previously (*12*). Cases detected during 2021–2023 were collected as part of the surveillance initiative undertaken by the US National Institute of Allergy and Infectious Diseases–Centers for Research in Emerging Infectious Diseases Network initiative. The Coordinating Research on Emerging Arboviral Threats Encompassing the Neotropics in Panama and the Armed Forces Health Surveillance Division, Global Emerging Infections Surveillance Branch (ProMIS ID no. P0052_23_NM), undertake acute febrile surveillance across the country. The dataset included demographic characteristics, clinical symptoms, severity of infection, and sick contacts. We also collected geographic coordinates of alphavirus-positive households when available. We condensed duplicate or similar signs and symptoms into composite variables, which provided a better representation of the symptomatology, then used those variables to compare clinical manifestations across the main arboviral infections in Panama, including MADV, VEEV, DENV, CHIKV, and ZIKV. The Panamanian Ministry of Health (protocol no. 2077 and protocol no. 365/CBI/ICGES/2023, approved on

November 30, 2023), and the Gorgas Memorial Institute institutional review board (protocol nos. 335/CBI/ICGES/21, 073/CBI/ICGES/21, and 138/CBI/ICGES/22, approved on March 19, 2021) approved the use of human data and samples from outbreaks.

Comparison of Arboviral Symptoms

To account for low statistical power, we grouped confirmed MADV and VEEV infections into a single category. We defined encephalitic alphavirus cases as all laboratory-confirmed alphavirus infections reported in Panama during 1961–2023. We compared encephalitic alphavirus infections to DENV, ZIKV, and CHIKV. We obtained a DENV dataset from a cross-sectional study in 2009 and a ZIKV dataset from 2016. Both DENV and ZIKV datasets were provided by the São José do Rio Preto Health Service in São Paulo State, Brazil, and were published elsewhere (*13*). We obtained CHIKV data from CHIKV surveillance in the state of Amazonas, and the City of Recife, Pernambuco, Brazil, during 2015–2020 (*14*) (Appendix).

Statistical Methods

Initially, we included a total of 121 variables associated with participants' symptomatology in the database; we categorized and grouped the variables by specific clinical criteria for each virus. We constructed composite symptoms based on clinical syndromic categorization by consensus of 2 independent physicians following alphavirus clinical guidelines. We further reduced symptoms using exploratory factor analysis and principal component analysis. We excluded variables with 0 variance by using a Kaiser-Meyer-Olkin threshold of 0.6 (Appendix Table 1). Ultimately, we reduced signs and symptoms to 14 variables used in the analysis.

To evaluate MADV- and VEEV-associated signs and symptoms, we conducted multivariable logistic regression analysis, controlling for age and

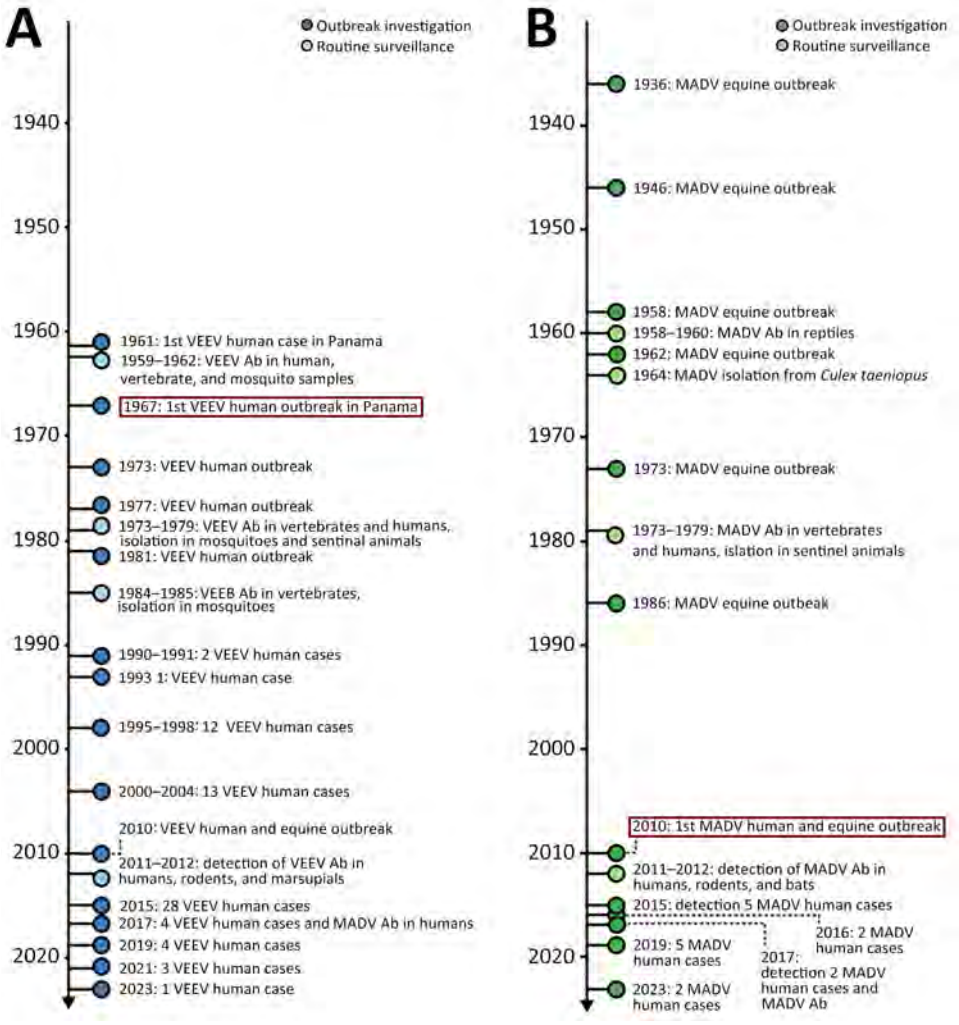

Figure 2. Timeline showing history of VEEV and MADV in humans animals, and vectors, Panama. Timeline shows incidence during 1961–2023. A) VEEV incidence; B) MADV incidence. Ab, antibodies; MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.

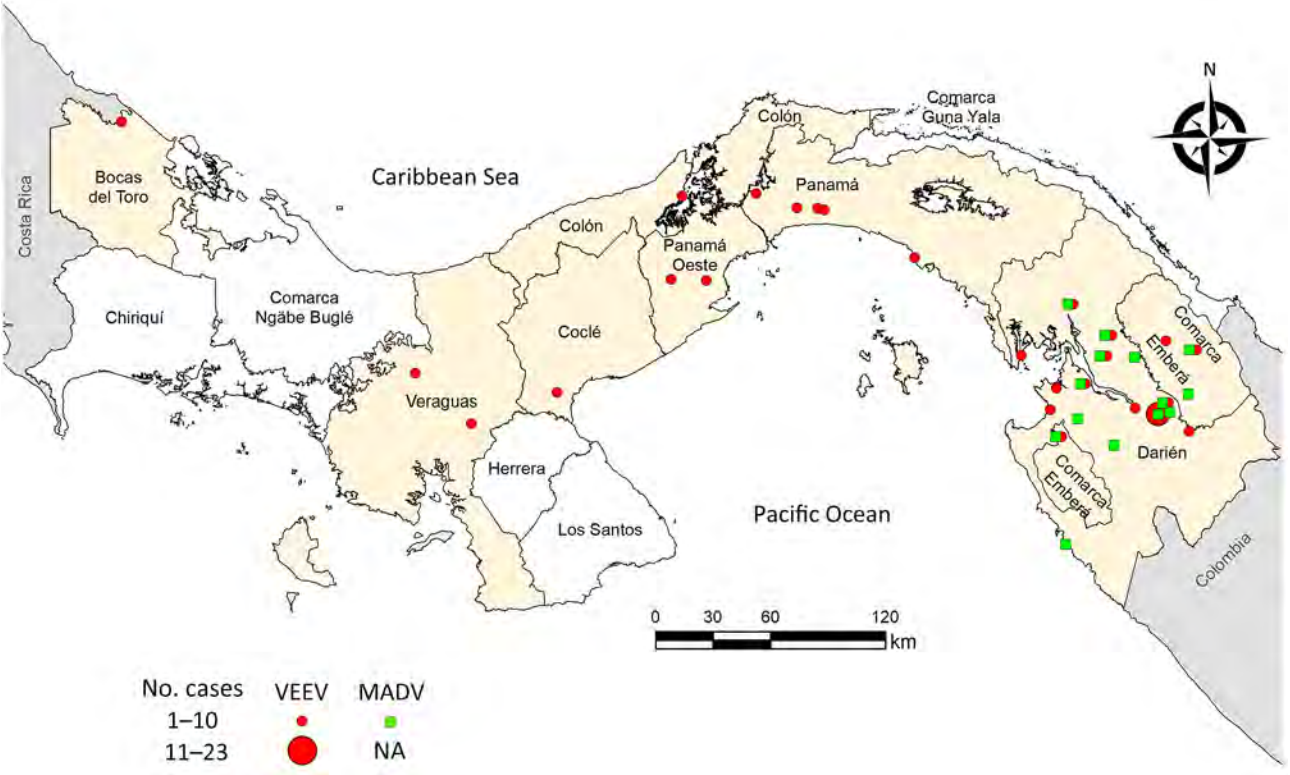

Figure 3. Locations of recorded cases in a study of characteristics of MADV and VEEV infections, Panama, 1961–2023. Green squares represent MADV cases and red circles VEEV cases. MADV cases were reported only in the eastern Panama region, in the province of Darien. MADV cases detected outside Darien, in Chiriquí, Comarca Näbe Bugle, and Herrera were reported in members of the border police working in the Darien Province, who at time of symptom onset were in their home region. MADV, Madariaga virus; NA, not applicable; VEEV, Venezuelan equine encephalitis virus.

biologic sex. We used univariate logistic regressions to evaluate the composite symptoms associated with alphavirus infection (MADV and VEEV) and those reported in DENV, ZIKV, and CHIKV infections. We selected variables using a nested log-likelihood ratio test. We excluded variables with >10% missing data from the final analysis. We expressed the associations between specific symptoms and viral infection as odds ratios; we considered $p<0.05$ statistically significant. We used Stata version 17 (Statacorp, https://www.stata.com) and R Studio version 2023.12.1+402.pro1 (Posit, https://www.rstudio.com) for statistical analysis.

Results

MADV and VEEV Epidemiology

During 1961–2023, Panama recorded 168 laboratory-confirmed MADV or VEEV infections. For VEEV infections, 131 cases were confirmed, of which 60 (46%) were detected during outbreaks and 71 (54%) were identified through arbovirus surveillance (Table; Figure 2, panel A). For MADV infections, 37 cases were confirmed, of which 34 (92%) were identified during outbreaks and 3 (8.1%) were detected through passive arbovirus surveillance (Figure 2, panel B). Detailed clinical and epidemiologic information was accessible for 132/168 (79%) human alphavirus encephalitis infections, comprising 36 (27%) MADV infections and 96 (73%) VEEV infections. The breakdown of age distribution revealed that MADV occurred more often in children, whereas most VEEV cases occurred in adults (Table).

All human MADV infections were reported from the Darien province. VEEV infections were reported throughout Panama, but most (63%) reports were also from Darien (Figure 3). The peak of MADV cases occurred during the 2010 outbreak in Darien, which had 13 laboratory-confirmed cases. The highest number (n = 28) of VEEV cases occurred in 2015 (Figure 2, panel A). Among the MADV case-patients, 23 were male and 13 were female. Three cases exhibited mild disease, 11 moderate, and 17 had severe clinical manifestations, resulting in a mild-to-severe ratio of 3:17. A total of 56 VEEV infections had recorded sex information; 39 case-patients

were male and 17 female. Severity assessment was possible in 45 VEEV cases; 10 (22%) cases were classified as mild, 25 (56%) as moderate, and 10 (22%) as severe, resulting in a mild-to-severe ratio of 1:1. Clinical data were incomplete for 5 MADV and 51 VEEV cases. One (1/36 [2.8%]) MADV fatality and 8 (8/95 [8.4%]) VEEV fatalities were reported.

MADV and VEEV Laboratory Testing

We conducted a retrospective analysis to identify the diagnostic methods employed for detecting VEEV and MADV infections during 1961–2023. MADV infections were identified nearly exclusively (n = 26 [92%]) by ELISA IgM, except a single case (8.1%) detected by RT-PCR on brain tissue after autopsy. VEEV infections were mostly identified through viral isolation (n = 67 [51%]), ELISA IgM (n = 45 [34%]), and RT-PCR (n = 19 [15%]) (Appendix Table 2).

VEEV and MADV Clinical Presentation

The most frequently documented signs or symptoms of MADV and VEEV infections included fever, headache, and vomiting. Neurologic symptoms were more common in MADV infections and slightly more common among male patients. Less common signs and symptoms, including diarrhea, pharyngitis, hemorrhage, and rash, were more prevalent in VEEV infections (Figure 4).

Fever was consistently reported for both viruses, both sexes, and all age groups (Figures 5, 6). Headaches were also consistently reported in patients infected by both viruses but increased in frequency concurrent with age. Neurologic symptoms were more frequent in MADV cases in the 0–5 and 6–20 years of age groups; in contrast, neurologic symptoms were reported in the >5 years of age group of VEEV cases. The frequency of neurologic symptoms was also higher in male case-patients with MADV infections,

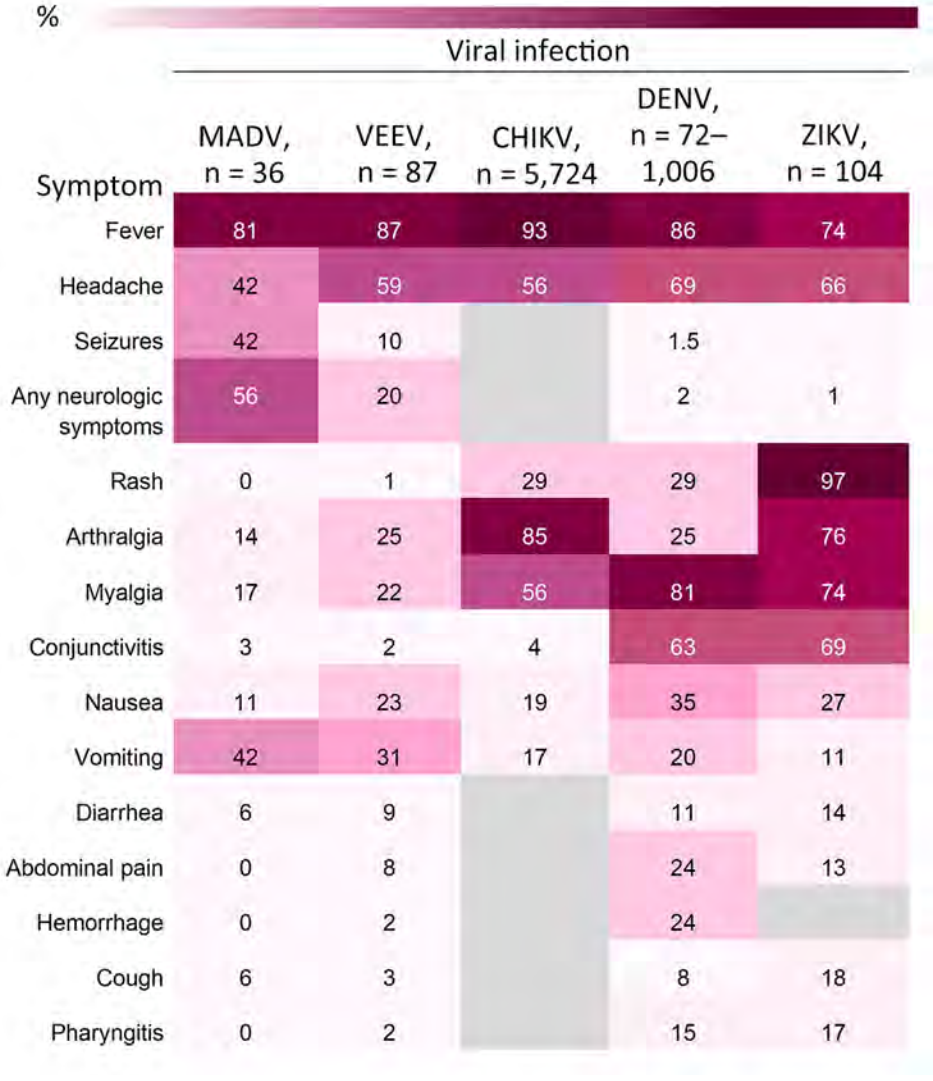

Figure 4. Heatmap of frequency of signs and symptoms by viral infection in South America used in study of characteristics of MADV and VEEV, Panama. Datasets from alphavirus cases in Panama (1961–2023), and DENV (2009), CHIKV (2015–2020), and ZIKV (2016) infection cases from Brazil were used to provide more complete symptom data. Gray blocks denote missing data. Neurologic symptoms included seizures, focal sensory or motor deficits, and diminished level of consciousness. CHIKV, chikungunya virus; DENV, dengue virus, MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus; ZIKV, Zika virus.

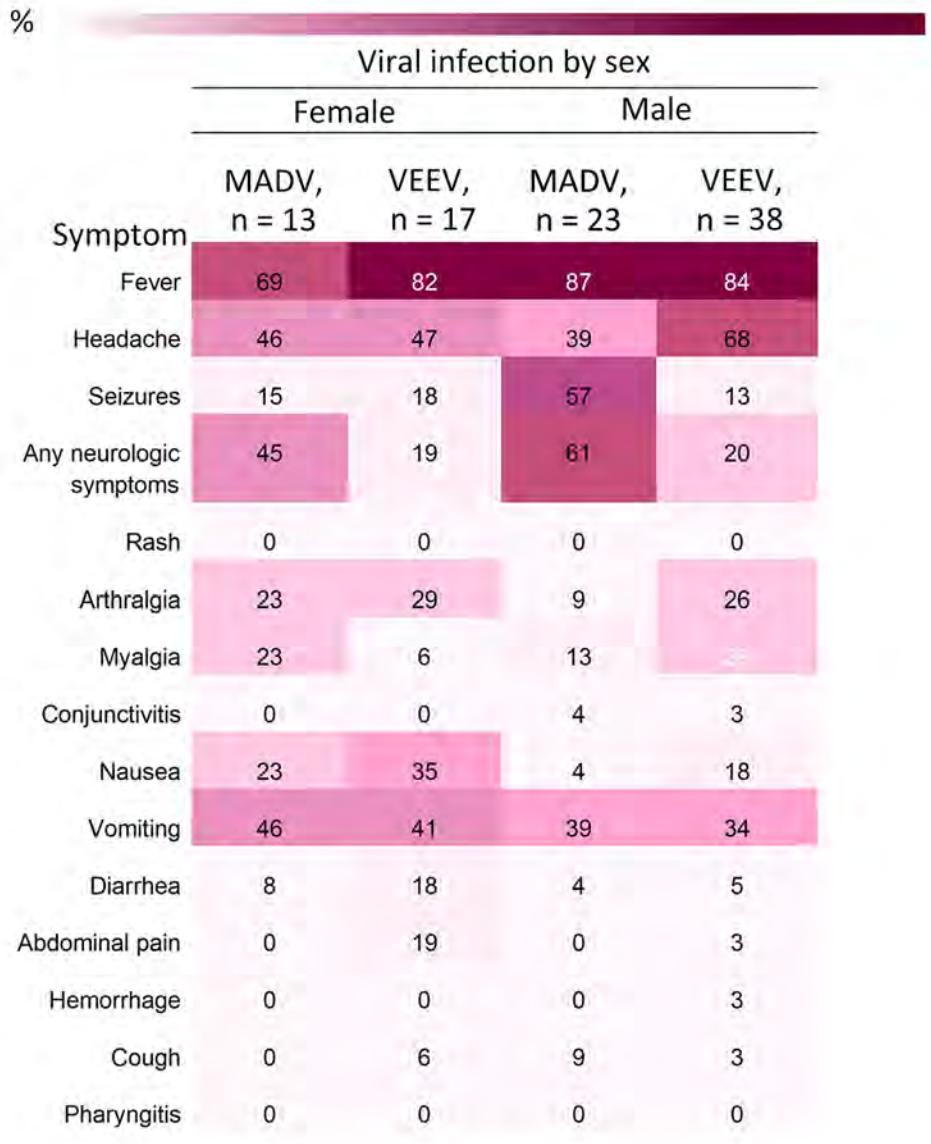

Figure 5. Heatmap of frequency of signs and symptoms by sex and viral infection in study of characteristics of MADV and VEEV virus infections, Panama. Cases reported during 1961–2023. Neurologic symptoms included seizures, focal sensory or motor deficits, and diminished level of consciousness. MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus

but equally distributed among VEEV case-patients (Figure 5). Myalgia, arthralgia, and nausea were more commonly seen in VEEV case-patients, and frequency increased with age; the highest frequency was reported in the ≥21 years of age group (Figure 6). Abdominal pain was reported only among VEEV cases, was more common in female case-patients, and was reported exclusively in the ≥21 years of age group. Conjunctivitis was seen exclusively in the ≥21 years of age group for MADV infections. Of note, diarrhea was equally distributed among VEEV cases of both sexes until age 20; only male case-patients reported diarrhea in the ≥21 years of age group.

Logistic regression analysis controlling for sex and age showed that seizures and vomiting were associated with MADV infections more than VEEV infections (Appendix Table 3). At the multivariable level, after variable selection processes, only seizures remained statistically significant when comparing MADV with VEEV (Figure 7, panel A; Appendix Table 3).

Encephalitic Alphavirus versus DENV, ZIKV, and CHIKV Infection

In multivariate analyses, dominant clinical syndromes differed by pathogen (Figure 7). Encephalitic alphavirus infections were more likely to include arthralgia and vomiting than DENV infections, and more likely to include fever and vomiting than ZIKV infections. Broadly, nausea and vomiting distinguished encephalitic alphavirus infections from CHIKV infections. We identified additional differences (Appendix Tables 4–6).

Discussion

In this epidemiologic study, we provided a comprehensive assessment of VEEV and MADV cases in Panama. We summarized and contextualized the clinical findings of human cases of MADV and VEEV in Panama, and identified symptoms that could be considered suggestive of MADV and VEEV infection when compared with other endemic arboviral infections in the region. We have shown that MADV and VEEV cases disproportionally affected males, and that MADV occurs more often in children, whereas most VEEV cases occur in adults.

Whether sex-related or age-related susceptibility differences of VEEV and MADV are caused by the lack of preexisting immunity or different exposure risks (e.g., occupational) is unclear. VEEV has been present in Panama since the mid-20th century, when the virus was isolated from a fatal human case in 1961 (*15*). The first recorded human outbreak of VEEV in Panama occurred in 1967 in US soldiers training on the western shores of Gatun Lake (*16*). Since then, VEEV outbreaks have been periodically reported in humans. Although equine cases of MADV have been documented in Panama since 1936 (*17*), instances of human cases were infrequent before 2010, despite active human surveillance during outbreaks and widespread mosquito isolations (*8*,*18*,*19*). A 2012 study on MADV and VEEV seropositivity in humans demonstrated an increasing prevalence of antibodies for VEEV with age, demonstrating that the virus is endemic in the region (*20*). The same trend was not observed for MADV, which suggested that the virus recently emerged in humans during the 2010 outbreak. MADV may have gained human virulence since 2010 (*6*), which may explain why we continue to see human cases. Children may be more susceptible to MADV because of lack of preexisting immunity or an immature immune system. The primary risk factors for human exposure to both viruses were found to be farming and fishing (*20*); spending more time outside performing those activities may put boys and men at an increased risk for exposure to infected mosquitoes.

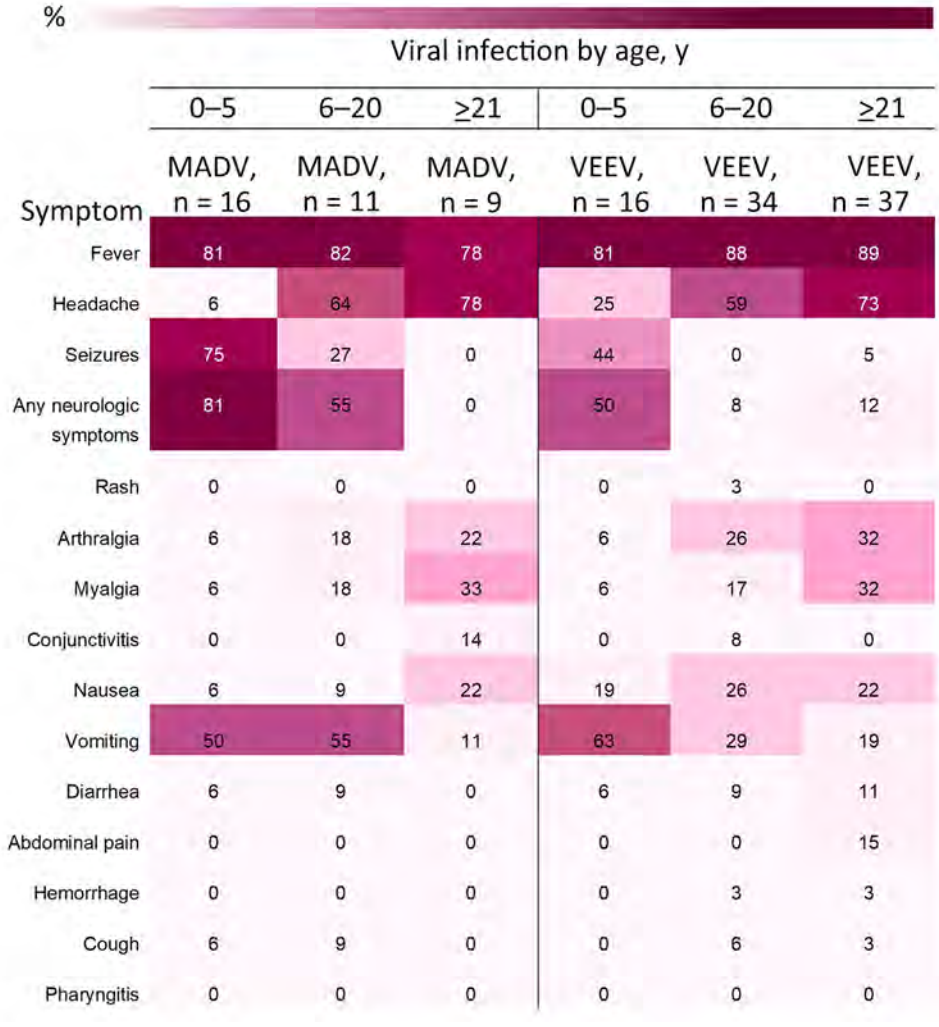

Figure 6. Heatmap of frequency of signs and symptoms by age and viral infection in study of characteristics of MADV and VEEV infections, Panama. Cases reported during 1961–2023**.** Neurologic symptoms included seizures, focal sensory or motor deficits, and diminished level of consciousness. MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus.

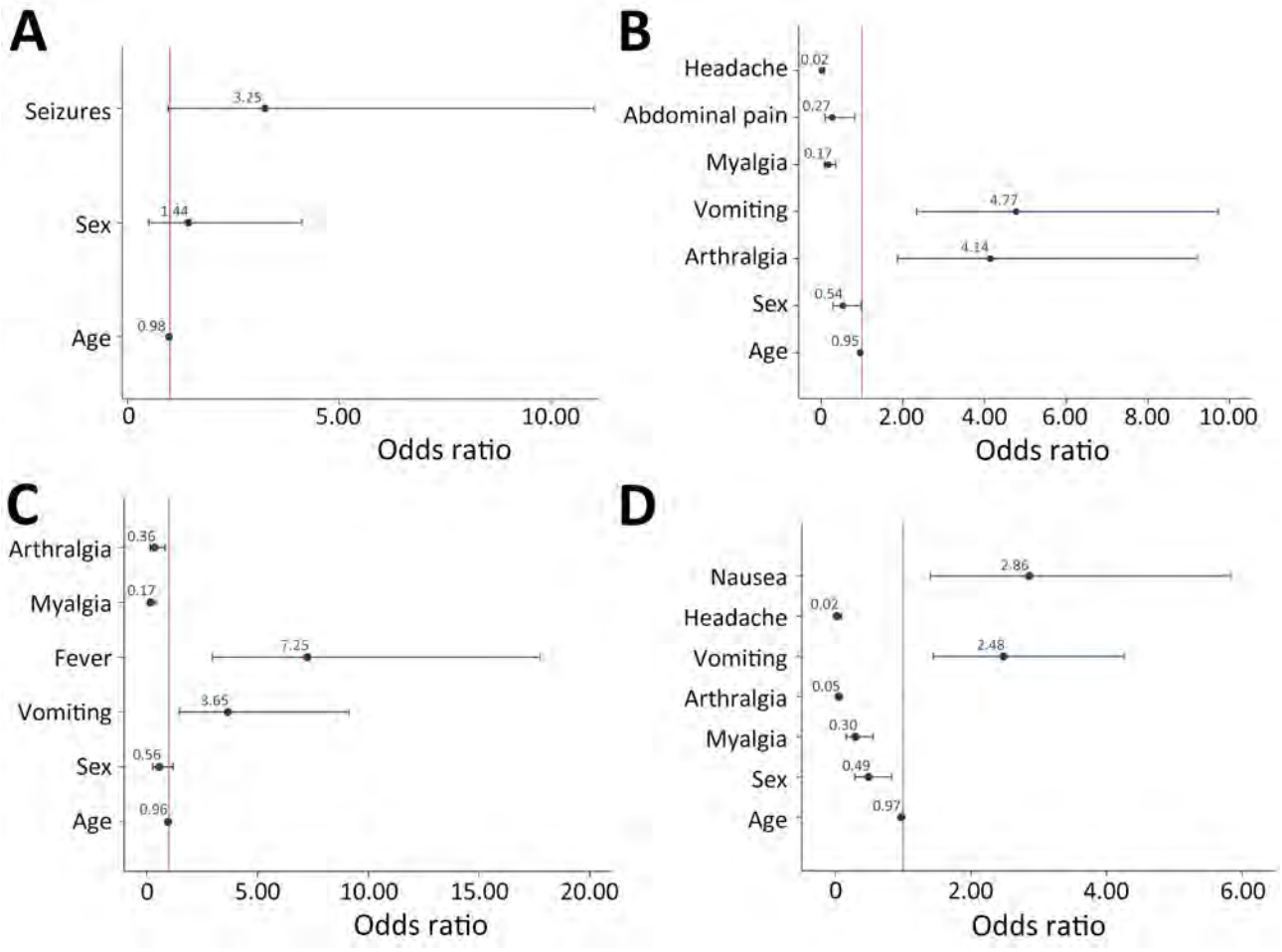

Figure 7. Multivariable logistic regression analysis of associated symptoms of encephalitic alphavirus infections and other arbovirus infections in study of characteristics of MADV and VEEV infections, Panama. A) MADV versus VEEV infection; B) MADV and VEEV versus DENV infection; C) MADV and VEEV versus ZIKV infection; D) MADV and VEEV versus CHIKV infection. MADV cases reported during 1961–2023**.** Dot represents odds ratio and whiskers indicate 95% CI. The red vertical line represents an odds ratio of 1, indicating that the odds of the event are the same in both groups. CHIKV, chikungunya virus; DENV, dengue virus, MADV, Madariaga virus; VEEV, Venezuelan equine encephalitis virus; ZIKV, Zika virus.

Our results highlight the need for continued surveillance for VEEV and MADV to better understand the 2 viruses and the differences between VEEV and MADV infection.

Darien Province in Panama is a hotspot for VEEV and MADV activity, especially for more recent outbreaks. All MADV human infections have occurred in that region, whereas VEEV infections have occurred throughout Panama. Darien is a remote region in eastern Panama near the Colombia border that is inhabited primarily by Indigenous communities. The region contains swamps and forest habitats that can support the enzootic transmission cycle of VEEV and MADV, which involves rodents and mosquitoes. Both viruses have the same mosquito vectors within the subgenus *Culex* (*Melanoconion*), and potentially the same rodent reservoir (*21*). The Darien Province also has a high number of refugee and migrant crossings; the United Nations reported >500,000 crossings in 2023 (*21*). Human migration through the region could result in more cases and potential spread to other regions; the MADV cases detected outside Darien were in members of the border police working in Darien Province whose symptoms did not develop until they returned to their home regions. Although our study reports 168 confirmed human cases of encephalitic alphavirus infection in Panama, the true burden of disease is likely underestimated, which is highlighted by the recent finding that 11.9% of dengue-like disease patients had VEEV infections (*22*).

The first limitation of our study is that the tests used for regular alphavirus diagnostics were in-house tests; an RT-PCR was recently developed

(*22*). Alphavirus infections in this cohort were diagnosed with a variety of tests over time as more robust methodologies were adopted. The diagnostic test performance of legacy tests performed before 2022 is not known, and misclassification bias might exist among the relevant cases. Clinical information on alphavirus infections was documented using forms that might not capture detailed clinical and laboratory parameters for both VEEV and MADV infections, and clinical data entry was incomplete. Second, encephalitic alphavirus cases often occur in rural or remote areas with limited healthcare systems and resources, which could mean the number of cases is underestimated. Third, the limited sample size could affect statistical power and conclusions, particularly for less frequent symptoms. Fourth, the clinical outcome could be virus strain–dependent and thus vary geographically. Finally, we compared symptoms of encephalitic alphavirus infection with those of DENV, ZIKV, and CHIKV infections from cohorts in Brazil; although the genetic background and social conditions in Panama may differ from those in Brazil, symptoms of DENV, ZIKV, and CHIKV infections appear to be similar across different populations (*23*–*26*).

In summary, outbreaks of MADV and VEEV are expected to continue, highlighting the need for continued surveillance efforts in Panama and other parts of Central and South America. Our findings could serve as a valuable tool for clinical and epidemiologic decision making in regions characterized by endemic arboviral circulation and limited laboratory capacity.

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Metagenomic Nanopore Sequencing of Tickborne Pathogens, Mongolia

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We performed nanopore-based metagenomic screening on 885 ticks collected from 6 locations in Mongolia and divided the results into 68 samples: 23 individual samples and 45 pools of 2–12 tick samples each. We detected bacterial and parasitic pathogens *Anaplasma ovis*, *Babesia microti*, *Coxiella burnetii*, *Borrelia miyamotoi*, *Francisella tularensis* subsp. *holarctica* and *novicida*, *Spiroplasma ixodetis*, *Theileria equi*, and *Rickettsia* spp., including *R. raoultii*, *R. slovaca*, and *R. canadensis*. We identified the viral pathogens Crimean-Congo hemorrhagic fever virus (2.9%), recently described Alongshan virus (ALSV) (2.9%), and Beiji nairovirus (5.8%). We assembled ALSV genomes, and maximum-likelihood analyses revealed clustering with viruses reported in humans and ticks from China. For ALSV, we identified surface glycoprotein markers associated with isolates from Asia viruses hosted by *Ixodes persulcatus* ticks. We also detected 20 virus species of unknown public health impact, including a near-complete Yanggou tick virus genome. Our findings demonstrate that nanopore sequencing can aid in detecting endemic and emerging tickborne pathogens.

A diverse spectrum of microbial agents, including viruses, bacteria, and protozoans, some with substantial health consequences or economic burden, can be transmitted to humans and animals by ticks (*1*). Surveillance plays a critical role in timely identification of tickborne pathogens and subsequent assessment of potential public health threats. Tickborne diseases constitute a major public and veterinary health threat in Mongolia, where a substantial portion of the population follows a pastoral lifestyle, including practices that involve frequent interactions with livestock animals and exposure to ticks (*2*). In this study, we used a combined pathogen screening strategy incorporating generic amplification and metagenomic sequencing in ticks collected from Mongolia. Research was conducted under an Institutional Animal Care and Use Committee–approved animal use protocol (protocol no. 21-01) in an American Association for Accreditation of Laboratory Animal Care International–accredited facility with a Public Health Services Animal Welfare Assurance and in compliance with the Animal Welfare Act and other federal statutes and regulations relating to laboratory animals.

Methods

We collected adult ticks by environmental dragging or removed ticks from sheep (*Ovis aries*) at 6 locations from the Bayan-Khongor, Selenge, and Umnugovi provinces in Mongolia during April 2021–May 2022. We morphologically identified, e-vouchered, and processed the tick samples as described elsewhere (*3*). We used nucleic acids from individual ticks for DNA

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barcoding and generic virus screening (*3*–*5*), then used the results to randomly assign each to a single or pooled sample (pool), in which we combined 5 μL of individual tick nucleic acids according to species and locality into 2–12 ticks per pool (Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0128-App1.pdf).

We performed nanopore-based metagenomic sequencing as described elsewhere (*3*). We performed similarity searches, de novo assembly, and read mapping on reads of ≥200 bp in Geneious Prime version 2022.2.1 (https://www.geneious.com). For mapping and pairwise comparisons, we used *Rickettsia* reference genomes (Appendix 2 Table 1, https://wwwnc.cdc.gov/EID/article/30/14/24-0128-App2.xlsx).

Results

Study Cohort and Pathogen Detection

We included 885 adult ticks in the study (Appendix 2 Table 1). We obtained uploaded DNA barcode sequences, specimen images, and collection data from information publicly available in the Barcode of Life Database (BOLD Systems, https://www.boldsystems.org) under project MONTK: Ticks of Mongolia (associated records MONTK001-23 through MONTK1128-23), where data are freely accessible. We separated 377 (42.6%) screened ticks into single-tick samples and 508 (57.4%) into multitick pools. We evaluated 68 samples, including 23 (33.8%) single-tick samples and 45 (66.2%) tick pools using nanopore-based metagenomic sequencing (Table 1; Appendix 1 Figure 1). We recovered no pathogens from *Hyalomma asiaticum* ticks from 2 (8.6%) single-tick samples or 6 (13.3%) pools.

Bacteria and Protozoa

Spotted fever group (SFG) *Rickettsia* and rickettsial endosymbionts were the most prevalent tickborne bacteria (Table 1). We identified the infecting species as *R. canadensis* in 5 single-tick samples (5/14, 35.7%) and 2 pools (2/32, 6.2%), *R. raoultii* in 8 pools (8/33, 24.2%), and *R. slovaca* in 1 single-tick sample (1/14, 7.1%). We detected *R. canadensis* and *R. slovaca* in *Ixodes persulcatus* ticks and *R. raoultii* in pools of *Dermacentor nuttalli* ticks and from 1 single-tick *Hy. asiaticum* sample (Appendix 2 Tables 2, 3).

Assembly and maximum-likelihood analysis of the *R. canadensis* contig (GenBank accession no. PP158215) encompassing the *rplO*, *rpmD*, and *secY* regions from an *I. persulcatus* tick sample showed a differential grouping of the Mongolia sequence within the *R. canadensis* clade, distinct from the SFG (Appendix 1 Figure 2). Analysis of *mutS* and *uvrD* contigs from a *D. nuttalli* tick pool revealed similar clustering of those sequences with *R. raoultii* strains (Appendix 1 Figure 3). In the remaining samples, we could not identify specific species, presumably because multiple SFGs were present. In individual ticks, we documented coinfections with SFG and endosymbionts in 1 *Hy. asiaticum* and 8 *I. persulcatus* ticks. We assembled complete plasmid sequences of *R. raoultii* from 5 *D. nuttalli* tick pools, which revealed 94.3%–97.6% identity with *R. raoultii* strain Khabarovsk plasmid pRra3 (GenBank accession no. CP010972).

Table 1. Prevalence of microbial pathogens in b52 in a metagenomic nanopore sequencing study of tickborne pathogens, Mongolia*

Pathogens	*Dermacentor nuttalli* ticks		*Hyalomma asiaticum* ticks		*Ixodes persulcatus* ticks		Total no. (%)
	Single, n = 2	Pooled, n = 15	Single, n = 11	Pooled, n = 15	Single, n = 10	Pooled, n = 15	
Bacteria							
Anaplasma ovis	0	1	0	0	0	0	1 (1.4)
Coxiella burnetii	2	0	6	6	7	0	21 (30.0)
Coxiella spp. endosymbiont	0	7	0	0	0	0	7 (10.2)
Borrelia miyamatoi	0	0	0	1	0	0	1 (1.4)
B. turcica	0	0	0	0	0	1	1 (1.4)
Francisella tularensis subsp. *holarctica*	0	0	1	0	0	0	1 (1.4)
F. tularensis subsp. *novicida*	0	0	0	1	0	0	1 (1.4)
F. persica and *F. opportunistica*	0	0	10	12	0	0	22 (32.3)
Rickettsia spp. spotted fever group	1	15	3	2	10	15	46 (67.6)
Rickettsia spp. endosymbiont	0	7	1	0	8	15	31 (45.5)
Spiroplasma ixodetis	0	0	0	0	0	5	5 (7.3)
Viruses							
Alongshan virus	0	0	0	0	0	2	2 (2.9)
Beiji nairovirus	0	0	0	0	0	4	4 (5.8)
Crimean-Congo hemorrhagic fever virus	0	0	2	0	0	0	2 (2.9)
Parasites							
Babesia microti	0	0	2	0	1	1	4 (5.8)
Theileria equi	0	3	0	0	0	0	3 (4.4)

*Tick-associated opportunistic or endosymbiotic bacteria closely related to pathogenic species are provided for comparison.

We observed pathogenic and opportunistic *Francisella* species exclusively in *Hy. asiaticum* ticks. We detected *F. tularensis* subsp. *holarctica* in 1 single-tick sample and subsp. *novicida* in 1 tick pool, with an overall combined prevalence of 2.9% (2/68). Opportunistic species, including *F. persica* and *F. opportunistica*, were more common, identified in 32.3% of all samples, including those with pathogenic *Francisella* species.

We detected *Coxiella burnetii* in all 3 tick species examined, with an overall prevalence of 30.8%. Unlike other endosymbionts, *Coxiella*-like bacteria were less commonly detected (10.2%) and only in *D. nutalli* ticks. We detected *Borrelia miyamotoi*, an agent of tickborne relapsing fever, in *Hy. asiaticum* ticks (1 pool); *Borrelia turcica* was detected only in *I. persulcatus* ticks. Other tickborne bacteria identified included *Spiroplasma ixodetis*, detected in 5 (7.3%) *I. persulcatus* tick pools, and *Anaplasma ovis*, detected in 1 (1.4%) pool of *D. nutalli* ticks. Among tickborne protozoan parasites, we detected *Babesia microti*, causative agent of human piroplasmosis, in 5.8% and *Theileria equi*, causative agent of equine piroplasmosis, in 4.4% of samples. We identified *B. microti* piroplasm, which also causes human babesiosis, in single-tick and pooled *Hy. asiaticum* and *I. persulcatus* tick samples; we detected *T. equi* only in *D. nutalli* tick pools (Table 1).

Viruses

We detected 3 tickborne viruses of human health concern: Crimean-Congo hemorrhagic fever virus (CCHFV) (family Nairoviridae, *Orthonairovirus hemorrhagiae*), Beiji nairovirus (BJNV) (family Nairoviridae, *Norwavirus beijiense*), and Alongshan virus (ALSV) (unclassified species of family Flaviviridae) (Table 1) (*6*). After preliminary reactivity in generic virus screening, we detected CCHFV in only 2 (2.9%) individual *Hy. asiaticum* ticks. Available sequence information revealed reads of CCHFV small and medium genome segments and displayed high identities to CCHFV genomes from the Inner Mongolia Autonomous Region of China (Appendix 2 Table 2). Reads and contigs of BJNV large and small segments in 4 (5.8%) *I. persulcatus* tick pools showed high pairwise identities with isolates previously characterized elsewhere (Appendix 2 Table 3). Finally, we detected ALSV in 2 (2.9%) pools of *I. persulcatus* ticks. We were able to generate sequences of all ALSV genome segments from both pools (Appendix 2 Table 3), and assemble the coding regions in pool b52, tentatively designated ALSV-Mongolia-b52 (GenBank accession nos. PP125347–50). Phylogenic construction revealed clustering of individual segments with ALSV documented from the Inner Mongolia Autonomous Region and Heilongjiang Province in China (Appendix 1 Figures 4–7). Pairwise comparisons based on complete glycoprotein sequences encoded on segment 2 located the ALSV-Mongolia-b52 strain within the Asia subgroup of the *I. persulcatus* tick isolates (Table 2) (*7*).

We identified sequences of 20 additional virus species belonging to 6 virus families, none of which are currently known to cause symptomatic disease in humans or animals (Appendix 1 Table 2). We recovered complete or near-complete coding sequences of Yanggou tick virus (unclassified species of family

Table 2. Amino acid substitutions in the Alongshan virus VP1a, VP1b, and nuORF proteins compared with ALSV-Mongolia-b52 in a metagenomic nanopore sequencing study of tickborne pathogens, Mongolia*

Virus protein	Amino acid position	*Ixodes ricinus* tick group	*Ixodes persulcatus* tick group		ALSV-Mongolia-b52
			Europe subgroup	Asia subgroup	
VP1a	8	Ala	Ala	Thr	Thr
	72	Val	Ala	Ala	Ala
	115	Ala	Val	Val	Val
	135	Val	Lys	Lys	Lys
	138	Pro	Ser	Ser	Ser
	153	Lys	Arg	Arg	Arg
	210	Gly	Gly	Ser	Ser
	216	Thr	Ala	Ala	Ala
	321	Val	Val	Thr	Thr
	460	Thr	Met	Thr	Thr
	472	Arg	His	His	His
	476	Arg	Arg	Gln	Gly
VP1b	58	Met	Met	Leu	Leu
	112	Ile	Ile	Val	Val
	127	Lys	Lys	Arg	Arg
	135	Ser	Ser	Gly	Gly
	216	Val	Ile	Ile	Ile
nuORF	4	Lys	Lys	Gly	Gly
	15	Asp	Asp	Asn	Asn
	132	Thr	Ala	Thr	Thr

*ALSV, Alongshan virus; nuORF, novel upstream open reading frame; VP, virus capsid protein.

Flaviviridae) from 1 *D. nuttalli* tick pool (YGTV-Mongolia-b77; GenBank accession nos. PP125351–54), distinctly clustered with related viruses in the maximum-likelihood analysis (Appendix 1 Figures 4–7).

Discussion

In this study, we used a metagenomic sequencing–based approach to detect and characterize tickborne pathogens agnostically in comparable numbers of single-tick and pooled tick samples, representing 3 tick species endemic to Mongolia. We detected pathogenic bacteria, viruses, or parasites in 86.7% of samples with a predominance of bacterial pathogens (Table 1). The most frequently observed bacteria were SFG *Rickettsia* (67.6%) and related endosymbionts (45.5%). Bacteria of the genus *Rickettsia*, gram-negative obligate intracellular bacteria, account for most bacterial infections transmitted by ticks (*8*). SFG includes >30 distinct *Rickettsia* species associated mainly with symptoms of spotted fever. Previous reports using various detection approaches have documented several *Rickettsia* species associated with spotted fever in Mongolia and the neighboring Inner Mongolia Autonomous Region of China (*2*). We identified *R. raoultii*, *R. slovaca*, and *R. canadensis* in single-tick and pooled tick samples from Mongolia. Initially reported in *Haemaphysalis leporispalustris* ticks from Canada, *R. canadensis* was also detected in *Haemaphysalis japonica* ticks from far eastern Russia, *Haemaphysalis flava* ticks from South Korea, and *Haemaphysalis longicornis* and *I. persulcatus* ticks from China. Monophyletic clustering based on *ompB* and *gltA* genes, observed in this study and elsewhere, suggests that *R. canadensis* constitutes an independent group (*9*). Although its pathogenicity is unclear, serologic evidence of *R. canadensis* exposure in humans has been documented (*10*), making the pathogen a potential agent of tickborne infections in endemic regions, including Mongolia.

Other bacterial pathogens detected in ticks, including *A. ovis*, *C. burnetii*, and *B. miyamotoi*, have been documented previously in various regions in Mongolia (*2*). We further detected *F. tularensis* subsp. *holarctica*, a subspecies that causes human tularemia, and *F. tularensis* subsp. *novicida* in *Hy. asiaticum* ticks collected from different locations. In contrast to tularemia agents, *F. tularensis* subsp. *novicida* rarely causes human disease and, even then, mostly involving persons with coexisting medical conditions or immunosuppression (*11*). Cases of human tularemia have been reported in Mongolia; therefore, our findings documenting the presence of *Francisella* species bacteria associated with human infections helps define the epidemiology of this pathogen. Finally, we identified the intracellular mollicute *S. ixodetis* in pooled *I. persulcatus* tick samples in Mongolia. Although members of the genus *Spiroplasma* are vertically transmitted endosymbionts of Ixodid ticks, cases of acute febrile illness caused by *S. ixodetis* have been documented in both immunocompromised and immunocompetent adults with frequent tick exposure (*12*). In addition to those bacteria, we identified the Piroplasmorida apicomplexans parasites *B. microti* and *T. equi*, previously reported to circulate in various locations in Mongolia (*2*). Despite the presence of those and other potentially pathogenic species, no information on human infections is currently available.

Tickborne viral pathogens detected in the study include CCHFV, BJNV, and ALSV in 11.7% (8/68) of samples. We identified sequences of CCHFV in low abundance in individual *Hy. asiaticum* tick samples (Appendix 2 Table 2). Previous screenings carried out in various regions have described a low frequency of CCHFV in *Hy. asiaticum* ticks (6). Of note, relatively high CCHFV seroprevalence in humans has been documented, as well as detectable antibodies in various mammals that might serve as reservoirs, although no human cases have been documented to date (*6*). BJNV has recently been included as a species in *Norwavirus* genus (family Nairoviridae), members of which lack the medium genome segment encoding for the structural glycoproteins in other nairoviruses, such as CCHFV (*6*). BJNV was described in tick-associated human febrile disease of unknown etiology from the Inner Mongolia Autonomous Region of China, further displaying pathogenicity in cell lines and experimental infections (*13*). Virus-specific antibodies were detected with a prevalence of up to 54.6% in human convalescent serum, as well as in sheep and cattle from the region. Detection of virus nucleic acids were reported from several tick species in China (*13*). BJNV was the most prevalent tickborne virus in our study at a 5.8% detection rate; we found the virus exclusively in *I. persulcatus* tick pools (Table 1).

ALSV is another recently described virus with human health effects. It was originally described in in patients in the Inner Mongolia Autonomous Region and Heilongjiang Province of China with febrile diseases and a history of tick bites, followed by seroconversion (*14*). ALSV particles are enveloped, possessing positive-sense single-stranded RNA genomes in 4 segments and classified in the Jingmenvirus group of *Flaviviridae* because of nonstructural protein homologies. ALSV has been reported in various tick species collected in several countries in Eurasia and showed evidence of viral replication and exposure in sheep, cattle, and deer (*7*,*15*). We detected ALSV in 2 *I. persulcatus* tick pools and assembled the prototype virus

genome (ALSV-Mongolia-b52). Maximum-likelihood analyses revealed grouping of ALSV-Mongolia-b52 with viruses reported from humans and ticks from China. Further analysis of the putative virus VP1a and VP1b surface glycoprotein sequences revealed amino acid markers associated with ASLV isolates from Asia hosted by *I. persulcatus* ticks (Table 2) (*7*). Those findings confirm the presence of ASLV in Mongolia and indicate *I. persulcatus* ticks are a probable vector. As with BJNV and many other tickborne infections, human ALSV infections are reported as tick bite–associated, nonspecific febrile illnesses. Diagnostic assays are imperative to determine the public health burden of those emergent human pathogens.

Among limitations, the study cohort was composed of 3 tick species that represent frequently documented tickborne pathogen vectors, including those species mostly observed in the northern part of Mongolia that is covered by boreal forest (*I. persulcatus* ticks), in the northern and central steppes (*D. nuttalli* ticks), and in the southern aimag areas (*Hy. asiaticum* ticks) (*2*). Nevertheless, we have described several other tick species within the genera *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus*, although some have been observed only rarely and include scarce information on public health effects (*2*). Our screening strategy included molecular barcoding and generic virus screening for individual ticks, whereas we pooled randomly selected samples of identical tick species and collection site. We performed microbial characterization of prescreened single-tick and pooled tick samples using metagenomic sequencing. Despite random assignment of ticks for individual or pooled screening, target genome quantity and infection rates of particular microorganisms might further hamper detection and might not correlate with actual prevalence. Therefore, we presented only pathogen detection rates and did not calculate the maximum-likelihood estimates or minimum infection rates, which are frequently generated based on data from target-specific assays. Nevertheless, incorporating morphology and barcoding for accurate species identification and generic testing as needed, combined with metagenomic sequencing for accurate pathogen characterization is an efficient strategy. Because of limited sample availability, we could not perform a systematic comparison of single-tick versus pooled tick testing or pathogen detection rates between collection sites. However, our findings demonstrate the utility of a metagenomic sequencing approach for detecting and characterizing both endemic and emerging pathogens, especially for locations with limited pathogen information.

In conclusion, we documented several bacterial and viral pathogens, some of which were initially described in ticks from Mongolia. Although the public health impact of those pathogens remains unclear, our findings demonstrate that nanopore sequencing can aid in detecting endemic and emerging tickborne pathogens.

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Disclaimer

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Comprehensive Surveillance of Severe Fever with Thrombocytopenia Syndrome Virus in Patients with Acute Febrile Illness, Wild Rodents, and Trombiculid Larval Mites, Thailand

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Infection with severe fever with thrombocytopenia syndrome (*Bandavirus dabieense*) virus poses a substantial public health threat because of its high mortality rates and severe complications. The virus is prevalent in Asia, although data from Thailand are scarce. Our study confirmed the virus in 1.6% of acute febrile illness patients and specific antibodies in 3% of archived samples since 2015 in Thailand. Nationwide zoonotic surveillance identified the virus in 8 rodent species and 4 chigger genera. Our findings underscore the importance of raising awareness among healthcare providers and the general public about the symptoms, risks, and prevention strategies associated with severe fever with thrombocytopenia syndrome virus infection. Ongoing surveillance of the virus in human and animal populations is essential for monitoring its prevalence, distribution, and potential for emergence.

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a virulent virus with a triple-segmented, negative-sense, single-stranded RNA genome. Taxonomically *Bandavirus dabieense*, the virus belongs to the genus *Bandavirus*, family *Phenuiviridae*. SFTSV poses a substantial public health challenge because of the lack of a vaccine or effective therapies and high mortality rates in previously healthy persons (*1*–*3*). First discovered in China in 2009 (*4*), SFTSV has since been reported in China, South Korea, and Japan and more recently in Vietnam, Myanmar, Pakistan, Taiwan, and Thailand (*5*–*12*). The virus is classified into genotypes A–F, each having distinct geographic variations in virulence and pathogenicity (*13*–*15*).

SFTSV is predominantly transmitted through tick bites, specifically by the Asian longhorned tick *Haemaphysalis longicornis*, known for its wide host range, vector competency for various pathogens, and extensive geographic distribution (*16*). Additional competent vectors include *Haemaphysalis flava* (*17*), *Ixodes sinensis* (*18*), and ≥8 other implicated tick species (*19*,*20*). Tick-bite prevention is considered the primary means of preventing SFTSV infection. Evidence also implicates mites in SFTSV transmission, particularly those in the family *Trombiculidae*, including *Leptotrombidium scutellare* and *Leptotrombidium deliense*, and family *Laelapidae*, including *Laelaps echidninus* (*21*,*22*).

Human-to-human transmission of SFTSV occurs through direct contact with infected blood or bodily

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fluids (*23*). Animal-to-human transmission is occasionally reported though contact with ill animals (*12*,*24*). The role of wild and domesticated animals has garnered considerable interest because of their potential involvement as pathogen reservoirs. In addition, the presence of viral RNA or specific antibodies has been confirmed in 10 domestic (*20*,*25*–*27*) and ≥10 wild animal species (*28*–*30*). This broad host and vector involvement underlines the complex epidemiology of SFTSV, posing major challenges in developing targeted public health strategies and mitigating the effect of this virus. Among vertebrate reservoirs of concern, rodents receive considerable attention because of their close proximity to humans.

Although SFTSV in Thailand was documented in 2020 (*12*), evidence suggests that the presence of SFTSV dates back to 2019 (*31*). Subsequent analysis of patients clinically suspected of having viral infection confirmed the presence of SFTSV genome segments belonging to genotype B (*32*). One study in Thailand investigated dogs as amplifying hosts, and the nucleotide sequence of SFTSV found in 1 dog appears closely related to genotype B or J3 (*33*). Although evidence from human and animal studies indicates the presence of SFTSV in multiple provinces of Thailand, primarily in Bangkok and its neighboring regions (Figure 1), the understanding of its distribution and potential hotspots remains incomplete. Comprehensive surveillance, particularly from regions with extensive farming or agricultural activities, where these habitats could serve as zoogeographic transmission points and disease hotspots, is imperative to guide effective prevention and control strategies.

The Study

To better understand the epidemiologic effect of SFTSV in Thailand, our study evaluated the prevalence of SFTSV RNA and specific antibodies in serum samples from 2,425 patients with undifferentiated acute febrile illness (AFI). The patients were admitted to Chum Phae Hospital in Khon Kaen Province, northeastern Thailand, during 2015–2021. We performed RNA detection by using quantitative reverse transcription PCR (qRT-PCR) targeting the partial nonstructural (NS) protein–encoding gene of the small segment (Appendix, https://wwwnc.cdc.gov/EID/article/30/14/24-0163-App1.pdf). We subsequently screened all SFTSV RNA-positive samples for other vectorborne pathogens endemic in Southeast Asia, including dengue, chikungunya, and Zika virus, by using the ZDC Multiplex RT-PCR Assay Kit (Bio-Rad, https://www.bio-rad.com) and bacterial pathogens, including *Rickettsia* and *Orientia* spp., by following a previously described protocol (*34*). We verified all qRT-PCR products for the expected size and subjected them to nucleotide sequencing by using the barcode-tagged sequencing method (Bionics, https://www.bionicsro.co.kr). We conducted phylogenetic analysis by using MEGA version 11 (https://www.megasoftware.net). The resulting nucleotide sequences of SFTSV amplified from the samples have been deposited into the GenBank database (accession nos. PP782658–61).

We detected SFTSV RNA in 38 of 2,425 AFI patients, resulting in a positivity rate of 1.6%. The median age of SFTSV RNA–positive patients was 47.2 years (15.9–86.7 years) (first–third interquartile range [IQR_{1-3}] 29–64 years). A total of 36.8% of the patients had agriculture-related occupations; male:female ratio was 1.4:1.0 (Table 1). The SFTSV RNA–positive cases did not exhibit a discernible seasonal pattern. We observed no co-positivity between SFTSV and other pathogens. SFTSV RNA–positive patients often visited the hospital with fever (38.0°C [range 36.1°C–40.3°C]); 20.5% experienced headaches, and 12.8% reported dizziness. We observed thrombocytopenia and variations in hemoconcentration in 3 cases (Appendix Figure 1). Of note, all patients recovered without severe complications. We successfully obtained only 2 nucleotide sequences from SFTSV RNA–positive patients, both of which demonstrated a close genetic relationship to genotype D. The strains shared 99.1% nucleotide similarity to strain LN2012–41 (GenBank accession no. KF887433) previously identified in a patient in China in 2012 (Figure 2).

We used an indirect ELISA specific for nucleoprotein (NP) of SFTSV to detect SFTSV IgM and SFTSV IgG in all 2,425 serum samples of AFI patients, according to manufacturer instructions (Bore Da Biotech, http://boreda.com). The average optical density at 450 nm (OD_{450}) of the positive controls provided by the kit was 0.233 for SFTSV IgM and 0.172 for SFTSV IgG. We considered samples with OD_{450} values exceeding those cutoffs to be seropositive (Appendix Figure 2). Serologic analysis revealed that 16 patients (0.7%) were seropositive for SFTSV IgM and 54 patients (2.2%) were seropositive for SFTSV IgG; 3 patients (0.1%) had detectable levels of both antibodies (Appendix Figure 2). Those findings resulted in an overall seropositive rate of 3% among the study population (Table 1). The median age of SFTSV IgM–seropositive patients was 63.8 years (IQR_{1-3} 20.6–68.1 years) and for SFTSV IgG–seropositive patients was 14 years (IQR_{1-3} 8.4–51.5 years). We observed no co-positive results between ELISA and qRT-PCR in the tested samples.

We evaluated the practicality of the paper-based lateral flow immunochromatography rapid test (SFTSV RDT; Bore Da Biotech) for potential use in prescreening of the viral NP at the point-of-care. Of the 38 SFTSV RNA–positive serum samples, 33 samples had sufficient quantities and were included in the analysis. The SFTSV RDT accurately detected the viral NP when the samples had a qRT-PCR cycle threshold of <37 or an average of 2.72×10^4 copies/mL serum observed in our study, demonstrating a positive concordance rate of 89.5% between both assays. Control tests with serum from healthy donors and patient serum samples previously confirmed positive for dengue virus 1–4, chikungunya virus, *Rickettsia typhi*, or *Orientia tsutsugamushi* showed no cross-reactivity. The overall agreement between

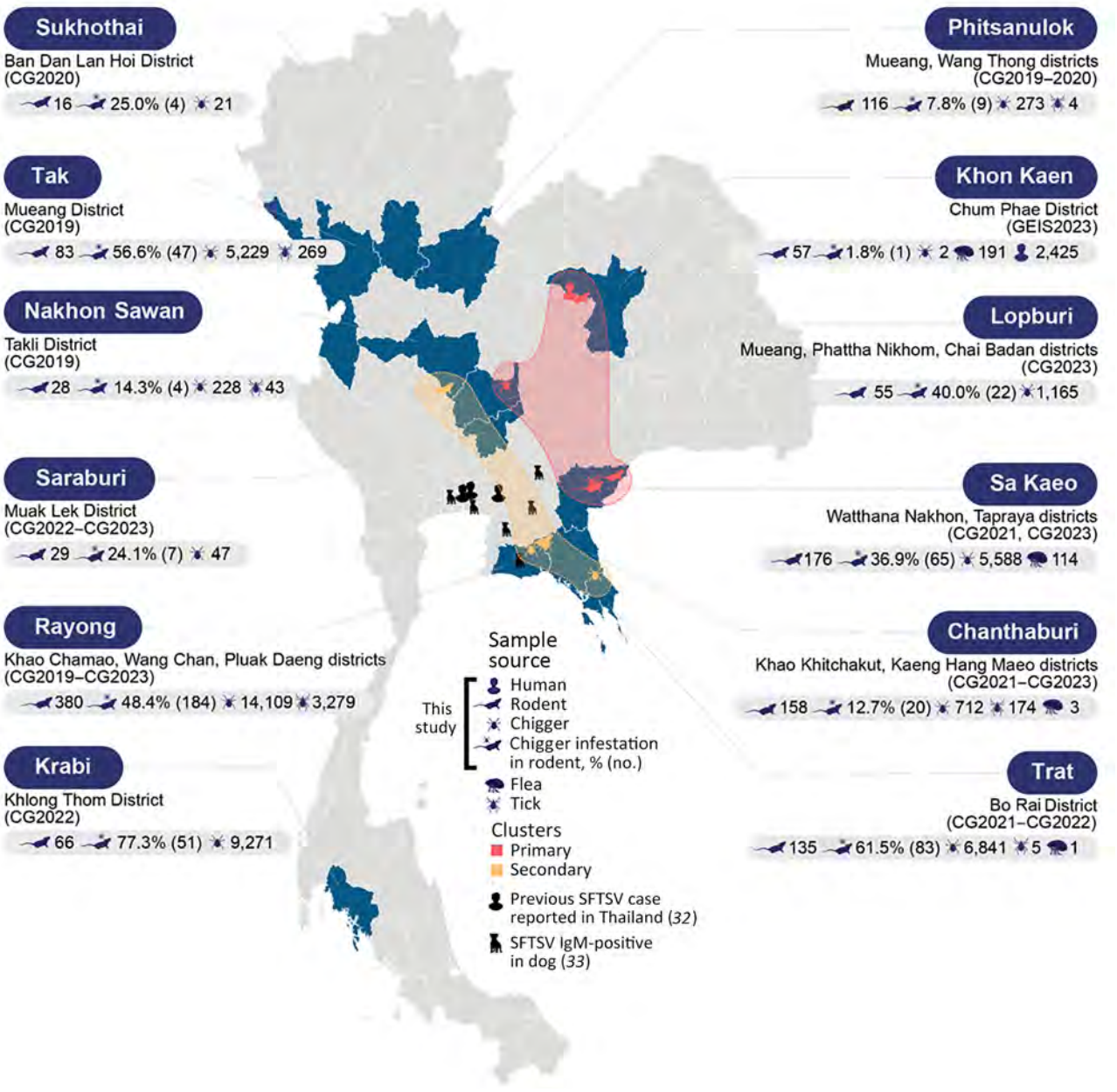

Figure 1. Geospatial clustering of SFTSV, Thailand, 2015–2021. Blue areas on the map represent the surveillance locations in this study. Icons indicate the types of host species and chiggers that tested positive for SFTSV. Data were consolidated to include previous reports of locations where SFTSV-positive patients and dogs were identified, aligning with the current locations of positive samples. Clusters were determined using the K-means clustering method. Primary clusters, highlighted in red, denote regions with a high overall prevalence of SFTSV across all hosts or are considered high-risk areas. Secondary clusters, highlighted in yellow, indicate areas with potential transmission dynamics, particularly involving animal hosts. SFTSV, severe fever with thrombocytopenia syndrome virus.

Table 1. Demographic disparities in acute febrile illness patients with SFTSV RNA–positive and specific antibodies–positive samples, Thailand, 2015–2021*

Variable	Total no. (%)	SFTSV positive	
		No. (%) qRT-PCR positive	No. (%) ELISA positive
Sex			
F	1,113 (45.9)	10 (26.3)	24 (32.9)
M	1,105 (45.6)	23 (60.5)	17 (23.3)
No information	207 (8.5)	5 (13.2)	32 (43.8)
Age range, y			
>15	1,474 (60.8)	0	0
0–30	584 (24.1)	8 (21.1)	20 (27.4)
31–40	34 (1.4)	7 (18.4)	3 (4.1)
41–50	36 (1.5)	1 (2.6)	3 (4.1)
51–60	38 (1.6)	4 (10.5)	3 (4.1)
61–70	20 (0.8)	5 (13.2)	7 (9.6)
>70	32 (1.3)	6 (15.8)	5 (6.8)
No information	207 (8.5)	7 (18.4)	32 (43.8)
Year			
2015	252 (10.4)	2 (5.3)	0
2016	99 (4.1)	1 (2.6)	0
2017	260 (10.7)	0	0
2018	203 (8.4)	2 (5.3)	15 (20.5)
2019	680 (28)	15 (39.5)	24 (32.9)
2020	486 (20)	1 (2.6)	2 (2.7)
2021	238 (9.8)	11 (28.9)	0
No information	207 (8.5)	6 (15.8)	32 (43.8)
Month			
January	150 (6.2)	2 (5.3)	1 (1.4)
February	154 (6.4)	8 (21.1)	0
March	196 (8.1)	6 (15.8)	0
April	131 (5.4)	0	2 (2.7)
May	100 (4.1)	0	6 (8.2)
June	160 (6.6)	11 (28.9)	6 (8.2)
July	313 (12.9)	0	9 (12.3)
August	370 (15.3)	1 (2.6)	6 (8.2)
September	183 (7.5)	1 (2.6)	1 (1.4)
October	186 (7.7)	0	5 (6.8)
November	168 (6.9)	2 (5.3)	4 (5.5)
December	107 (4.4)	1 (2.6)	1 (1.4)
No information	207 (8.5)	6 (15.8)	32 (43.8)
Season			
Hot, Mar–May	427 (17.6)	6 (15.8)	27 (37)
Rainy, Jun–Oct	1212 (50)	13 (34.2)	6 (8.2)
Winter, Nov–Feb	579 (23.9)	13 (34.2)	8 (11)
No information	207 (8.5)	6 (15.8)	32 (43.8)
Total	2,425	38 (1.6)	73 (3)

*qRT-PCR, quantitative reverse transcription PCR; SFTSV, severe fever with thrombocytopenia syndrome virus.

SFTSV RDT and qRT-PCR was substantial, having a κ value of 0.732, which validated its effectiveness (Appendix Table 3).

To investigate the role of rodents and chiggers in SFTSV transmission, we analyzed a total of 2,052 tissue samples from 1,019 wild rodents, representing 15 species across 7 genera and 4 families, by using qRT-PCR targeting the NS gene. We collected the samples during 2019–2023 as part of the rodentborne and ectoparasiteborne disease risk assessment program in Thailand (Appendix). Eleven rodents from 8 species were positive for SFTSV RNA, indicating an overall positivity rate of 1.1%. We collected the RNA-positive samples during 2019–2023 (Appendix Table 1) in Nakhon Sawan, Chanthaburi, Sa Kaeo, and Khon Kaen Provinces, and the positive rodent species included *Mus caroli* (Ryukyu mouse), *Menetes berdmorei* (Berdmore's squirrel), *Rattus norvegicus* (Norway rat), *Berylmys berdmorei* (small white-toothed rat), *Rattus exulans* (Polynesian rat), *Bandicota indica* (greater bandicoot rat), *Bandicota savilei* (Savile's bandicoot rat), and *Rattus tanezumi* complex (Asian house rat) (Table 2). Of the 1,019 rodents analyzed, we observed the highest SFTSV RNA prevalence in lungs (0.4% [4 rodents]) and spleen (0.4% [4 rodents]), followed by kidneys (0.3% [3 rodents]). Phylogenetic analysis of the partial sequence of the NS gene obtained from 2 rodents indicated a close relationship to genotype D, sharing 97.3% and 99.1% nucleotide similarity to the strain LN2012–41 from China and being nearly identical to SFTSV sequences obtained from AFI patients (Figure 2). The high genetic similarity observed across

different locations and periods suggests a potential widespread distribution of SFTSV in Thailand. However, this observation might also be influenced by the limitation of using only 124-bp nucleotide sequences for phylogenetic analysis. To ensure the validity of our findings and to rule out the possibility of cross-contamination, we performed nucleic acid extraction of samples and qRT-PCR in 2 separate laboratories

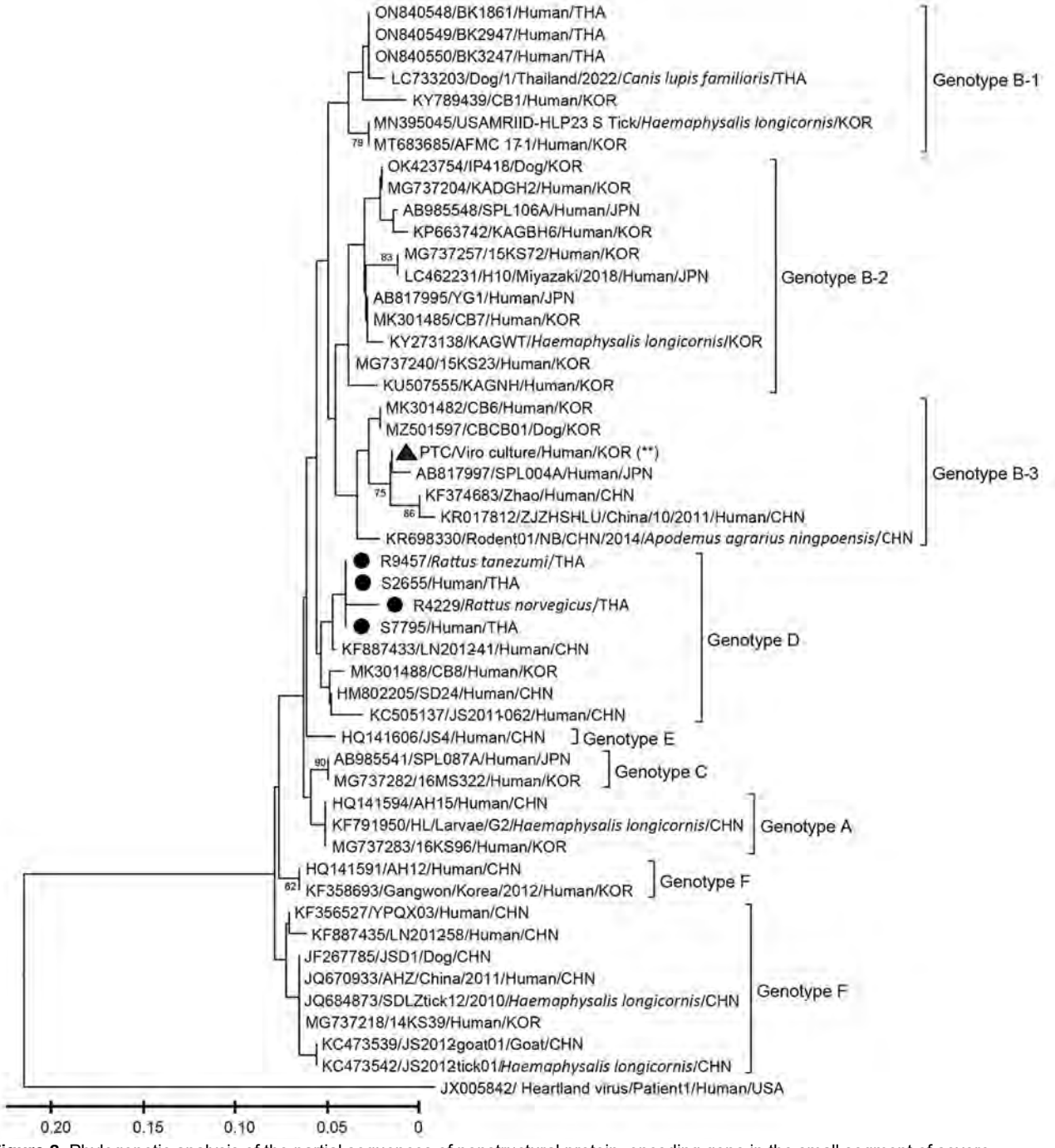

Figure 2. Phylogenetic analysis of the partial sequences of nonstructural protein–encoding gene in the small segment of severe fever with thrombocytopenia syndrome virus identified in Thailand, 2015–2021 and other countries. Black circles indicate nucleotide sequences identified in this study; black triangle indicates nucleotide sequence of the positive control originated from SFTSV patients in South Korea. The sequences are described by GenBank accession numbers/strain names/hosts/3-letter country code. Bootstrap values of nodes, based on 1,000 bootstrapping replicates, associated with the definition of genotypes are indicated in the principal nodes. Scale bar indicates phylogenetic distance of 0.05 nucleotide substitutions per site.

Table 2. Positivity rates of SFTSV RNA detected in wild rodents, Thailand, 2015–2021*

Family	Species	No. (%) positive	SFTSV RNA–positive, by tissue			Average RNA level, copies/mL
			Lung	Liver	Spleen	
Muridae	*Rattus tanezumi* rat	1/559 (0.2)	–	1	–	4.07×10^4
	R. exulans rat	1/98 (1)	–	–	1	3.05×10^3
	R. novegicus rat	1/16 (6.3)	1	–	–	5.73×10^3
	Mus cervicolor mouse	0/6	–	–	–	–
	M. caroli mouse	1/7 (14.3)	1	–	–	4.03×10^3
	Bandicota indica rat	2/113 (1.8)	1	1	–	4.89×10^3
	B. savileii rat	2/121 (1.7)	–	2	–	1.01×10^4
	Maxomys surifer rat	0/23	–	–	–	–
	Niviventer fulvescens rat	0/5	–	–	–	–
	Berylmys berdmorei rat	1/23 (4.3)	–	–	1	1.71×10^4
	B. bowersi rat	0/1	–	–	–	–
	Chiromyscus chiropus rat	0/1	–	–	–	–
Tupaiidae	*Tupaia belangeri* shrew	0/19	–	–	–	–
	T. glis shrew	0/9	–	–	–	–
Sciuridae	*Menetes berdmorei* squirrel	2/19 (10.5)	1	–	1	4.06×10^3
3 families	15 species	11/11,019 (1.1)	4	4	3	

*SFTSV, severe fever with thrombocytopenia syndrome virus; –, negative result.

by using different stocks of samples and reagents for confirmation. In addition, we prepared the positive control for the assay by using virus culture stock from an SFTSV RNA–positive case in South Korea that displayed a genetic distance from our positive samples.

From the analysis of 573 individual chiggers retrieved from 155 wild rodents, we detected SFTSV RNA in 8 chiggers (1.4%), which had an average SFTSV RNA level of 2.40×10^4 copies/chigger (range 5.80×10^3–70×10^4 copies/chigger) (Appendix Table 2). We retrieved the SFTSV RNA–positive chiggers from 6 rodents from Sa Kaeo, Chanthaburi, Lopburi, Rayong, and Trat provinces. Phylogenetic analysis of cytochrome oxidase subunit I gene sequences confirmed the correct genus assignment for SFTSV RNA–positive chiggers and revealed close relatedness to *Gahrliepia* (*walchia*) (4 samples), *Blankaartia acuscutellaris* (1 sample), and *Schoengastia kanhaensis* mites (1 sample) (Appendix Figure 3). Two SFTSV RNA–positive chiggers from the genus *Leptotrombidium* had insufficient sample quantities for further analysis. In addition, 4 of 8 SFTSV RNA–positive chiggers showed co-positivity for unidentified *Rickettsia*, but none tested positive for *O. tsutsugamushi*.

Discussion

Our study confirmed the presence of SFTSV RNA in AFI patients, wild rodents, and chiggers across multiple locations in Thailand and identified a notable seroprevalence of SFTSV-specific antibodies among AFI patients, highlighting a substantial yet underrecognized prevalence of SFTSV. The analysis of samples spanning multiple years, including the detection of SFTSV RNA in AFI patients in 2015, suggests that SFTSV has been circulating in Thailand since 2015, which predates the SFTSV detections reported in other Southeast Asia countries, including Vietnam in 2017 (*35*), Thailand in 2019 (*12*,*31*) and Myanmar during 2018–2019 (*11*). Our findings provide additional evidence of the existence of SFTSV genotype D, indicating that ≥2 genotypes have been identified recently in Thailand. Positive cases in patients and animals have been identified across several provinces in multiple regions, including Chachoengsao, Samutprakan, and Chonburi Provinces in the central region; Nakhon Sawan Province in the northern region; Chanthaburi, Sa Kaeo, Prachinburi, Rayong, and Chonburi Provinces in the eastern region; and Khon Kaen Province in the northeastern region (Figure 1) (*31*–*33*). This extensive distribution of the virus signifies a widespread and longstanding effect, necessitating ongoing surveillance and enhanced diagnostic measures to fully comprehend the disease ecology and transmission dynamics and to manage public health interventions effectively.

We observed that the prevalence of SFTSV RNA in wild rodents in our study was similar to the rate reported in China, where 0.7% of rodents tested positive. The species in China included *Apodemus agrarius* (striped field mouse), *Crocidura lasiura* (Ussuri white-toothed shrew), *R. norvegicus* (brown rat), and *Mus musculus* (house mouse) (*22*). A subsequent study from the same authors reported a higher prevalence of 32.3% and positive species including *A. agrarius* mice, *Tscherskia triton* (greater long-tailed hamster), and *M. musculus* mice (*14*). The primary difference between our study and previous studies lies in the surveillance sites and periods. In our study, rodents were captured from semirural areas near communities and were primarily collected during the dry season in Thailand within a 2–3-month timeframe

at selected sites. During this period, agricultural activities in some locations are reduced, which could potentially limit rodent abundance and their exposure to a broader range of ectoparasites, including ticks. To more accurately determine whether rodents serve as reservoirs of SFTSV and better understand the complex dynamics of SFTSV transmission, comprehensive surveillance studies involving collection of multiple rodent species across diverse geographic locations, seasons, and rodent species, coupled with serologic analysis, are crucial. Incorporating factors such as habitat diversity, agricultural practices, and climatic conditions into future research will also contribute to a comprehensive understanding of the ecologic niche of SFTSV.

Of note, we detected SFTSV RNA in chiggers infesting rodents that tested negative for the virus. This unexpected finding suggests a potential lack of direct rodent-to-chigger transmission. Given that chiggers feed on liquefied host skin tissue rather than blood, accidental acquisition of the virus during feeding appears less likely. Experimental studies supporting this notion demonstrated that chiggers feeding on *O. tsutsugamushi*–infected hosts failed to transmit the pathogen to their offspring (*36*). Alternatively, chiggers may acquire the virus through cofeeding with infected conspecifics and subsequently transmit the virus transovarially to their offspring, a phenomenon that has been successfully demonstrated in establishing new lines of *O. tsutsugamushi*–infected chiggers in previous research at our institute (*37*). Furthermore, reported co-positivity for SFTSV and *O. tsutsugamushi* in patients from South Korea and Myanmar (*11*,*38*,*39*) suggests a potential epidemiologic intersection between these pathogens, possibly enabled by shared vector species or overlapping habitats (*40*). The co-concurrence of these infections complicates diagnosis and underscores the importance of integrated surveillance systems to monitor these and potentially other cocirculating pathogens.

In conclusion, although the role of chiggers in SFTSV transmission remains unclear, the widespread distribution and abundance of chiggers, especially in the Asia–Pacific region, leads to frequent exposure to chiggerborne pathogens. Our findings suggest that the epidemiology of SFTSV may be more complex than previously understood, involving several potential vectors, reservoir hosts, and interactions with other pathogens. This apparent complexity underscores the need for comprehensive surveillance and research to better understand and mitigate the risks associated with this emerging infectious disease.

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Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted under an approved animal use protocol in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the National Research Councils Guide for the Care and Use of Laboratory Animals (*41*). The investigators have adhered to the policies for protection of human subjects as prescribed in Army Regulation 70–25 (*42*).

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Carlos J. (Anderson) Andreson (1905–1978), *Serology*, 1943, Watercolor on paper, 33 in × 36 in/84.8 cm × 91.4 cm. Accession no. 88-159-B. Gift of Abbott Laboratories; public domain image courtesy of Naval History and Heritage Command, Washington, DC, United States.

Military Personnel who Advance Global Surveillance for Infectious Diseases

M. Shayne Gallaway, Jessica Radzio-Basu

Soon after World War II began, Abbott Laboratories commissioned Carlos J. Andreson to document medical advancements in US Naval hospitals and showcase the contributions of doctors and scientists to the war effort. Andreson was an American painter, illustrator, graphic artist, and Works Progress Administration artist. He was born in Midvale, Utah, USA, in 1905, as Carlos J. Anderson. Early in his career, he changed the spelling of his last name to Andreson to distinguish himself from other artists with the last name Anderson. His works are currently part of the collections at the Metropolitan Museum of Art, National Museum of American Art, Utah State Fine Arts Collection, and Springville Museum of Art.

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Andreson was among the artists commissioned through the New Deal initiatives in the 1930s, which were aimed at providing economic relief during the Great Depression. In particular, the Federal Art Project, under the Works Progress Administration, resulted in the creation of approximately 200,000 works of art across the United States. As part of that effort, Andreson created a series of 24 historical building paintings and drawings. Completed as part of that series was Andreson's watercolor painting *Serology* (1943), featured on the cover of this supplement.

The work depicts Navy personnel preparing specimens for study in a serology laboratory. In the 1940s, serology was a groundbreaking laboratory technique that played a crucial role in the medical community's race to find effective treatments amid global upheaval. Serology involved examining blood serum (and other fluids) for the presence of antibodies to specific pathogens. However, at the time, accurate diagnosis using serology was complicated by a lack of standardization, nonspecific reactions, and cross-reacting antibodies. An incomplete understanding of the immune system and the concept of antibody classes made interpretation of results difficult.

During World War II, art played a vital role; it was used for education, public health campaigns, and morale-building. *Serology* highlights the painstaking efforts of laboratory workers and researchers who struggled to make sense of where, when, and how diseases moved through populations, bolstering disease surveillance efforts that would change the practice of public health.

The early 1940s were marked by significant challenges in public health resulting from the global spread of infectious diseases and the beginning of World War II. Considerable challenges and doubts surrounded disease surveillance. During that period and throughout military history, the combination of disease and nonbattle injuries (DNBI) accounted for large numbers of casualties. The proportion of deaths from DNBIs versus battle injuries decreased significantly from the US Civil War (1861–1865; 60%) to World War II (25%); however, most Army hospital admissions (95%) during 1941–1945 still resulted from DNBIs. In the 21st Century, DNBIs remain the leading cause of illness and death in conflicts involving the US military. During the 5 major operations making up the Global War on Terrorism (2001–2021), the estimated incidence rate for diseases (e.g., behavioral health, chronic, ill-defined, infectious, respiratory) were almost 3 times higher than the incidence rates for nonbattle injuries and battle injuries.

Since 1946, the US Department of Defense has operated overseas laboratories alongside host-country agencies with the purpose of studying and surveilling infectious diseases of mutual interest during periods of conflict and peace. Those laboratories have made substantial contributions to global health by developing medical countermeasures, assisting with public health emergency responses, and fostering collaborations and friendships within their various regional areas of operation. Serologic tests have proved essential for Department of Defense laboratories to detect infectious diseases such as syphilis, malaria, typhoid fever, and tuberculosis among and within various geographic populations. Globally based US military field hospital laboratories have used serologic testing to manage outbreaks that could infect thousands of troops and render them incapable of serving.

Modern serology-based techniques have evolved from the procedures that Andreson depicted in his painting; the techniques have overcome early limitations associated with diagnosing disease, sample collection, and differentiation of an antibody response (i.e., vaccine vs. natural infection). Modern serology is a key tool used for analyzing human infectious diseases and has applications for public health, disease prevention, clinical diagnosis, and disease management.

Andreson's painting *Serology* reflects the microscopic interactions between pathogens and the immune system and invites viewers to appreciate laboratory processes and the complexity of nascent science through art. Andreson's portrayal of a laboratory scene echoes the frustrations of a world still grappling with the rapid spread of disease. The muted palette reflects the somber reality of the limitations in medical technology and public health infrastructure of the time. The interaction of shapes suggests constant observation, symbolizing the early efforts of disease surveillance, which were hampered by limited knowledge and resources. Andreson captures the scientific breakthroughs of the time but also the profound challenges faced by those seeking to control and prevent disease during that period. The artwork's layered complexity and color tones reflect the concerns generated by incomplete understanding of diseases. The painting serves as a commentary on the scientific advancements of the time and a tribute to the resilience of those striving to improve public health in a time of crisis.

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